ISSN 1007-9327
CN 14-1219/R

World Journal of Gastroenterology®

Indexed and Abstracted in:

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, *Index Medicus*, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology and Hepatology*, CAB Abstracts and Global Health.
ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

Volume 14 Number 33
September 7, 2008
World J Gastroenterol
2008 September 7; 14(33): 5105-5232

Online Submissions
wjg.wjgnet.com
www.wjgnet.com
Printed on Acid-free Paper
世界胃肠病学杂志

A Weekly Journal of Gastroenterology and Hepatology

World Journal of Gastroenterology®

Editorial Board

2007-2009

Published by The WJG Press and Baishideng
Room 903, Ocean International Center, Building D
No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Fax: +86-10-8538-1893 E-mail: wjg@wjgnet.com http://www.wjgnet.com

The World Journal of Gastroenterology Editorial Board consists of 1208 members, representing a team of worldwide experts in gastroenterology and hepatology. They are from 60 countries, including Albania (1), Argentina (4), Australia (39), Austria (10), Belarus (1), Belgium (15), Brazil (2), Bulgaria (1), Canada (28), Chile (1), China (60), Croatia (2), Cuba (1), Czech (2), Denmark (7), Egypt (4), Estonia (1), Finland (4), France (44), Germany (108), Greece (9), Hungary (2), Iceland (1), India (12), Iran (3), Ireland (4), Israel (8), Italy (96), Japan (176), Lebanon (3), Lithuania (1), Macedonia (1), Malaysia (3), Mexico (6), Monaco (1), Morocco (1), The Netherlands (26), New Zealand (1), Nigeria (1), Norway (3), Pakistan (2), Peru (1), Poland (6), Portugal (1), Russia (3), Saudi Arabia (2), Serbia (1), Singapore (4), Slovakia (2), Slovenia (1), South Africa (2), South Korea (14), Spain (38), Sweden (15), Switzerland (13), Turkey (8), United Arab Emirates (1), United Kingdom (83), United States (316) and Uruguay (2).

Andrew V Biankin, *Sydney*
Filip Braet, *Sydney*
Andrew D Clouston, *Sydney*
Graham Cooksley, *Queensland*
Darrell HG Crawford, *Brisbane*
Adrian G Cummins, *Woodville South*
Guy D Eslick, *Sydney*
Michael A Fink, *Melbourne*
Robert JL Fraser, *Daw Park*
Peter Raymond Gibson, *Victoria*
Jacob George, *Westmead*
Mark D Gorrell, *Sydney*
Yik-Hong Ho, *Townsville*
Gerald J Holtmann, *Adelaide*
Michael Horowitz, *Adelaide*
John E Kellow, *Sydney*
Rupert Leong, *Concord*
Geoffrey W McCaughan, *Sydney*
Finlay A Macrae, *Victoria*
Daniel Markovich, *Brisbane*
Phillip S Oates, *Perth*
Jacqui Richmond, *Victoria*
Stephen M Riordan, *Sydney*
Ian C Roberts-Thomson, *Adelaide*
Devanshi Seth, *Camperdown*
Arthur Shulkes, *Melbourne*
Ross C Smith, *Sydney*
Kevin J Spring, *Brisbane*
Huy A Tran, *New South Wales*
Debbie Trinder, *Fremantle*
Martin J Veysey, *Gosford*
Daniel L Worthley, *Bedford*

Austria

Peter Ferenci, *Vienna*
Valentin Fuhrmann, *Vienna*
Alfred Gangl, *Vienna*
Christoph Gasche, *Vienna*
Kurt Lenz, *Linz*
Markus Peck-Radosavljevic, *Vienna*
Rudolf E Stauber, *Auenbruggerplatz*
Herbert Tilg, *Innsbruck*
Michael Trauner, *Graz*
Harald Vogelsang, *Vienna*
Guenter Weiss, *Innsbruck*

Belarus

Yury K Marakhouski, *Minsk*

Belgium

Rudi Beyaert, *Gent*
Bart Rik De Geest, *Leuven*
Inge I Depoortere, *Leuven*
Olivier Detry, *Liège*
Benedicte Y De Winter, *Antwerp*
Karel Geboes, *Leuven*
Thierry Gustot, *Brussels*
Yves J Horsmans, *Brussels*
Geert G Leroux-Roels, *Ghent*
Louis Libbrecht, *Leuven*
Etienne M Sokal, *Brussels*
Marc Peeters, *De Pintelaan*
Gert A Van Assche, *Leuven*
Yvan Vandenplas, *Brussels*
Eddie Wisse, *Keerbergen*

Brazil

Heitor Rosa, *Goiania*
Ana Cristina Simões e Silva, *Belo Horizonte*

Bulgaria

Zahariy Krastev, *Sofia*

Canada

Fernando Alvarez, *Québec*
David Armstrong, *Ontario*
Jeffrey P Baker, *Toronto*
Olivier Barbier, *Québec*
Nancy Baxter, *Toronto*
Matthew Bjerknes, *Toronto*
Frank J Burczynski, *Manitoba*
Michael F Byrne, *Vancouver*
Wang-Xue Chen, *Ottawa*
Chantal Guillemette, *Québec*
Samuel S Lee, *Calgary*
Gary A Levy, *Toronto*
Andrew L Mason, *Alberta*
John K Marshall, *Ontario*
Donna-Marie McCafferty, *Calgary*
Thomas I Michalak, *St. John's*
Gerald Y Minuk, *Manitoba*
Paul Moayyedi, *Hamilton*
Kostas Pantopoulos, *Québec*
William G Paterson, *Kingston*
Eldon Shaffer, *Calgary*
Morris Sherman, *Toronto*
Martin Storr, *Calgary*
Elena F Verdu, *Ontario*
John L Wallace, *Calgary*
Eric M Yoshida, *Vancouver*

Chile

Silvana Zanlungo, *Santiago*

China

Henry LY Chan, *Hongkong*
Xiao-Ping Chen, *Wuhan*
Zong-Jie Cui, *Beijing*
Da-Jun Deng, *Beijing*
Er-Dan Dong, *Beijing*
Sheung-Tat Fan, *Hong Kong*
Jin Gu, *Beijing*
Xin-Yuan Guan, *Pokfulam*
De-Wu Han, *Taiyuan*
Ming-Liang He, *Hong Kong*
Wayne HC Hu, *Hong Kong*
Chee-Kin Hui, *Hong Kong*
Ching-Lung Lai, *Hong Kong*
Kam Chuen Lai, *Hong Kong*
James YW Lau, *Hong Kong*
Yuk-Tong Lee, *Hong Kong*
Suet-Yi Leung, *Hong Kong*
Wai-Keung Leung, *Hong Kong*
John M Luk, *Pokfulam*
Chung-Mau Lo, *Hong Kong*
Jing-Yun Ma, *Beijing*
Ronnie Tung Ping Poon, *Hong Kong*
Lun-Xiu Qin, *Shanghai*
Yu-Gang Song, *Guangzhou*
Qin Su, *Beijing*
Wai-Man Wong, *Hong Kong*
Hong Xiao, *Shanghai*
Dong-Liang Yang, *Wuhan*
Winnie Yeo, *Hong Kong*
Yuan Yuan, *Shenyang*
Man-Fung Yuen, *Hong Kong*
Jian-Zhong Zhang, *Beijing*
Xin-Xin Zhang, *Shanghai*
Bo-Jian Zheng, *Hong Kong*
Shu Zheng, *Hangzhou*

Croatia

Tamara Cacev, *Zagreb*
Marko Duvnjak, *Zagreb*

Cuba

Damian C Rodriguez, *Havana*

Czech

Milan Jirsa, *Praha*
Pavel Trunečka, *Prague*

Denmark

Peter Bytzer, *Copenhagen*
Asbjørn M Drewes, *Aalborg*
Hans Gregersen, *Aalborg*
Jens H Henriksen, *Hvidovre*
Claus P Hovendal, *Odense*
Fin S Larsen, *Copenhagen*
Søren Møller, *Hvidovre*

Egypt

Abdel-Rahman El-Zayadi, *Giza*
Amr M Helmy, *Cairo*
Ayman Yosry, *Cairo*

Estonia

Riina Salupere, *Tartu*

Finland

Irma E Jarvela, *Helsinki*
Katri M Kaukinen, *Tampere*
Minna Nyström, *Helsinki*
Pentti Sipponen, *Espoo*

France

Bettaieb Ali, *Dijon*
Corlu Anne, *Rennes*
Denis Ardid, *Clermont-Ferrand*
Charles P Balabaud, *Bordeaux*
Soumeya Bekri, *Rouen*
Jacques Belghiti, *Clichy*
Jacques Bernuau, *Clichy Cedex*
Pierre Brissot, *Rennes*
Patrice P Cacoub, *Paris*
Franck Carbonnel, *Besancon*
Laurent Castera, *Pessac*
Bruno Clément, *Rennes*
Benoit Coffin, *Colombes*
Jacques Cosnes, *Paris*
Thomas Decaens, *Cedex*

Francoise L Fabiani, *Angers*
Gérard Feldmann, *Paris*
Jean Fioramonti, *Toulouse*
Jean-Noël Freund, *Strasbourg*
Jean-Paul Galmiche, *Nantes*
Catherine Guettier, *Villejuif*
Chantal Housset, *Paris*
Juan L Iovanna, *Marseille*
Rene Lambert, *Lyon*
Patrick Marcellin, *Paris*
Philippe Mathurin, *Lille*
Tamara Matysiak–Budnik, *Paris*
Francis Mégraud, *Bordeaux*
Richard Moreau, *Clichy*
Thierry Piche, *Nice*
Raoul Poupon, *Paris*
Jean Rosenbaum, *Bordeaux*
Dominique Marie Roulot, *Bobigny*
Thierry Poynard, *Paris*
Jean-Philippe Salier, *Rouen*
Didier Samuel, *Villejuif*
Jean-Yves Scoazec, *Lyon*
Khalid A Tazi, *Clichy*
Emmanuel Tiret, *Paris*
Baumert F Thomas, *Strasbourg*
Marie-Catherine Vozenin-brotons, *Villejuif*
Jean-Pierre H Zarski, *Grenoble*
Jessica Zucman-Rossi, *Paris*

Germany

Hans-Dieter Allescher, *G-Partenkirchen*
Martin Anlauf, *Kiel*
Rudolf Arnold, *Marburg*
Max G Bachem, *Ulm*
Thomas F Baumert, *Freiburg*
Daniel C Baumgart, *Berlin*
Hubert Blum, *Freiburg*
Thomas Bock, *Tuebingen*
Katja Breitkopf, *Mannheim*
Dunja Bruder, *Braunschweig*
Markus W Büchler, *Heidelberg*
Christa Buechler, *Regensburg*
Reinhard Buettner, *Bonn*
Elke Cario, *Essen*
Uta Dahmen, *Essen*
Christoph F Dietrich, *Bad Mergentheim*
Arno J Dormann, *Koeln*
Rainer J Duchmann, *Berlin*
Volker F Eckardt, *Wiesbaden*
Paul Enck, *Tuebingen*
Fred Fändrich, *Kiel*
Ulrich R Fölsch, *Kiel*
Helmut Friess, *Heidelberg*
Peter R Galle, *Mainz*
Nikolaus Gassler, *Aachen*
Andreas Geier, *Aachen*
Markus Gerhard, *Munich*
Wolfram H Gerlich, *Giessen*
Dieter Glebe, *Giessen*
Burkhard Göke, *Munich*
Florian Graepler, *Tuebingen*
Axel M Gressner, *Aachen*
Veit Gülberg, *Munich*
Rainer Haas, *Munich*
Eckhart G Hahn, *Erlangen*
Stephan Hellmig, *Kiel*
Martin Hennenberg, *Bonn*
Johannes Herkel, *Hamburg*
Klaus R Herrlinger, *Stuttgart*
Eva Herrmann, *Homburg/Saar*
Eberhard Hildt, *Berlin*
Joerg C Hoffmann, *Berlin*
Ferdinand Hofstaedter, *Regensburg*
Werner Hohenberger, *Erlangen*
Jörg C Kalff, *Bonn*
Ralf Jakobs, *Ludwigshafen*
Jutta Keller, *Hamburg*
Andrej Khandoga, *Munich*
Sibylle Koletzko, *München*
Stefan Kubicka, *Hannover*
Joachim Labenz, *Siegen*
Frank Lammert, *Bonn*
Thomas Langmann, *Regensburg*
Christian Liedtke, *Aachen*
Matthias Löhr, *Mannheim*
Christian Maaser, *Muenster*
Ahmed Madisch, *Dresden*
Peter Malfertheiner, *Magdeburg*
Michael P Manns, *Hannover*
Helmut Messmann, *Augsburg*
Stephan Miehlke, *Dresden*
Sabine Mihm, *Göttingen*
Silvio Nadalin, *Essen*
Markus F Neurath, *Mainz*
Johann Ockenga, *Berlin*
Florian Obermeier, *Regensburg*
Gustav Paumgartner, *Munich*
Ulrich KS Peitz, *Magdeburg*
Markus Reiser, *Bochum*
Emil C Reisinger, *Rostock*
Steffen Rickes, *Magdeburg*
Tilman Sauerbruch, *Bonn*
Dieter Saur, *Munich*
Hans Scherubl, *Berlin*
Joerg Schirra, *Munich*
Roland M Schmid, *München*
Volker Schmitz, *Bonn*
Andreas G Schreyer, *Regensburg*
Tobias Schroeder, *Essen*
Henning Schulze-Bergkamen, *Mainz*
Hans Seifert, *Oldenburg*
Norbert Senninger, *Muenster*
Manfred V Singer, *Mannheim*
Gisela Sparmann, *Rostock*
Christian J Steib, *München*
Jurgen M Stein, *Frankfurt*
Ulrike S Stein, *Berlin*
Manfred Stolte, *Bayreuth*
Christian P Strassburg, *Hannover*
Wolfgang R Stremmel, *Heidelberg*
Harald F Teutsch, *Ulm*
Robert Thimme, *Freiburg*
Hans L Tillmann, *Leipzig*
Tung-Yu Tsui, *Regensburg*
Axel Ulsenheimer, *Munich*
Patrick Veit-Haibach, *Essen*
Claudia Veltkamp, *Heidelberg*
Siegfried Wagner, *Deggendorf*
Henning Walczak, *Heidelberg*
Heiner Wedemeyer, *Hannover*
Fritz von Weizsacker, *Berlin*
Jens Werner, *Heidelberg*
Bertram Wiedenmann, *Berlin*
Reiner Wiest, *Regensburg*
Stefan Wirth, *Wuppertal*
Stefan JP Zeuzem, *Homburg*

Greece

Alexandra A Alexopoulou, *Athens*
George N Dalekos, *Larissa*
Christos Dervenis, *Athens*
Melanie Maria Deutsch, *Athens*
Tsianos Epameinondas, *Ioannina*
Elias A Kouroumalis, *Heraklion*
George Papatheodoridis, *Athens*
Spiros Sgouros, *Athens*

Hungary

Peter L Lakatos, *Budapest*
Zsuzsa Szondy, *Debrecen*

Iceland

Hallgrimur Gudjonsson, *Reykjavik*

India

Philip Abraham, *Mumbai*
Rakesh Aggarwal, *Lucknow*
Kunissery A Balasubramanian, *Vellore*
Deepak Kumar Bhasin, *Chandigarh*
Sujit K Bhattacharya, *Kolkata*
Yogesh K Chawla, *Chandigarh*
Radha K Dhiman, *Chandigarh*
Sri Prakash Misra, *Allahabad*
Ramesh Roop Rai, *Jaipur*
Nageshwar D Reddy, *Hyderabad*
Rakesh Kumar Tandon, *New Delhi*

Iran

Seyed-Moayed Alavian, *Tehran*
Reza Malekzadeh, *Tehran*
Seyed A Taghavi, *Shiraz*

Ireland

Billy Bourke, *Dublin*
Ronan A Cahill, *Cork*
Anthony P Moran, *Galway*

Israel

Simon Bar-Meir, *Hashomer*
Abraham R Eliakim, *Haifa*
Zvi Fireman, *Hadera*
Yaron Ilan, *Jerusalem*
Avidan U Neumann, *Ramat-Gan*
Yaron Niv, *Pardesia*
Ran Oren, *Tel Aviv*
Ami D Sperber, *Beer-Sheva*

Italy

Giovanni Addolorato, *Roma*
Luigi E Adinolfi, *Naples*
Domenico Alvaro, *Rome*
Mario Angelico, *Rome*
Vito Annese, *San Giovanni Rotond*
Filippo Ansaldi, *Genoa*
Adolfo F Attili, *Roma*
Giovanni Barbara, *Bologna*
Claudio Bassi, *Verona*
Gabrio Bassotti, *Perugia*
Pier M Battezzati, *Milan*
Stefano Bellentani, *Carpi*
Antonio Benedetti, *Ancona*
Mauro Bernardi, *Bologna*
Livia Biancone, *Rome*
Luigi Bonavina, *Milano*
Flavia Bortolotti, *Padova*
Giuseppe Brisinda, *Rome*
Elisabetta Buscarini, *Crema*
Giovanni Cammarota, *Roma*

Antonino Cavallari, *Bologna*
Giuseppe Chiarioni, *Valeggio*
Michele Cicala, *Rome*
Massimo Colombo, *Milan*
Amedeo Columbano, *Cagliari*
Massimo Conio, *Sanremo*
Dario Conte, *Milano*
Gino R Corazza, *Pavia*
Francesco Costa, *Pisa*
Antonio Craxi, *Palermo*
Silvio Danese, *Milan*
Roberto de Franchis, *Milano*
Roberto De Giorgio, *Bologna*
Maria Stella De Mitri, *Bologna*
Giovanni D De Palma, *Naples*
Fabio Farinati, *Padua*
Giammarco Fava, *Ancona*
Francesco Feo, *Sassari*
Fiorucci Stefano, *Perugia*
Andrea Galli, *Firenze*
Valeria Ghisett, *Turin*
Gianluigi Giannelli, *Bari*
Edoardo G Giannini, *Genoa*
Paolo Gionchetti, *Bologna*
Fabio Grizzi, *Milan*
Salvatore Gruttadauria, *Palermo*
Mario Guslandi, *Milano*
Pietro Invernizzi, *Milan*
Ezio Laconi, *Cagliari*
Giacomo Laffi, *Firenze*
Giovanni Maconi, *Milan*
Lucia Malaguarnera, *Catania*
Emanuele D Mangoni, *Napoli*
Paolo Manzoni, *Torino*
Giulio Marchesini, *Bologna*
Fabio Marra, *Florence*
Marco Marzioni, *Ancona*
Giuseppe Mazzella, *Bologna*
Mario U Mondelli, *Pavia*
Giuseppe Montalto, *Palermo*
Giovanni Monteleone, *Rome*
Giovanni Musso, *Torino*
Gerardo Nardone, *Napoli*
Valerio Nobili, *Rome*
Fabio Pace, *Milano*
Luisi Pagliaro, *Palermo*
Francesco Pallone, *Rome*
Fabrizio R Parente, *Milan*
Maurizio Parola, *Torino*
Francesco Perri, *San Giovanni Rotondo*
Raffaele Pezzilli, *Bologna*
Alberto Pilotto, *San Giovanni Rotondo*
Alberto Piperno, *Monza*
Mario Pirisi, *Novara*
Anna C Piscaglia, *Roma*
Paolo Del Poggio, *Treviglio*
Gabriele B Porro, *Milano*
Piero Portincasa, *Bari*
Cosimo Prantera, *Roma*
Bernardino Rampone, *Siena*
Oliviero Riggio, *Rome*
Claudio Romano, *Messina*
Marco Romano, *Napoli*
Gerardo Rosati, *Potenza*
Mario Del Tacca, *Pisa*
Gloria Taliani, *Rome*
Pier A Testoni, *Milan*
Enrico Roda, *Bologna*
Domenico Sansonno, *Bari*
Vincenzo Savarino, *Genova*
Vincenzo Stanghellini, *Bologna*
Giovanni Tarantino, *Naples*
Roberto Testa, *Genoa*
Dino Vaira, *Bologna*
Anna Linda Zignego, *Florence*

Japan

Kyoichi Adachi, *Izumo*
Yasushi Adachi, *Sapporo*
Taiji Akamatsu, *Matsumoto*
Sk Md Fazle Akbar, *Ehime*
Takafumi Ando, *Nagoya*
Akira Andoh, *Otsu*
Taku Aoki, *Tokyo*
Masahiro Arai, *Tokyo*
Tetsuo Arakawa, *Osaka*
Yasuji Arase, *Tokyo*
Masahiro Asaka, *Sapporo*
Hitoshi Asakura, *Tokyo*
Takeshi Azuma, *Fukui*
Yoichi Chida, *Fukuoka*
Takahiro Fujimori, *Tochigi*
Jiro Fujimoto, *Hyogo*
Kazuma Fujimoto, *Saga*
Mitsuhiro Fujishiro, *Tokyo*
Yoshihide Fujiyama, *Otsu*
Hiroyuki Fukui, *Tochigi*
Hiroyuki Hanai, *Hamamatsu*
Naohiko Harada, *Fukuoka*
Makoto Hashizume, *Fukuoka*
Tetsuo Hayakawa, *Nagoya*
Toru Hiyama, *Higashihiroshima*
Kazuhide Higuchi, *Osaka*
Keisuke Hino, *Ube*
Keiji Hirata, *Kitakyushu*
Yuji Iimuro, *Nishinomiya*
Kenji Ikeda, *Tokyo*
Toru Ikegami, *Fukuoka*
Kenichi Ikejima, *Bunkyo-ku*
Fumio Imazeki, *Chiba*
Yutaka Inagaki, *Kanagawa*
Yasuhiro Inokuchi, *Yokohama*
Haruhiro Inoue, *Yokohama*
Masayasu Inoue, *Osaka*
Hiromi Ishibashi, *Nagasaki*
Shunji Ishihara, *Izumo*
Toru Ishikawa, *Niigata*
Kei Ito, *Sendai*
Masayoshi Ito, *Tokyo*
Hiroaki Itoh, *Akita*
Ryuichi Iwakiri, *Saga*
Yoshiaki Iwasaki, *Okayama*
Terumi Kamisawa, *Tokyo*
Hiroshi Kaneko, *Aichi-Gun*
Shuichi Kaneko, *Kanazawa*
Takashi Kanematsu, *Nagasaki*
Mitsuo Katano, *Fukuoka*
Junji Kato, *Sapporo*
Mototsugu Kato, *Sapporo*
Shinzo Kato, *Tokyo*
Norifumi Kawada, *Osaka*
Sunao Kawano, *Osaka*
Mitsuhiro Kida, *Kanagawa*
Yoshikazu Kinoshita, *Izumo*
Tsuneo Kitamura, *Chiba*
Seigo Kitano, *Oita*
Kazuhiko Koike, *Tokyo*
Norihiro Kokudo, *Tokyo*
Satoshi Kondo, *Sapporo*
Shoji Kubo, *Osaka*
Shigeki Kuriyama, *Kagawa*[2]
Katsunori Iijima, *Sendai*
Masato Kusunoki, *Tsu Mie*
Shin Maeda, *Tokyo*
Shigeru Marubashi, *Suita*
Masatoshi Makuuchi, *Tokyo*
Osamu Matsui, *Kanazawa*
Yasuhiro Matsumura, *Chiba*
Yasushi Matsuzaki, *Tsukuba*
Kiyoshi Migita, *Omura*
Kenji Miki, *Tokyo*
Tetsuya Mine, *Kanagawa*
Hiroto Miwa, *Hyogo*
Masashi Mizokami, *Nagoya*
Yoshiaki Mizuguchi, *Tokyo*
Motowo Mizuno, *Hiroshima*
Morito Monden, *Suita*
Hisataka S Moriwaki, *Gifu*
Yasuaki Motomura, *Iizuka*
Yoshiharu Motoo, *Kanazawa*
Naofumi Mukaida, *Kanazawa*
Kazunari Murakami, *Oita*
Kunihiko Murase, *Tusima*
Hiroaki Nagano, *Suita*
Masahito Nagaki, *Gifu*
Masaki Nagaya, *Kawasaki*
Yujl Naito, *Kyoto*
Atsushi Nakajima, *Yokohama*
Hisato Nakajima, *Tokyo*
Hiroki Nakamura, *Yamaguchi*
Shotaro Nakamura, *Fukuoka*
Mikio Nishioka, *Niihama*
Shuji Nomoto, *Nagoya*
Susumu Ohmada, *Maebashi*
Hirohide Ohnishi, *Akita*
Masayuki Ohta, *Oita*
Tetsuo Ohta, *Kanazawa*
Kazuichi Okazaki, *Osaka*
Katsuhisa Omagari, *Nagasaki*
Saburo Onishi, *Nankoku*
Morikazu Onji, *Ehime*
Satoshi Osawa, *Hamamatsu*
Masanobu Oshima, *Kanazawa*
Hiromitsu Saisho, *Chiba*
Hidetsugu Saito, *Tokyo*
Yutaka Saito, *Tokyo*
Isao Sakaida, *Yamaguchi*
Michiie Sakamoto, *Tokyo*
Yasushi Sano, *Chiba*
Hiroki Sasaki, *Tokyo*
Iwao Sasaki, *Sendai*
Motoko Sasaki, *Kanazawa*
Chifumi Sato, *Tokyo*
Shuichi Seki, *Osaka*
Hiroshi Shimada, *Yokohama*
Mitsuo Shimada, *Tokushima*
Tomohiko Shimatan, *Hiroshima*
Hiroaki Shimizu, *Chiba*
Ichiro Shimizu, *Tokushima*
Yukihiro Shimizu, *Kyoto*
Shinji Shimoda, *Fukuoka*
Tooru Shimosegawa, *Sendai*
Tadashi Shimoyama, *Hirosaki*
Ken Shirabe, *Iizuka City*
Yoshio Shirai, *Niigata*
Katsuya Shiraki, *Mie*
Yasushi Shiratori, *Okayama*
Masayuki Sho, *Nara*
Yasuhiko Sugawara, *Tokyo*
Hidekazu Suzuki, *Tokyo*
Minoru Tada, *Tokyo*
Tadatoshi Takayama, *Tokyo*
Tadashi Takeda, *Osaka*
Koji Takeuchi, *Kyoto*
Kiichi Tamada, *Tochigi*
Akira Tanaka, *Kyoto*
Eiji Tanaka, *Matsumoto*
Noriaki Tanaka, *Okayama*
Shinji Tanaka, *Hiroshima*
Hideki Taniguchi, *Yokohama*
Kyuichi Tanikawa, *Kurume*
Akira Terano, *Shimotsugagun*
Hitoshi Togash, *Yamagata*
Shinji Togo, *Yokohama*
Kazunari Tominaga, *Osaka*
Takuji Torimura, *Fukuoka*

Minoru Toyota, *Sapporo*
Akihito Tsubota, *Chiba*
Takato Ueno, *Kurume*
Naomi Uemura, *Tokyo*
Shinichi Wada, *Tochigi*
Hiroyuki Watanabe, *Kanazawa*
Toshio Watanabe, *Osaka*
Yuji Watanabe, *Ehime*
Toshiaki Watanabe, *Tokyo*
Chun-Yang Wen, *Nagasaki*
Satoshi Yamagiwa, *Niigata*
Koji Yamaguchi, *Fukuoka*
Takayuki Yamamoto, *Yokkaichi*
Takashi Yao, *Fukuoka*
Masashi Yoneda, *Tochigi*
Hiroshi Yoshida, *Tokyo*
Masashi Yoshida, *Tokyo*
Norimasa Yoshida, *Kyoto*
Hitoshi Yoshiji, *Nara*
Kentaro Yoshika, *Toyoake*
Yasunobu Yoshikai, *Fukuoka*
Masahide Yoshikawa, *Kashihara*
Katsutoshi Yoshizato, *Higashihiroshima*

Lebanon

Bassam N Abboud, *Beirut*
Ala I Sharara, *Beirut*
Joseph D Boujaoude, *Beirut*

Lithuania

Limas Kupcinskas, *Kaunas*

Macedonia

Vladimir C Serafimoski, *Skopje*

Malaysia

Andrew Seng Boon Chua, *Ipoh*
Khean-Lee Goh, Kuala *Lumpur*
Jayaram Menon, *Sabah*

Mexico

Diego Garcia-Compean, *Monterrey*
Eduardo R Marin-Lopez, *Jesús García*
Nahum Méndez-Sánchez, *Mexico*
Saúl Villa-Treviño, *México*

Monaco

Patrick Rampal, *Monaco*

Morocco

Abdellah Essaid, *Rabat*

The Netherlands

Ulrich Beuers, *Amsterdam*
Gerd Bouma, *Amsterdam*
Lee Bouwman, *Leiden*
J Bart A Crusius, *Amsterdam*
NKH de Boer, *Amsterdam*
Koert P de Jong, *Groningen*
Henrike Hamer, *Maastricht*
Frank Hoentjen, *Haarlem*
Janine K Kruit, *Groningen*
Ernst J Kuipers, *Rotterdam*
CBHW Lamers, *Leiden*
Ton Lisman, *Utrecht*
Yi Liu, *Amsterdam*
Jeroen Maljaars, *Maastricht*
Servaas Morré, *Amsterdam*
Chris JJ Mulder, *Amsterdam*
Michael Müller, *Wageningen*
Amado S Peña, *Amsterdam*
Robert J Porte, *Groningen*
Ingrid B Renes, *Rotterdam*
Andreas Smout, *Utrecht*
Paul E Sijens, *Groningen*
Reinhold W Stockbrugger, *Maastricht*
Luc JW van der Laan, *Rotterdam*
Karel van Erpecum, *Utrecht*
Gerard P VanBerge-Henegouwen,*Utrecht*

New Zealand

Ian D Wallace, *Auckland*

Nigeria

Samuel B Olaleye, *Ibadan*

Norway

Trond Berg, *Oslo*
Tom H Karlsen, *Oslo*
Helge L Waldum, *Trondheim*

Pakistan

Muhammad S Khokhar, *Lahore*
Syed MW Jafri, *Karachi*

Peru

Hector H Garcia, *Lima*

Poland

Tomasz Brzozowski, *Cracow*
Robert Flisiak, *Bialystok*
Hanna Gregorek, *Warsaw*
Dariusz M Lebensztejn, *Bialystok*
Wojciech G Polak, *Wroclaw*
Marek Hartleb, *Katowice*

Portugal

Miguel C De Moura, *Lisbon*

Russia

Vladimir T Ivashkin, *Moscow*
Leonid Lazebnik, *Moscow*
Vasiliy I Reshetnyak, *Moscow*

Saudi Arabia

Ibrahim A Al Mofleh, *Riyadh*
Ahmed Helmy, *Riyadh*

Serbia

Dusan M Jovanovic, *Sremska Kamenica*

Singapore

Bow Ho, *Singapore*
Khek-Yu Ho, *Singapore*
Fock Kwong Ming, *Singapore*
Francis Seow-Choen, *Singapore*

Slovakia

Silvia Pastorekova, *Bratislava*
Anton Vavrecka, *Bratislava*

Slovenia

Sasa Markovic, *Ljubljana*

South Africa

Rosemar Joyce Burnett, *Pretoria*
Michael C Kew, *Parktown*

South Korea

Byung Ihn Choi, *Seoul*
Ho Soon Choi, *Seoul*
Marie Yeo, *Suwon*
Sun Pyo Hong, *Gyeonggi-do*
Jae J Kim, *Seoul*
Jin-Hong Kim, *Suwon*
Myung-Hwan Kim, *Seoul*
Chang Hong Lee, *Seoul*
Jong Kyun Lee, *Seoul*
Eun-Yi Moon, *Seoul*
Jae-Gahb Park, *Seoul*
Dong Wan Seo, *Seoul*
Dong Jin Suh, *Seoul*
Byung Chul Yoo, *Seoul*

Spain

Juan G Abraldes, *Barcelona*
Agustin Albillos, *Madrid*
Raul J Andrade, *Málaga*
Luis Aparisi, *Valencia*
Fernando Azpiroz, *Barcelona*
Ramon Bataller, *Barcelona*
Josep M Bordas, *Barcelona*
Xavier Calvet, *Sabadell*
Jordi Camps, *Catalunya*
Andres Cardenas, *Barcelona*
Vicente Carreño, *Madrid*
Jose Castellote, *Barcelona*
Antoni Castells, *Barcelona*
Vicente Felipo, *Valencia*
Juan C Garcia-Pagán, *Barcelona*
Jaime B Genover, *Barcelona*
Javier P Gisbert, *Madrid*
Jaime Guardia, *Barcelona*
Isabel Fabregat, *Barcelona*
Mercedes Fernandez, *Barcelona*
Angel Lanas, *Zaragoza*
Juan-Ramón Larrubia, *Guadalajara*
Laura Lladóa, *Barcelona*
María IT López, *Jaén*
Juan R Malagelada, *Barcelona*
José M Mato, *Derio*
Juan F Medina, *Pamplona*
Miguel A Muñoz-Navas, *Pamplona*
Julian Panes, *Barcelona*
Miguel M Perez, *Valencia*
Miguel Perez-Mateo, *Alicante*

Josep M Pique, *Barcelona*
Jesús M Prieto, *Pamplona*
Sabino Riestra, *Pola De Siero*
Luis Rodrigo, *Oviedo*
Manuel Romero-Gómez, *Sevilla*
Joan Roselló-Catafau, *Barcelona*

Sweden

Einar S Björnsson, *Gothenburg*
Curt Einarsson, *Huddinge*
Per M Hellström, *Stockholm*
Ulf Hindorf, *Lund*
Elisabeth Hultgren-Hörnquist, *Örebro*
Anders E Lehmann, *Mölndal*
Hanns-Ulrich Marschall, *Stockholm*
Lars C Olbe, *Molndal*
Lars A Pahlman, *Uppsala*
Matti Sallberg, *Stockholm*
Magnus Simrén, *Göteborg*
Xiao-Feng Sun, *Linköping*
Ervin Tóth, *Malmö*
Weimin Ye, *Stockholm*
Christer S von Holstein, *Lund*

Switzerland

Chrish Beglinger, *Basel*
Pierre A Clavien, *Zurich*
Jean-Francois Dufour, *Bern*
Franco Fortunato, *Zürich*
Jean L Frossard, *Geneva*
Gerd A Kullak-Ublick, *Zurich*
Pierre Michetti, *Lausanne*
Francesco Negro, *Genève*
Bruno Stieger, *Zurich*
Radu Tutuian, *Zurich*
Stephan R Vavricka, *Zurich*
Gerhard Rogler, *Zurich*
Arthur Zimmermann, *Berne*

Turkey

Yusuf Bayraktar, *Ankara*
Figen Gurakan, *Ankara*
Aydin Karabacakoglu, *Konya*
Serdar Karakose, *Konya*
Hizir Kurtel, *Istanbul*
Osman C Ozdogan, *Istanbul*
Özlem Yilmaz, *Izmir*
Cihan Yurdaydin, *Ankara*

United Arab Emirates

Sherif M Karam, *Al-Ain*

United Kingdom

David H Adams, *Birmingham*
Simon Afford, *Birmingham*
Navneet K Ahluwalia, *Stockport*
Ahmed Alzaraa, *Manchester*
Lesley A Anderson, *Belfast*
Charalambos G Antoniades, *London*
Anthony TR Axon, *Leeds*
Qasim Aziz, *Manchester*
Nicholas M Barnes, *Birmingham*
Jim D Bell, *London*
Mairi Brittan, *London*
Alastair D Burt, *Newcastle*
Simon S Campbell, *Manchester*
Simon R Carding, *Leeds*
Paul J Ciclitira, *London*
Eithne Costello, *Liverpool*
Tatjana Crnogorac-Jurcevic, *London*
Harry Dalton, *Truro*
Amar P Dhillon, *London*
William Dickey, *Londonderry*
James E East, *London*
Emad M El-Omar, *Aberdeen*
Ahmed M Elsharkawy, *Newcastle Upon Tyne*
Annette Fristscher-Ravens, *London*
Elizabeth Furrie, *Dundee*
Daniel R Gaya, *Edinburgh*
Subrata Ghosh, *London*
William Greenhalf, *Liverpool*
Indra N Guha, *Southampton*
Peter C Hayes, *Edinburgh*
Gwo-Tzer Ho, *Edinburgh*
Anthony R Hobson, *Salford*
Lesley A Houghton, *Manchester*
Stefan G Hübscher, *Birmingham*
Robin Hughes, *London*
Pali Hungin, *Stockton*
David P Hurlstone, *Sheffield*
Rajiv Jalan, *London*
Janusz AZ Jankowski, *Oxford*
Brian T Johnston, *Belfast*
David EJ Jones, *Newcastle*
Roger Jones, *London*
Michael A Kamm, *Harrow*
Peter Karayiannis, *London*
Laurens Kruidenier, *Harlow*
Patricia F Lalor, *Birmingham*
Chee Hooi Lim, *Midlands*
Hong-Xiang Liu, *Cambridge*
Yun Ma, *London*
Kenneth E L McColl, *Glasgow*
Stuart AC McDonald, *London*
Dermot P Mcgovern, *Oxford*
Giorgina Mieli-Vergani, *London*
Nikolai V Naoumov, *London*
John P Neoptolemos, *Liverpool*
James Neuberger, *Birmingham*
Philip Noel Newsome, *Birmingham*
Mark S Pearce, *Newcastle Upon Tyne*
Stephen P Pereira, *London*
D Mark Pritchard, *Liverpool*
Sakhawat Rahman, *London*
Stephen E Roberts, *Swansea*
Marco Senzolo, *Padova*
Soraya Shirazi-Beechey, *Liverpool*
Robert Sutton, *Liverpool*
Simon D Taylor-Robinson, *London*
Paris P Tekkis, *London*
Ulrich Thalheimer, *London*
David G Thompson, *Salford*
Nick P Thompson, *Newcastle*
David Tosh, *Bath*
Frank I Tovey, *London*
Chris Tselepis, *Birmingham*
Diego Vergani, *London*
Geoffrey Warhurst, *Salford*
Alastair John Watson, *Liverpool*
Peter J Whorwell, *Manchester*
Roger Williams, *London*
Karen L Wright, *Bath*
Min Zhao, *Foresterhill*

United States

Manal F Abdelmalek, *Durham*
Gary A Abrams, *Birmingham*
Maria T Abreu, *New York*
Reid B Adams, *Virginia*
Golo Ahlenstiel, *Bethesda*
BS Anand, *Houston*
Frank A Anania, *Atlanta*
M Ananthanarayanan, *New York*
Gavin E Arteel, *Louisville*
Jasmohan S Bajaj, *Milwaukee*
Subhas Banerjee, *Palo Alto*
Peter A Banks, *Boston*
Jamie S Barkin, *Miami Beach*
Kim E Barrett, *San Diego*
Marc D Basson, *Detroit*
Anthony J Bauer, *Pittsburgh*
Wallace F Berman, *Durham*
Timothy R Billiar, *Pittsburgh*
Edmund J Bini, *New York*
David G Binion, *Milwaukee*
Jennifer D Black, *Buffalo*
Herbert L Bonkovsky, *Charlotte*
Carla W Brady, *Durham*
Andrea D Branch, *New York*
Robert S Bresalier, *Houston*
Alan L Buchman, *Chicago*
Ronald W Busuttil, *Los Angeles*
Alan Cahill, *Philadelphia*
John M Carethers, *San Diego*
David L Carr-Locke, *Boston*
Maurice A Cerulli, *New York*
Ravi S Chari, *Nashville*
Jiande Chen, *Galveston*
Xian-Ming Chen, *Omaha*
Xin Chen, *San Francisco*
Ramsey Chi-man Cheung, *Palo Alto*
William D Chey, *Ann Arbor*
John Y Chiang, *Rootstown*
Parimal Chowdhury, *Arkansas*
Raymond T Chung, *Boston*
James M Church, *Cleveland*
Ram Chuttani, *Boston*
Mark G Clemens, *Charlotte*
Ana J Coito, *Los Angeles*
Vincent Coghlan, *Beaverton*
David Cronin II, *New Haven*
John Cuppoletti, *Cincinnati*
Mark J Czaja, *New York*
Peter V Danenberg, *Los Angeles*
Kiron M Das, New *Brunswick*
Conor P Delaney, *Cleveland*
Jose L del Pozo, *Rochester*
Sharon DeMorrow, *Temple*
Deborah L Diamond, *Seattle*
Douglas A Drossman, *Chapel Hill*
Katerina Dvorak, *Tucson*
Bijan Eghtesad, *Cleveland*
Hala El-Zimaity, *Houston*
Michelle Embree-Ku, *Providence*
Sukru Emre, New *Haven*
Douglas G Farmer, *Los Angeles*
Alessio Fasano, *Baltimore*
Mark A Feitelson, *Philadelphia*
Ariel E Feldstein, *Cleveland*
Alessandro Fichera, *Chicago*
Robert L Fine, *New York*
Magali Fontaine, *Stanford*
Chris E Forsmark, *Gainesville*
Glenn T Furuta, *Aurora*
Chandrashekhar R Gandhi, *Pittsburgh*
Susan L Gearhart, *Baltimore*
Xupeng Ge, *Boston*
Xin Geng, *New Brunswick*
M Eric Gershwin, *Suite*
Jean-Francois Geschwind, *Baltimore*
Ignacio Gil-Bazo, *New York*
Shannon S Glaser, *Temple*
Ajay Goel, *Dallas*
Richard M Green, *Chicago*
Julia B Greer, *Pittsburgh*

James H Grendell, *New York*
David R Gretch, *Seattle*
Stefano Guandalini, *Chicago*
Anna S Gukovskaya, *Los Angeles*
Sanjeev Gupta, *Bronx*
David J Hackam, *Pittsburgh*
Stephen B Hanauer, *Chicago*
Gavin Harewood, *Rochester*
Margaret M Heitkemper, *Washington*
Alan W Hemming, *Gainesville*
Samuel B Ho, *San Diego*
Peter R Holt, *New York*
Colin W Howden, *Chicago*
Hongjin Huang, *Alameda*
Jamal A Ibdah, *Columbia*
Atif Iqbal, *Omaha*
Hajime Isomoto, *Rochester*
Hartmut Jaeschke, *Tucson*
Dennis M Jensen, *Los Angeles*
Cheng Ji, *Los Angeles*
Leonard R Johnson, *Memphis*
Michael P Jones, *Chicago*
Peter J Kahrilas, *Chicago*
Anthony N Kalloo, *Baltimore*
Marshall M Kaplan, *Boston*
Neil Kaplowitz, *Los Angeles*
Serhan Karvar, *Los Angeles*
Rashmi Kaul, *Tulsa*
Jonathan D Kaunitz, *Los Angeles*
Ali Keshavarzian, *Chicago*
Miran Kim, *Providence*
Joseph B Kirsner, *Chicago*
Leonidas G Koniaris, *Miami*
Burton I Korelitz, *New York*
Robert J Korst, *New York*
Richard A Kozarek, *Seattle*
Alyssa M Krasinskas, *Pittsburgh*
Michael Kremer, *Chapel Hill*
Shiu-Ming Kuo, *Buffalo*
Paul Y Kwo, *Indianapolis*
Daryl Tan Yeung Lau, *Galvesto*
Stephen J Lanspa, *Omaha*
Joel E Lavine, *San Diego*
Bret Lashner, *Cleveland*
Dirk J van Leeuwen, *Lebanon*
Glen A Lehman, *Indianapolis*
Alex B Lentsch, *Cincinnati*
Andreas Leodolter, *La Jolla*
Gene LeSage, *Houston*
Josh Levitsky, *Chicago*
Cynthia Levy, *Gainesville*
Ming Li, *New Orleans*
Zhiping Li, *Baltimore*
Zhe-Xiong Lian, *Davis*
Lenard M Lichtenberger, *Houston*
Gary R Lichtenstein, *Philadelphia*
Otto Schiueh-Tzang Lin, *Seattle*
Martin Lipkin, *New York*
Chen Liu, *Gainesville*
Edward V Loftus, *Rocheste*
Robin G Lorenz, *Birmingham*
Michael R Lucey, *Madison*
James D Luketich, *Pittsburgh*
Guangbin Luo, *Cheveland*
Henry T Lynch, *Omaha*
Patrick M Lynch, *Houston*
John S Macdonald, *New York*
Bruce V MacFadyen, *Augusta*
Willis C Maddrey, *Dallas*
Ashok Malani, *Los Angeles*
Mercedes Susan Mandell, *Aurora*
Peter J Mannon, *Bethesda*
Charles M Mansbach, *Tennessee*
John F Di Mari, *Texas*
John M Mariadason, *Bronx*
Jorge A Marrero, *Ann Arbor*
Paul Martin, *New York*
Paulo Ney Aguiar Martins, *Boston*
Wendy M Mars, *Pittsburgh*
Laura E Matarese, *Pittsburgh*
Richard W McCallum, *Kansas*
Beth A McCormick, *Charlestown*
Lynne V McFarland, *Washington*
Kevin McGrath, *Pittsburgh*
Harihara Mehendale, *Monroe*
Ali Mencin, *New York*
Fanyin Meng, *Ohio*
Stephan Menne, *New York*
Didier Merlin, *Atlanta*
Howard Mertz, *Nashville*
George W Meyer, *Sacramento*
George Michalopoulos, *Pittsburgh*
James M Millis, *Chicago*
Fabrizio Michelassi, *New York*
Albert D Min, *New York*
Pramod K Mistry, *New Haven*
Emiko Mizoguchi, *Boston*
Smruti R Mohanty, *Chicago*
Satdarshan S Monga, *Pittsburgh*
Timothy H Moran, *Baltimore*
Peter L Moses, *Burlington*
Steven F Moss, *Providence*
Andrew J Muir, *Durham*
Milton G Mutchnick, *Detroit*
Masaki Nagaya, *Boston*
Victor Navarro, *Philadelphia*
Laura E Nagy, *Cleveland*
Hiroshi Nakagawa, *Philadelphia*
Douglas B Nelson, *Minneapolis*
Justin H Nguyen, *Florida*
Patrick G Northup, *Charlottesville*
Christopher O'Brien, *Miami*
Robert D Odze, *Boston*
Brant K Oelschlager, *Washington*
Curtis T Okamoto, *Los Angeles*
Stephen JD O'Keefe, *Pittsburgh*
Dimitry Oleynikov, *Omaha*
Stephen J Pandol, *Los Angeles*
Georgios Papachristou, *Pittsburgh*
Pankaj J Pasricha, *Galveston*
Zhiheng Pei, *New York*
Michael A Pezzone, *Pittsburgh*
CS Pitchumoni, *New Brunswiuc*
Paul J Pockros, *La Jolla*
Jay Pravda, *Gainesville*
Massimo Raimondo, *Jacksonville*
GS Raju, *Galveston*
Raymund R Razonable, *Minnesota*
Murray B Resnick, *Providence*
Adrian Reuben, *Charleston*
Douglas K Rex, *Indianapolis*
Victor E Reyes, *Galveston*
Basil Rigas, *New York*
Yehuda Ringel, *Chapel Hill*
Richard A Rippe, *Chapel Hill*
Maribel Rodriguez-Torres, *Santurce*
Marcos Rojkind, *Washington*
Philip Rosenthal, *San Francisco*
Barry Rosser, *Jacksonville Florida*
Hemant K Roy, *Evanston*
Sammy Saab, *Los Angeles*
Shawn D Safford, *Norfolk*
Dushyant V Sahani, *Boston*
Bruce E Sands, *Boston*
James M Scheiman, *Ann Arbor*
Eugene R Schiff, *Miami*
Nicholas J Shaheen, *Chapel Hill*
Vanessa M Shami, *Charlottesville*
Prateek Sharma, *Kansas City*
Harvey L Sharp, *Minneapolis*
Stuart Sherman, *Indianapolis*
Shivendra Shukla, *Columbia*
Alphonse E Sirica, *Virginia*
Shanthi V Sitaraman, *Atlanta*
Stuart J Spechler, *Dallas*
Shanthi Srinivasan, *Atlanta*
Michael Steer, *Boston*
Peter D Stevens, *New York*
Charmaine A Stewart, *Rochester*
Christian D Stone, *Saint Louis*
Gary D Stoner, *Columbus*
R Todd Stravitz, *Richmond*
Liping Su, *Chicago*
Christina Surawicz, *Seattle*
Robert W Summers, *Iowa City*
Wing-Kin Syn, *Durham*
Gyongyi Szabo, *Worcester*
Yvette Taché, *Los Angeles*
Seng-Lai Tan, *Seattle*
Andrzej S Tarnawski, *Orange*
K-M Tchou-Wong, *New York*
Jonathan P Terdiman, *San Francisco*
Neil D Theise, *New York*
Christopher C Thompson, *Boston*
Swan N Thung, *New York*
Michael Torbenson, *Baltimore*
Natalie J Torok, *Sacramento*
RA Travagli, *Baton Rouge*
George Triadafilopoulos, *Stanford*
Chung-Jyi Tsai, *Lexington*
Janet Elizabeth Tuttle-Newhall, *Durham*
Andrew Ukleja, *Florida*
Michael F Vaezi, *Nashville*
Hugo E Vargas, *Scottsdale*
Arnold Wald, *Wisconsin*
Scott A Waldman, *Philadelphia*
Jian-Ying Wang, *Baltimore*
Timothy C Wang, *New York*
Irving Waxman, *Chicago*
Steven A Weinman, *Galveston*
Steven D Wexner, *Weston*
Keith T Wilson, *Baltimore*
Jacqueline L Wolf, *Boston*
Jackie Wood, *Ohio*
George Y Wu, *Farmington*
Jian Wu, *Sacramento*
Samuel Wyllie, *Houston*
Wen Xie, *Pittsburgh*
Vijay Yajnik, *Boston*
Vincent W Yang, *Atlanta*
Francis Y Yao, *San Francisco*
Hal F Yee, *San Francisco*
Xiao-Ming Yin, *Pittsburgh*
Min You, *Tampa*
Zobair M Younossi, *Virginia*
Liqing Yu, *Winston-Salem*
David Yule, *Rochester*
Ruben Zamora, *Pittsburgh*
Michael E Zenilman, *New York*
Zhi Zhong, *Chapel Hill*
Michael A Zimmerman, *Colorado*
Stephen D Zucker, *Cincinnati*

Uruguay

Henry Cohen, *Montevideo*

[1]Passed away on October 20, 2007
[2]Passed away on June 11, 2007

National Journal Award
2005

World Journal of Gastroenterology®

Weekly Established in October 1995

Volume 14 Number 33
September 7, 2008

Contents

Contents

World Journal of Gastroenterology
Volume 14 **Number** 33 **September** 7, 2008

World Journal of Gastroenterology
Volume 14 **Number** 33 **September** 7, 2008

Contents

NAME OF JOURNAL
World Journal of Gastroenterology

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

EDITING
Editorial Board of *World Journal of Gastroenterology,* Room 903, Ocean International Center, Building D, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

PUBLISHING
The WJG Press and Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Ocean International Center, Building D, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

PRINTING
Beijing Kexin Printing House

OVERSEAS DISTRIBUTOR
Beijing Bureau for Distribution of Newspapers and Journals (Code No. 82-261)
China International Book Trading Corporation PO Box 399, Beijing, China (Code No. M4481)

PUBLICATION DATE
September 7, 2008

EDITOR-IN-CHIEF
Lian-Sheng Ma, *Beijing*

SUBSCRIPTION
RMB 50 Yuan for each issue, RMB 2400 Yuan for one year

CSSN
ISSN 1007-9327
CN 14-1219/R

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5105

World J Gastroenterol 2008 September 7; 14(33): 5105-5109
World Journal of Gastroenterology ISSN 1007-9327

REVIEW

Recurrence of cholestatic liver disease after living donor liver transplantation

Sumihito Tamura, Yasuhiko Sugawara, Junichi Kaneko, Junichi Togashi, Yuichi Matsui, Noriyo Yamashiki, Norihiro Kokudo, Masatoshi Makuuchi

Sumihito Tamura, Yasuhiko Sugawara, Junichi Kaneko, Junichi Togashi, Yuichi Matsui, Noriyo Yamashiki, Norihiro Kokudo, Artificial Organ and Transplantation Division, Department of Surgery, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
Masatoshi Makuuchi, Japanese Red Cross Medical Center, 4-1-22 Hiroo, Shibuya-ku, Tokyo 150-8935, Japan
Author contributions: Tamura S and Sugawara Y designed the study and wrote the paper; Kaneko J, Togashi J, Matsui Y, Yamashiki N and Kokudo N performed the study; Makuuchi M supervised the project.
Supported by A Grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and Grants-in-aid for Research on HIV/AIDS and Research on Measures for Intractable Diseases from the Ministry of Health, Labor and Welfare of Japan, No. B18390341
Correspondence to: Yasuhiko Sugawara, MD, Artificial Organ and Transplantation Division, Department of Surgery, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. yasusuga-tky@umin.ac.jp
Telephone: +81-3-38155411 Fax: +81-3-56843989
Received: January 30, 2008 Revised: July 25, 2008
Accepted: August 1, 2008
Published online: September 7, 2008

Abstract

End-stage liver disease, due to cholestatic liver diseases with an autoimmune background such as primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC), is considered a good indication for liver transplantation. Excellent overall patient and graft outcomes, based mostly on the experience from deceased donor liver transplantation (DDLT), have been reported. Due to the limited number of organ donations from deceased donors in most Asian countries, living donor liver transplantation (LDLT) is the mainstream treatment for end-stage liver disease, including that resulting from PBC and PSC. Although the initial experiences with LDLT for PBC and PSC seem satisfactory or comparable to that with DDLT, some aspects, including the timing of transplantation, the risk of recurrent disease, and its long-term clinical implications, require further evaluation. Whether or not the long-term outcomes of LDLT from a biologically related donor are equivalent to that of DDLT requires further observations. The clinical course following LDLT may be affected by the genetic background shared between the recipient and the living related donor.

Key words: Liver transplantation; Primary biliary cirrhosis; Primary sclerosing cholangitis; Living donor; Recurrence

Peer reviewers: Jaime Guardia, Professor, Internal Medicine and Liver Unit, Hospital Universitari 'Vall d'Hebron'. Universitat Autonoma de Barcelona, Hospital Universitari Vall d'Hebron, Pg Vall d'Hebron, 119, Barcelona 08035, Spain; Tadatoshi Takayama, Professor, Department of Digestive Surgery, Nihon University School of Medicine, 30-1 Oyaguchikami-machi, Itabashi-ku, Tokyo 173-8610, Japan

Tamura S, Sugawara Y, Kaneko J, Togashi J, Matsui Y, Yamashiki N, Kokudo N, Makuuchi M. Recurrence of cholestatic liver disease after living donor liver transplantation. *World J Gastroenterol* 2008; 14(33): 5105-5109 Available from: URL: http://www.wjgnet.com/1007-9327/14/5105.asp DOI: http://dx.doi.org/10.3748/wjg.14.5105

INTRODUCTION

Primary biliary cirrhosis (PBC) is characterized by the destruction of interlobular and septal bile ducts, leading to cholestasis and fibrosis[1]. Primary sclerosing cholangitis (PSC) is also a chronic cholestatic liver disease, characterized by inflammatory and fibrotic bile duct lesions forming multiple strictures and dilatations of the intra- and extrahepatic bile ducts[2-4].

Disordered immune regulation is considered to have a role in both PBC and PSC, though the specific immunologic mechanisms are yet to be clarified despite recent advancements[2,5]. In both PBC and PSC, there is a gradual progression of cholestasis, which results in end-stage liver disease (ESLD). In PBC, the administration of ursodeoxycholic acid (UDCA) starting at an early stage of the disease improves the prognosis and is, therefore, recommended[6,7]. On the other hand, effective medical treatment for PSC remains a matter of debate. UDCA administration, with or without the use of immunosuppressive agents, seems to have

a beneficial effect on liver function tests, assessed by blood chemistries. But, it is not clear whether there is a beneficial effect on delaying the progression of the disease and improving the overall survival rate[8].

Liver transplantation is the optimal treatment for patients with PBC or PSC presenting with ESLD and associated complications, such as severe manifestations of portal hypertension[5,8]. The short-term mortality rate, due to disease recurrence following transplantation, is lower in patients with PBC or PSC than in those with hepatitis C or hepatic malignancy. Longer follow-up, however, has revealed that both PBC and PSC can recur. But, the impact of a recurrence on long-term patient survival seems to be insignificant in deceased donor liver transplantation (DDLT)[9].

Living donor liver transplantation (LDLT) has been accepted with greater enthusiasm in Asia, especially in the Far East where organs from deceased donors remain extremely scarce. Technical innovations driven by necessity have enabled the development of LDLT and extended its indications to adult populations[10,11]. In November, 1993, the first successful LDLT for an adult recipient was performed at Shinshyu University by Makuuchi and colleagues[12]. A 53-year-old woman with end-stage cholestatic liver disease due to PBC received a left liver graft from her son. The patient remains the longest survivor of this epoch-making success.

In LDLT, there is no waiting time. There is no competition between recipients for the availability of organs, unlike in DDLT. Liver transplantation becomes an option as soon as a socially-acceptable and medically-suitable dedicated living donor becomes available[13]. Although a transplant with anatomically challenging features and smaller graft size may pose some difficulties, LDLT has some clear advantages when compared with DDLT. LDLT may be performed in a planned manner after thorough preparation at an earlier stage of the liver disease. In addition to the recipients' surgical risk, potential risks to the living donor cannot be overlooked. Transplantation should be considered when there is a clear survival benefit; that is, when the estimated risk of mortality for a patient on medical treatment alone exceeds that of the expected peri-operative mortality. We consider that LDLT becomes a beneficial option for patients with ESLD including that from PBC or PSC, who have a Model for End-stage Liver Disease (MELD) score of 15-17 (a calculated 3-mo mortality rate of approximately 5%), balancing the risk and benefit to the recipient and prospective living donor[14]. LDLT for patients with co-existing hepatocellular carcinoma may be considered at an earlier stage.

PBC

Whether the combined symptoms of intractable pruritus, lethargy, or osteoporosis encountered in PBC should be considered an indication for LDLT in patients without significant signs of portal hypertension or liver failure remains an open question. At our institution, the indication of LDLT for PBC is based on the potential for improvement or benefits in life expectancy. Unlike in DDLT[15,16], less importance may be placed on quality of life in LDLT. We have considered that quality of life issues in the recipient do not outweigh even the slightest possibility of donor mortality or morbidity that may result in disability. In addition to the above-mentioned MELD score, the results obtained from the Mayo model are taken into account[17]. Although the model was developed prior to the MELD era, and is not considered a factor for organ allocation in DDLT, we find that the Mayo model is very useful in the LDLT setting. As reported by Kim and colleagues[18,19], there is a window of opportunity appropriate for LDLT, expressed as risk scores of 6 to 7.8. Risk scores higher than 10 are related to longer hospitalization after LDLT and mortality[20,21]. When the risk of death by the natural disease course is predicted to outweigh the surgical risks of liver transplantation, LDLT may be planned and performed.

The 5-year survival rate after DDLT for PBC is approximately 80%[18,22]. The anti-mitochondrial antibody (AMA) status does not change after liver transplantation and, therefore, is not diagnostic of recurrent disease after transplantation[5]. PBC recurrence is currently defined according to the agreed criteria, including patient transplanted for PBC, persistence of AMA, and compatible liver biopsy[23]. The largest single-center series with a mean follow-up period of 56 mo indicated a rate of such confirmed recurrence to be 17% at a mean follow-up period of 36 mo after transplantation. Interestingly, although histologically recognizable recurrence of the disease may be common, its effect on survival seems insignificant in DDLT[22]. Liermann and colleagues[15,22] demonstrated that patients with recurrent disease tended to be younger and receive organs from younger donors, and to have a longer warm ischemia time. But, such differences were considered to be clinically insignificant. The effect of a human leukocyte antigen (HLA) match between the donor and recipient remains unknown.

PBC is reported to recur after LDLT. Hashimoto and colleagues presented the first series of recurrent PBC after LDLT[24]. All patients remained positive for AMA as in DDLT. The presence of mixed portal inflammatory infiltrates with granulomatous cholangitis was considered a definite histologic finding suggestive of recurrence. In 2 of 6 (33%) patients, such findings were established by protocol biopsy 1 to 2 years after LDLT. In the study, immunosuppression consisted of tacrolimus and steroids. Mycophenolate mofetil was not used. UDCA (600 mg/d) was administered to all patients.

The number of patients in Hashimoto's study[24] is too small to draw a definitive conclusion. The high rate of recurrence (33% in 2 years) is in sharp contrast with that in an earlier DDLT series. As recently reviewed by Neuberger, early reports in DDLT in which the recurrence of PBC was evaluated in a smaller patient population and a shorter observation period indicated a tendency to underestimate the risk of disease recurrence, a finding that was later reversed by a larger series with a longer follow-up period[15]. Compared to DDLT,

the history and volume of cases of LDLT for PBC is extremely limited. The rate of histologically confirmed recurrence at this very early stage is, therefore, somewhat disturbing.

Two single-center studies, both with 50 patients, were recently presented[21,25]. Hasegawa and colleagues[21] presented 3 and 5-year overall survival rates of 88% and 80%, respectively, with a median follow-up period of 35 mo. Before transplantation, AMA was positive in 42 patients (84%). AMA remained positive in 33 patients (66%) at 6 mo after transplantation. Multivariate analysis indicated that a lower updated Mayo risk score was a significant favorable factor for shorter hospitalization following LDLT, confirming the usefulness of the model in LDLT, as in DDLT[21]. In the study, periodic liver biopsies were not performed, and occasional biopsy in cases of abnormal liver function did not present with findings suggestive of recurrent PBC. Biopsy-proven acute cellular rejection was observed in 18 (36%) patients. Because histologic findings are indispensable for the diagnosis of recurrent PBC, the study provides little information on disease recurrence, though the excellent midterm patient survival warrants further application of LDLT for PBC.

On the other hand, Morioka and colleagues[25] presented 5-year overall survival rates of 67%. The recurrence of PBC was confirmed in 18% of patients within a median of 36 mo after LDLT (range 12-123 mo). The results of the study suggested that a lower number of HLA mismatches between donor and recipient, and a younger donor age resulted in better survival, though a lower number of HLA mismatches were also suggested to be a risk factor for PBC recurrence.

The study by Morioka and colleagues[25] included ABO incompatible cases, which were not included in the study by Hasegawa and colleagues[21], and recipients presented with higher MELD scores. The median MELD score in Hasegawa's study[21] was 13, whereas that in Morioka's series[25] was 23. Of the 50 patients, 14 (28%) died within 6 mo after LDLT in Morioka's series[25], whereas 3 (6%) died within 6 mo after LDLT in Hasegawa study[21]. It is not clear how the lower number of HLA mismatches affects short-term (6 mo) survival in the latter study. Recurrent PBC seems to be of less importance during this period as it was described to occur after 12 postoperative months. In fact, data more recently presented indicated that a simple comparison of HLA matching has little or no impact on survival[26]. Clearly, the impact of HLA mismatches on PBC recurrence in LDLT requires further study.

The most recent registry study from Japan, in which 221 PBC patients were analyzed, reports a 5-year survival rate of 79%. Histologic evaluation was available for 70 patients, among whom 7 presented with findings compatible with recurrent disease with a median follow-up period of 36 mo[27]. The information is limited, however, and it is, therefore, difficult to draw a universally acceptable conclusion on the overall long-term outcome of LDLT for PBC.

PSC

As for PBC, the indication for LDLT for PSC at our institution is based on the improved life expectancy. Although MELD score is considered more appropriate for DDLT, the new Mayo Model[28] may be helpful for deciding the optimal timing of LDLT and preparation once a living donor candidate becomes available. Development of the model was based upon a large cohort of patients followed for two decades. Histologic evaluation by liver biopsy is not required and the score is easily obtained from readily available clinical variables. Its validity among the Far East Asian population, however, requires further analysis. At our institution, we currently consider a MELD score > 15 and/or a Mayo risk score > 2.0 as good starting points to prepare for LDLT.

PSC is considered to be a good indication for DDLT with an excellent 5-year graft survival rate of approximately 80%. A higher rate of re-transplantation compared to that for other indications (9.6% *vs* 4.9% within 2 years), however, has been recognized. But, its relation with recurrent disease is unclear. Recurrence of PSC after DDLT has been reported at rates between 1% to 33%, depending on the diagnostic criteria and follow-up duration[8]. Graziadei and colleagues[29] proposed combined cholangiographic and hepatic histologic criteria with strict exclusion criteria (cases with ABO incompatibility between donor and recipient, nonanastomotic strictures before posttransplantation day 90, anastomotic strictures alone, hepatic artery complications, or ductopenic rejection were excluded) and reported that PSC recurs after DDLT in 20% with typical radiologic manifestations found within a year, and histologic presentation within 3 years after DDLT. In their study, 5-year patient and graft survival rates were comparable between patients with recurrent PSC and patients with PSC without signs of recurrence.

A case suspected of recurrent PSC following LDLT was first reported by Kita *et al*[30]. There have been sporadic case reports from Asian regions[31-34], as well as from the West[35-39], of LDLT for PSC from living related donors since then, but none reported recurrence; follow-up periods were less than 2 years in most case reports. Aside from the above case reports, cases of LDLT for PSC are found in moderate to large registries worldwide[40-44]. Moon *et al*[40] reported 2 cases of LDLT for PSC among their large series of 580 LDLTs. Soejima *et al*[41] also reported 2 cases of LDLT for PSC in their series of 52 LDLTs. Both, however, lacked a specific description of the long-term outcome. The largest registry analysis by Maheshwari *et al*[44] reported the outcome of 3309 PSC patients who underwent liver transplantation, among whom 69 underwent LDLT. Only 10 of those presented with a follow-up period longer than a year, however, and details regarding the outcome were not provided.

Thus, there is a significant lack of information specifically on the long-term outcome of LDLT for PSC, especially regarding recurrence. One explanation may be the rare incidence of PSC in the Far East

Table 1 Recurrence of PSC following LDLT

Series	No.	Rate of blood related donors	No. rec. PSC	Median period to dx. of rec.
Tokyo[47]	9	89% (5 out of 9)	4 (44%)	40 mo (range 14-66 mo)[2]
Kyoto[48]	22	82% (23 out of 28[1])	13 (59%)	31 mo (range 22-71 mo)[3]

No.: Number of patients; rec.: Recurrence; dx.: Diagnosis. [1]Twenty-eight patients underwent LDLT for PSC. Among them 22, (79%) survived for more than a year and were the subject of the analysis for recurrent PSC; [2]Diagnosis based on radiologic findings. No protocol liver biopsies were performed; [3]Diagnosis based on pathologic findings obtained from protocol liver biopsies and later confirmed by radiologic findings.

where LDLT is far more common than DDLT. Unlike in Nordic countries or in the United States, where PSC is one of the most important indications for liver transplantation[45], this indication accounts for less than 3% of the total liver transplantations performed in Japan[46].

Recently, two additional studies on LDLT for PSC were published from high volume centers in the region[47,48]. The Tokyo group described the outcome of 9 adult LDLT cases, of which 8 were living-related. The median follow-up period was 3.5 years after LDLT. No ABO blood type-incompatible cases were performed. The outcome in terms of patient survival was satisfactory with a 5-year rate of 90%. When recurrence of PSC was evaluated according to Graziadei's criteria[29], however, recurrent PSC was diagnosed in four patients. Rates of freedom from recurrent PSC at 1, 3, and 5 years were 100%, 73%, and 49%, respectively. The mean time to recurrence was 3.3 years. When limited to biologically related donor-recipient cases, recurrent PSC was diagnosed in 50% of cases. Interestingly, none of the patients presented with the HLA haplotypes associated with a higher susceptibility for developing PSC in the Caucasian population[47].

The Kyoto group reported 28 patients with PSC who underwent LDLT. Among the 22 patients who survived for more than a year, 13 (59%) presented with PSC recurrence with a mean follow-up period of 31 mo, 5 of whom died or required re-transplantation for graft failure. The HLA haplotypes that may affect recurrence of the hepatic condition remain unclear, although HLA-DR15 is positively associated with ulcerative colitis. The group concluded that unlike PBC, the recurrence of PSC adversely affects the outcome in LDLT[48]. Although there is a difference in the short-term mortality between the two reports, the high rate of recurrence reported in both studies requires further attention. Key features of the two series are summarized in Table 1. The clinical manifestations of recurrence described in both series seem more aggressive than those in patients that underwent LDLT for in PBC. The risk factors and susceptible genetic characteristics in LDLT remain unclear. Further prospective study with a protocol cholangiogram and genetic considerations with an HLA haplotype analysis is necessary.

CONCLUSION

LDLT provides a satisfactory short-to-midterm outcome for PBC and PSC. Both PBC and PSC, however, can recur after LDLT. The incidence of recurrence appears to be higher in LDLT compared to DDLT. The long-term impact of recurrence on survival, however, remains unknown at this point. PSC may present with a poorer long-term outcome, but further studies are necessary.

REFERENCES

1 **Kaplan MM**. Primary biliary cirrhosis. *N Engl J Med* 1996; **335**: 1570-1580
2 **Angulo P**, Lindor KD. Primary sclerosing cholangitis. *Hepatology* 1999; **30**: 325-332
3 **Lee YM**, Kaplan MM. Primary sclerosing cholangitis. *N Engl J Med* 1995; **332**: 924-933
4 **Portincasa P**, Vacca M, Moschetta A, Petruzzelli M, Palasciano G, van Erpecum KJ, van Berge-Henegouwen GP. Primary sclerosing cholangitis: updates in diagnosis and therapy. *World J Gastroenterol* 2005; **11**: 7-16
5 **Kaplan MM**, Gershwin ME. Primary biliary cirrhosis. *N Engl J Med* 2005; **353**: 1261-1273
6 **Lindor K**. Ursodeoxycholic acid for the treatment of primary biliary cirrhosis. *N Engl J Med* 2007; **357**: 1524-1529
7 **Corpechot C**, Carrat F, Bahr A, Chretien Y, Poupon RE, Poupon R. The effect of ursodeoxycholic acid therapy on the natural course of primary biliary cirrhosis. *Gastroenterology* 2005; **128**: 297-303
8 **LaRusso NF**, Shneider BL, Black D, Gores GJ, James SP, Doo E, Hoofnagle JH. Primary sclerosing cholangitis: summary of a workshop. *Hepatology* 2006; **44**: 746-764
9 **Faust TW**. Recurrent primary biliary cirrhosis, primary sclerosing cholangitis, and autoimmune hepatitis after transplantation. *Liver Transpl* 2001; **7**: S99-S108
10 **Sugawara Y**, Makuuchi M. Technical advances in living-related liver transplantation. *J Hepatobiliary Pancreat Surg* 1999; **6**: 245-253
11 **Imamura H**, Makuuchi M, Sakamoto Y, Sugawara Y, Sano K, Nakayama A, Kawasaki S, Takayama T. Anatomical keys and pitfalls in living donor liver transplantation. *J Hepatobiliary Pancreat Surg* 2000; **7**: 380-394
12 **Hashikura Y**, Makuuchi M, Kawasaki S, Matsunami H, Ikegami T, Nakazawa Y, Kiyosawa K, Ichida T. Successful living-related partial liver transplantation to an adult patient. *Lancet* 1994; **343**: 1233-1234
13 **Yamashiki N**, Sugawara Y, Tamura S, Kaneko J, Nojiri K, Omata M, Makuuchi M. Selection of liver-transplant candidates for adult-to-adult living donor liver transplantation as the only surgical option for end-stage liver disease. *Liver Transpl* 2006; **12**: 1077-1083
14 **Merion RM**, Schaubel DE, Dykstra DM, Freeman RB, Port FK, Wolfe RA. The survival benefit of liver transplantation. *Am J Transplant* 2005; **5**: 307-313
15 **Neuberger J**. Liver transplantation for primary biliary cirrhosis: indications and risk of recurrence. *J Hepatol* 2003; **39**: 142-148
16 **Navasa M**, Forns X, Sanchez V, Andreu H, Marcos V, Borras JM, Rimola A, Grande L, Garcia-Valdecasas JC, Granados A, Rodes J. Quality of life, major medical complications and hospital service utilization in patients with primary biliary cirrhosis after liver transplantation. *J Hepatol* 1996; **25**: 129-134
17 **Murtaugh PA**, Dickson ER, Van Dam GM, Malinchoc M, Grambsch PM, Langworthy AL, Gips CH. Primary biliary cirrhosis: prediction of short-term survival based on repeated patient visits. *Hepatology* 1994; **20**: 126-134

18 **Kim WR**, Wiesner RH, Therneau TM, Poterucha JJ, Porayko MK, Evans RW, Klintmalm GB, Crippin JS, Krom RA, Dickson ER. Optimal timing of liver transplantation for primary biliary cirrhosis. *Hepatology* 1998; **28**: 33-38

19 **Kim WR**, Wiesner RH, Poterucha JJ, Therneau TM, Benson JT, Krom RA, Dickson ER. Adaptation of the Mayo primary biliary cirrhosis natural history model for application in liver transplant candidates. *Liver Transpl* 2000; **6**: 489-494

20 **Sugawara Y**, Makuuchi M, Takayama T, Imamura H. Living-related liver transplantation for primary biliary cirrhosis. *Transplantation* 2001; **72**: 1087-1091

21 **Hasegawa K**, Sugawara Y, Imamura H, Ikeda M, Kokudo N, Makuuchi M. Living donor liver transplantation for primary biliary cirrhosis: retrospective analysis of 50 patients in a single center. *Transpl Int* 2005; **18**: 794-799

22 **Liermann Garcia RF**, Evangelista Garcia C, McMaster P, Neuberger J. Transplantation for primary biliary cirrhosis: retrospective analysis of 400 patients in a single center. *Hepatology* 2001; **33**: 22-27

23 **Hubscher SG**, Elias E, Buckels JA, Mayer AD, McMaster P, Neuberger JM. Primary biliary cirrhosis. Histological evidence of disease recurrence after liver transplantation. *J Hepatol* 1993; **18**: 173-184

24 **Hashimoto E**, Shimada M, Noguchi S, Taniai M, Tokushige K, Hayashi N, Takasaki K, Fuchinoue S, Ludwig J. Disease recurrence after living liver transplantation for primary biliary cirrhosis: a clinical and histological follow-up study. *Liver Transpl* 2001; **7**: 588-595

25 **Morioka D**, Egawa H, Kasahara M, Jo T, Sakamoto S, Ogura Y, Haga H, Takada Y, Shimada H, Tanaka K. Impact of human leukocyte antigen mismatching on outcomes of living donor liver transplantation for primary biliary cirrhosis. *Liver Transpl* 2007; **13**: 80-90

26 **Hashimoto T**, Sugawara Y, Makuuchi M. Impact of human leukocyte antigen mismatching on outcomes of living donor liver transplantation for primary biliary cirrhosis. *Liver Transpl* 2007; **13**: 938-939

27 **Yamagiwa S**, Ichida T. Recurrence of primary biliary cirrhosis and primary sclerosing cholangitis after liver transplantation in Japan. *Hepatol Res* 2007; **37** Suppl 3: S449-S454

28 **Kim WR**, Poterucha JJ, Wiesner RH, LaRusso NF, Lindor KD, Petz J, Therneau TM, Malinchoc M, Dickson ER. The relative role of the Child-Pugh classification and the Mayo natural history model in the assessment of survival in patients with primary sclerosing cholangitis. *Hepatology* 1999; **29**: 1643-1648

29 **Graziadei IW**, Wiesner RH, Batts KP, Marotta PJ, LaRusso NF, Porayko MK, Hay JE, Gores GJ, Charlton MR, Ludwig J, Poterucha JJ, Steers JL, Krom RA. Recurrence of primary sclerosing cholangitis following liver transplantation. *Hepatology* 1999; **29**: 1050-1056

30 **Kita Y**, Lake JR, Ferrell LD, Mori M, Roberts JP, Kakizoe S, Kiyosawa K, Tanaka E, Shiga J, Takikawa H, Inoue Y, Ohtake T, Ohtomo K, Yotsuyanagi H, Oka T, Harihara Y, Takayama T, Kubota K, Kawarasaki H, Hashikura Y, Kawasaki S, Ascher NL, Makuuchi M. Possible recurrence of primary sclerosing cholangitis following living-related liver transplantation: report of a case. *Transplant Proc* 1998; **30**: 3321-3323

31 **Oya H**, Sato Y, Yamamoto S, Takeishi T, Kobayashi T, Hatakeyama K. Living related donor liver transplantation for primary sclerosing cholangitis with hepatocellular carcinoma and Crohn's disease: a case report. *Transplant Proc* 2004; **36**: 2297-2298

32 **Kaibori M**, Uemoto S, Fujita S, Inomata Y, Egawa H, Asonuma K, Kiuchi T, Hayashi M, Nakamura M, Tanaka K. Native hepatectomy after auxiliary partial orthotopic liver transplantation. *Transpl Int* 1999; **12**: 383-386

33 **Ikegami T**, Nishizaki T, Yanaga K, Kakizoe S, Nomoto K, Ohta R, Minagawa R, Sugimachi K. Living-related auxiliary partial orthotopic liver transplantation for primary sclerosing cholangitis--subsequent removal of the native liver. *Hepatogastroenterology* 1999; **46**: 2951-2954

34 **Nakamura M**, Fuchinoue S, Nakajima I, Kitajima K, Tojimbara T, Takasaki K, Shiraga H, Ito K, Tanaka K, Agishi T. Three cases of sequential liver-kidney transplantation from living-related donors. *Nephrol Dial Transplant* 2001; **16**: 166-168

35 **Varotti G**, Gondolesi GE, Roayaie S, Suriawinata A, Soltys K, Fishbein TM, Schwartz ME, Miller C. Combined adult-to-adult living donor right lobe liver transplantation and pancreatoduodenectomy for distal bile duct adenocarcinoma in a patient with primary sclerosing cholangitis. *J Am Coll Surg* 2003; **197**: 765-769

36 **Akyildiz M**, Karasu Z, Cagirgan S, Kilic M, Tokat Y. Rituximab therapy for life-threatining immune hemolytic anemia in a liver transplant recipient: a case report. *Transplant Proc* 2004; **36**: 1492-1493

37 **Wachs ME**, Bak TE, Karrer FM, Everson GT, Shrestha R, Trouillot TE, Mandell MS, Steinberg TG, Kam I. Adult living donor liver transplantation using a right hepatic lobe. *Transplantation* 1998; **66**: 1313-1316

38 **Jabbour N**, Gagandeep S, Mateo R, Sher L, Henderson R, Selby R, Genyk Y. Live donor liver transplantation: staging hepatectomy in a Jehovah's Witness recipient. *J Hepatobiliary Pancreat Surg* 2004; **11**: 211-214

39 **Heimbach JK**, Menon KV, Ishitani MB, Nyberg SL, Jankowski CJ, Lindor KD, Rosen CB. Living donor liver transplantation using a right lobe graft in an adult with situs inversus. *Liver Transpl* 2005; **11**: 111-113

40 **Moon DB**, Lee SG. Adult-to-adult living donor liver transplantation at the Asan Medical Center. *Yonsei Med J* 2004; **45**: 1162-1168

41 **Soejima Y**, Harada N, Shimada M, Suehiro T, Minagawa R, Hiroshige S, Ninomiya M, Shiotani S, Nishizaki T. Perioperative management and complications in donors related to living-donor liver transplantation. *Surgery* 2002; **131**: S195-S199

42 **Solano E**, Khakhar A, Bloch M, Quan D, McAlister V, Ghent C, Wall W, Marotta P. Liver transplantation for primary sclerosing cholangitis. *Transplant Proc* 2003; **35**: 2431-2434

43 **Hayashi PH**, Forman L, Steinberg T, Bak T, Wachs M, Kugelmas M, Everson GT, Kam I, Trotter JF. Model for End-Stage Liver Disease score does not predict patient or graft survival in living donor liver transplant recipients. *Liver Transpl* 2003; **9**: 737-740

44 **Maheshwari A**, Yoo HY, Thuluvath PJ. Long-term outcome of liver transplantation in patients with PSC: a comparative analysis with PBC. *Am J Gastroenterol* 2004; **99**: 538-542

45 **Bjoro K**, Friman S, Hockerstedt K, Kirkegaard P, Keiding S, Schrumpf E, Olausson M, Oksanen A, Isoniemi H, Hjortrup A, Bergan A, Ericzon BG. Liver transplantation in the Nordic countries, 1982-1998: changes of indications and improving results. *Scand J Gastroenterol* 1999; **34**: 714-722

46 **http://jlts.umin.ac.jp/Registry(2005).pdf**

47 **Tamura S**, Sugawara Y, Kaneko J, Matsui Y, Togashi J, Makuuchi M. Recurrence of primary sclerosing cholangitis after living donor liver transplantation. *Liver Int* 2007; **27**: 86-94

48 **Haga H**, Miyagawa-Hayashino A, Taira K, Morioka D, Egawa H, Takada Y, Manabe T, Uemoto S. Histological recurrence of autoimmune liver diseases after living-donor liver transplantation. *Hepatol Res* 2007; **37** Suppl 3: S463-S469

S- Editor Li DL **L- Editor** Rippe RA **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5110

World J Gastroenterol 2008 September 7; 14(33): 5110-5114
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Emiko Mizoguchi, MD, PhD, *Series Editor*

Role of STAT3 in inflammatory bowel disease

Ken Sugimoto

Ken Sugimoto, Experimental Pathology, Massachusetts General Hospital, Harvard Medical School, Boston MA 02114, United States
Author contributions: Sugimoto K contributed all to this paper.
Correspondence to: Ken Sugimoto, MD, Experimental Pathology, Massachusetts General Hospital, Harvard Medical School, Boston MA 02114,
United States. sugiken@jg8.so-net.ne.jp
Telephone: +1-617-6433652 Fax: +1-617-7262365
Received: April 29, 2008 Revised: June 23, 2008
Accepted: June 30, 2008
Published online: September 7, 2008

Abstract

Signal transducers and activators of transcription 3 (STAT3) play an important role in various autoimmune disorders including inflammatory bowel disease (IBD). Recent studies have revealed that STAT3 activation plays distinctly different roles between innate immune responses and acquired immune responses in colitis. STAT3-mediated activation of acquired immune responses plays a pathogenic role in colitis by enhancing the survival of pathogenic T cells. In contrast, STAT3-mediated activation of innate responses contributes to the suppression of colitis. This review will summarize the current understanding of the roles of STAT3 in IBD and the potential of targeting STAT3 for the treatment of IBD, emphasizing recent observations.

Key words: Inflammatory bowel disease; STAT3; Inflammation; Innate immunity; Acquired immunity

Peer reviewers: Atsushi Mizoguchi, Assistant Professor, Department of Experimental Pathology, Massachusetts General Hospital, Simches 8234, 185 Cambridge Street, Boston MA 02114, United States; Frank Hoentjen, PhD, Free University Medical Center, Sumatrastraat 16, Haarlem 2022XL, Netherlands; Beth A McCormick, PhD, Department of Pediatric Gastroenterology and Nutrition, Massachusetts General Hospital/Harvard Medical School, 114 16th Street (114-3503), Charlestown 02129, United States; Anthony J Bauer, Professor, PhD, University of Pittsburgh, Department of Medicine/Gastroenterology, S-849 Scaife Hall, 3550 Terrace Street, Pittsburgh 15261, United States

Sugimoto K. Role of STAT3 in inflammatory bowel disease. *World J Gastroenterol* 2008; 14(33): 5110-5114 Available from: URL: http://www.wjgnet.com/1007-9327/14/5110.asp DOI: http://dx.doi.org/10.3748/wjg.14.5110

INTRODUCTION

Inflammatory bowel disease (IBD) is a group of idiopathic chronic relapsing immune-mediated inflammatory disorders, which is characterized by two major forms of intestinal inflammation: ulcerative colitis (UC) and Crohn's disease (CD)[1]. Although the precise mechanisms of the pathogenesis of IBD still remain unclear, accumulating studies have shown that multiple cytokines, including interleukins (ILs) and interferons, are involved in the pathogenesis of IBD[2,3]. Most of these cytokines activate various members of a family of cytoplasmic transcription factors such as signal transducers and activators of transcription (STATs)[4,5]. Indeed, several recent studies have focused on the role of STATs on IBD. Among STATs, the role of STAT3 in IBD has been most well documented by recent studies obtained from human IBD studies[6-8] and experimental IBD models[9-12]. These studies have revealed that STAT3 is in a unique position for pathogenesis of IBD, because STAT3 plays distinct roles between innate immune cells and acquired immune cells. This review will focus on the roles of STAT3 in IBD from a different point of view, namely, innate immune systems *versus* acquired immune systems.

STAT3 AND IBD PATIENTS

STAT3 was initially identified as an acute phase response factor, an inducible DNA binding protein that binds to the IL-6 responsive element within the promoters of hepatic acute phase protein genes[13]. Subsequent studies indicate that STAT3 becomes activated in response to a wide variety of cytokines and growth factors[4,5,14]. After ligation of their specific receptors by these cytokines and growth factors, STAT3 is immediately phosphorylated, resulting in their dimerization and migration to the nucleus to induce several gene expressions encoding molecules that play a role in a variety of biological functions such as cell growth, anti- and pro-apoptosis, cell motility, negative feedback loop (suppression of cytokine productions), regulatory cytokine production and anti-bacterial activity depending on cell types[4,5,14-17].

Although the role of STAT3 activation in the pathogenesis of IBD is still not very clear, the expression and activation of STAT3 protein in the mucosa of human IBD patients has been well studied. Lovato *et al* demonstrated constitutive activation of STAT3 in five out of seven CD patients in a small case series[6]. Musso *et al* demonstrated that STAT3 activation was confined to actively inflamed colons from not only CD, but also UC patients[7]. Accordingly, activated STAT3 was detected in isolated lamina propria mononuclear cells from inflamed IBD tissues, but not in peripheral blood mononuclear cells from control subjects or IBD patients. In this study, immunofluorescence demonstrated that the sources of activated STAT3 were macrophages and T lymphocytes, but not neutrophils. Mudter *et al* also showed that an increased amount of total STAT3 protein appeared in both UC and CD compared to noninflammatory control cells, and total STAT3 correlated with increased activated pSTAT3 in tissue sections from both UC and CD[8]. They also showed that increased pSTAT3 also directly correlated with the histological degree of inflammation of tissues. Importantly, this activation is observed in both acquired cells such as T cells and innate cells such as colonic epithelial cells (CECs).

THE ROLE OF STAT3 ON ACQUIRED IMMUNE CELLS IN IBD

Since classical knockout of the *STAT3* gene in mice resulted in early embryonic lethality[18], various cell type-specific STAT3 knockout mice were generated to elucidate the functional relevance of STAT3[15,19-23]. Acquired immune cells, especially $CD4^+$ T cells, have a considerable ability to induce the development and exacerbation of not only CD-like, but also UC-like experimental colitis[24-27]. Importantly, Takeda *et al* showed that T cell specific STAT3 knockout mice showed impaired T cell proliferation[19]. Moreover, they showed that STAT3 activation is involved in IL-6-dependent T cell proliferation through prevention of apoptosis. Atreya *et al* also showed that activation of IL-6/STAT3 cascade in lamina propria T cells can also induce prolonged survival of pathogenic T cells and, indeed, inactivation of this cascade contributes to the attenuation of chronic intestinal inflammation[9]. These observations indicate that activation of STAT3 in the acquired cells plays a pathogenic role in chronic colitis by inducing prolonged survival of pathogenic T cells and disruption of immune tolerance. Indeed, inactivation of IL-6 or leptin/STAT3 cascades results in the suppression of acquired immune (T cell) mediated colitis[9-12].

THE ROLE OF STAT3 ON INNATE IMMUNE CELLS IN IBD

Dysregulated host/microbial interactions have been postulated to play a crucial role in the development of IBD by inducing uncontrolled activation of acquired immune responses[24,28-30]. Innate immune responses mediated by CECs and phagocytic cells such as macrophages play a crucial role in the initial defense to maintain the appropriate host/microbial interactions[15,22]. Accumulating studies have demonstrated that several mechanisms are involved in the maintenance and/or enhancement of the barrier function by CECs[15,31,32]. Among these mechanisms, activation of STAT3 is considered to be an important pathway that can enhance and maintain not only the barrier function of epithelial cells, but also the activation of macrophage[15,22]. Indeed, disruption of innate immune responses by genetically engineered either inactivation of STAT3 cascade in the CECs, or else macrophages induce intestinal inflammation[15,21-23]. This observation is further supported by a recent study using a conditional STAT3 knockout mouse strain in which STAT3 deletion is induced during the adult life[23]. This mouse strain shows efficient elimination of STAT3 protein in innate cells (macrophages and CECs), but not acquired cells (T and B cells), and develops a lethal colitis immediately after the deletion of STAT3 in the adult life. These findings indicate that, in contrast to acquired immune cells, activation of STAT3 in innate cells plays a regulatory role in colitis. Interestingly, inactivation of STAT3 in both CECs and macrophages results in lethal damage[18,23], whereas such fatal inflammation is not observed in mice in which the inactivation is specifically induced in either CECs or macrophages[15,22]. This raises a possibility that transient inactivation of STAT3 in both CEC and macrophage may cause lethal damage.

THE ROLE OF STAT3-ACTIVATORS ON IBD

Several cytokines and growth factors including IL-6, IL-7, IL-9, IL-10, IL-11, IL-15, IFN-α/β, leukemia inhibitory factor (LIF), hepatocyte growth factor (HGF), oncostatin M (OSM), leptin and growth hormone (GH) have been identified to activate STAT3 cascade with a different efficiency depending on the cell types[4,5,14]. Among these, roles of IL-6, IL-11, IL-15, HGF, GH and leptin in experimental colitis have been demon strated[9-12,33-36]. For example, IL-6, IL-15 and leptin play a pathogenic role in colitis by activating STAT3 predominantly in the acquired immune cells[9,10,12,36,37] (Figure 1).

In contrast, GH and IL-11, both of which activate STAT3 predominantly in CECs, contribute to the suppression of experimental colitis[33-35]. Of note, human trials utilizing GH, IL-11 and anti-IL-6R mAb have been conducted and have shown that the strategies using these products are beneficial to IBD patients[38-40] (Figure 1).

IL-22 has recently been shown to be preferentially expressed by the Th17 subset[41,42] and has been demonstrated as a strong activator of STAT3[43-45]. Recently, we have demonstrated that IL-22 contributes to rapid amelioration of local inflammation associated with a Th2-mediated colitis through activation of STAT3 in CECs[46] (Figure 1). Since IL-22 specifically targets innate immune pathways[16,45-47], selective activation of

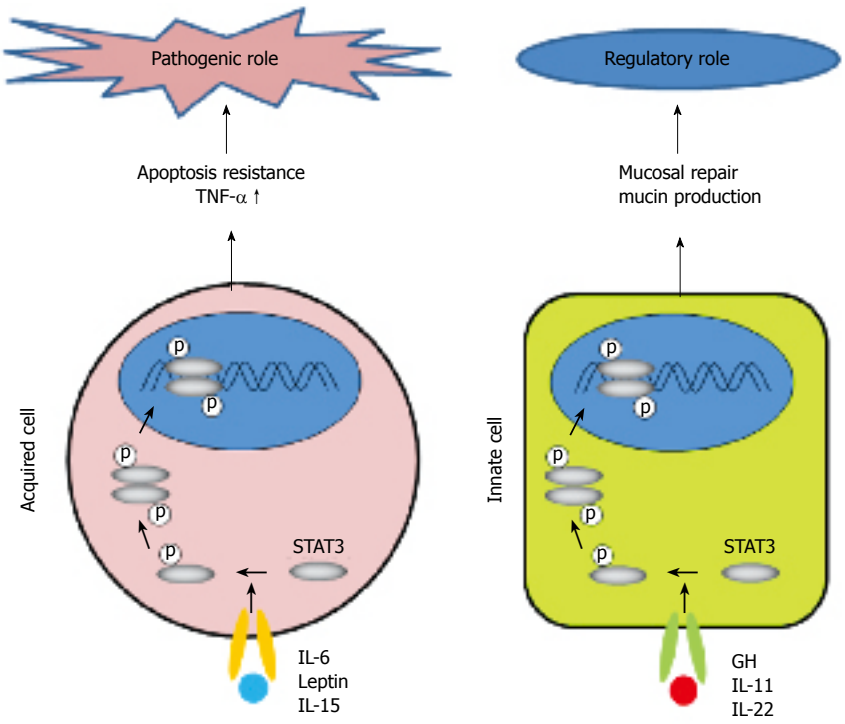


Figure 1 STAT3 plays distinct roles between innate immune cells and acquired immune cells. STAT3-mediated activation of acquired immune responses plays a pathogenic role in colitis by enhancing survival of pathogenic T cells and by inducing TNF-α. In contrast, STAT3-mediated activation of innate responses contributes to the suppression of colitis by enhancing the mucosal repair and by inducing mucin production.

	STAT3 activator	Target cell	Effect	Regulatory or pathogenic	Reference
Acquired	IL-6	$CD4^+$ T cell	Apoptosis resistance	Pathogenic	9
	Leptin	IEL, LPMC	TNF-α ↑, MIP-1α ↑, MIP-2 ↑	Pathogenic	12
	IL-15	$CD8^+$ T cell	IFN-γ ↑, TNF-α ↑	Pathogenic	36
Innate	GH	CEC	Mucosal repair	Regulatory	33
	IL-11	CEC	Crypt cell proliferation after injury	Regulatory	40
	IL-22	CEC	Mucin production	Regulatory	46

STAT3 in CECs, but not acquired immune cells by IL-22 may contribute to amelioration or chronic Th2-mediated colitis.

These findings suggest that it is possible to activate or inactivate STAT3 in specific cell populations for the treatment of human IBD.

THE ROLE OF MOLECULES INDUCED BY STAT3 ON IBD

STAT3 has been shown to induce anti-apoptotic genes such as *bcl-2* and *bcl-xl* in T cells, and increase to resistance of lamina propria pathogenic T cells to apoptosis[9]. In contrast, STAT3 can induce several molecules involved in the regulation of inflammation in CECs. Recent studies have demonstrated that STAT3 is required for the induction of IL-10, a well-known regulatory cytokine that is involved in the suppression of several inflammatory processes[48] including experimental colitis[15,49,50]. Local, but not systemic administration of this cytokine is of benefit in human IBD[51,52]. Impaired IL-10 production in macrophage by the STAT3 deletion is involved in the development of colitis[15], and enhanced production of this cytokine by CEC contributes to the suppression of colitis[53]. Importantly, IL-22, a strong activator of STAT3, has been shown to induce IL-10 production by CEC lines[45]. In addition to IL-10, STAT3 activation can also induce the expression of suppressors of cytokine signaling (SOCS) 3 that has been shown to play an important role in the suppression of acute colitis[11,17]. Furthermore, STAT3 has been recently demonstrated to induce trefoil factors that regulate intestinal epithelial homeostasis[22]. Moreover, IL-22-mediated STAT3 activation contributes to the improvement of colitis-associated mucus layer destruction associated with goblet cell depletion by enhancing the production of membrane-bound mucins (MUC1, -3, -10, and -13)[46]. Membrane-bound mucins form a static external barrier at the epithelial surface and are stored in goblet cell vacuoles[54-56]. Therefore, our recent study suggests that the enhanced mucus barrier formation participates in the IL-22/STAT3-mediated attenuation of Th2-mediated colitis[46].

CONCLUSION

Recent data indicate that STAT3 is one of the crucial targets for the treatment of IBD. However, as the receptors of these cytokines and growth factors are present on both innate and acquired cells, activation of STAT3 is likely to occur in both cell types[5,14,34-36,57,58]. Therefore, as the function of STAT3 is a double-edged sword, careful attention should be directed toward the cell population that is being targeted when one contemplates STAT3 inhibition or activation in human IBD[59].

REFERENCES

1 **Podolsky DK**. Inflammatory bowel disease. *N Engl J Med* 2002; **347**: 417-429
2 **Strober W**, Fuss IJ, Blumberg RS. The immunology of mucosal models of inflammation. *Annu Rev Immunol* 2002; **20**: 495-549
3 **Mudter J**, Neurath MF. Il-6 signaling in inflammatory bowel disease: pathophysiological role and clinical relevance. *Inflamm Bowel Dis* 2007; **13**: 1016-1023
4 **Rawlings JS**, Rosler KM, Harrison DA. The JAK/STAT signaling pathway. *J Cell Sci* 2004; **117**: 1281-1283
5 **Levy DE**, Darnell JE Jr. Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol* 2002; **3**: 651-662
6 **Lovato P**, Brender C, Agnholt J, Kelsen J, Kaltoft K, Svejgaard A, Eriksen KW, Woetmann A, Odum N. Constitutive STAT3 activation in intestinal T cells from patients with Crohn's disease. *J Biol Chem* 2003; **278**: 16777-16781
7 **Musso A**, Dentelli P, Carlino A, Chiusa L, Repici A, Sturm A, Fiocchi C, Rizzetto M, Pegoraro L, Sategna-Guidetti C, Brizzi MF. Signal transducers and activators of transcription 3 signaling pathway: an essential mediator of inflammatory bowel disease and other forms of intestinal inflammation. *Inflamm Bowel Dis* 2005; **11**: 91-98
8 **Mudter J**, Weigmann B, Bartsch B, Kiesslich R, Strand D, Galle PR, Lehr HA, Schmidt J, Neurath MF. Activation pattern of signal transducers and activators of transcription (STAT) factors in inflammatory bowel diseases. *Am J Gastroenterol* 2005; **100**: 64-72
9 **Atreya R**, Mudter J, Finotto S, Mullberg J, Jostock T, Wirtz S, Schutz M, Bartsch B, Holtmann M, Becker C, Strand D, Czaja J, Schlaak JF, Lehr HA, Autschbach F, Schurmann G, Nishimoto N, Yoshizaki K, Ito H, Kishimoto T, Galle PR, Rose-John S, Neurath MF. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis *in vivo*. *Nat Med* 2000; **6**: 583-588
10 **Yamamoto M**, Yoshizaki K, Kishimoto T, Ito H. IL-6 is required for the development of Th1 cell-mediated murine colitis. *J Immunol* 2000; **164**: 4878-4882
11 **Suzuki A**, Hanada T, Mitsuyama K, Yoshida T, Kamizono S, Hoshino T, Kubo M, Yamashita A, Okabe M, Takeda K, Akira S, Matsumoto S, Toyonaga A, Sata M, Yoshimura A. CIS3/SOCS3/SSI3 plays a negative regulatory role in STAT3 activation and intestinal inflammation. *J Exp Med* 2001; **193**: 471-481
12 **Siegmund B**, Lehr HA, Fantuzzi G. Leptin: a pivotal mediator of intestinal inflammation in mice. *Gastroenterology* 2002; **122**: 2011-2025
13 **Wegenka UM**, Buschmann J, Lutticken C, Heinrich PC, Horn F. Acute-phase response factor, a nuclear factor binding to acute-phase response elements, is rapidly activated by interleukin-6 at the posttranslational level. *Mol Cell Biol* 1993; **13**: 276-288
14 **Akira S**. Roles of STAT3 defined by tissue-specific gene targeting. *Oncogene* 2000; **19**: 2607-2611
15 **Takeda K**, Clausen BE, Kaisho T, Tsujimura T, Terada N, Forster I, Akira S. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity* 1999; **10**: 39-49
16 **Wolk K**, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R. IL-22 increases the innate immunity of tissues. *Immunity* 2004; **21**: 241-254
17 **Yasukawa H**, Sasaki A, Yoshimura A. Negative regulation of cytokine signaling pathways. *Annu Rev Immunol* 2000; **18**: 143-164
18 **Takeda K**, Noguchi K, Shi W, Tanaka T, Matsumoto M, Yoshida N, Kishimoto T, Akira S. Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proc Natl Acad Sci USA* 1997; **94**: 3801-3804
19 **Takeda K**, Kaisho T, Yoshida N, Takeda J, Kishimoto T, Akira S. Stat3 activation is responsible for IL-6-dependent T cell proliferation through preventing apoptosis: generation and characterization of T cell-specific Stat3-deficient mice. *J Immunol* 1998; **161**: 4652-4660
20 **Sano S**, Itami S, Takeda K, Tarutani M, Yamaguchi Y, Miura H, Yoshikawa K, Akira S, Takeda J. Keratinocyte-specific ablation of Stat3 exhibits impaired skin remodeling, but does not affect skin morphogenesis. *EMBO J* 1999; **18**: 4657-4668
21 **Welte T**, Zhang SS, Wang T, Zhang Z, Hesslein DG, Yin Z, Kano A, Iwamoto Y, Li E, Craft JE, Bothwell AL, Fikrig E, Koni PA, Flavell RA, Fu XY. STAT3 deletion during hematopoiesis causes Crohn's disease-like pathogenesis and lethality: a critical role of STAT3 in innate immunity. *Proc Natl Acad Sci USA* 2003; **100**: 1879-1884
22 **Tebbutt NC**, Giraud AS, Inglese M, Jenkins B, Waring P, Clay FJ, Malki S, Alderman BM, Grail D, Hollande F, Heath JK, Ernst M. Reciprocal regulation of gastrointestinal homeostasis by SHP2 and STAT-mediated trefoil gene activation in gp130 mutant mice. *Nat Med* 2002; **8**: 1089-1097
23 **Alonzi T**, Newton IP, Bryce PJ, Di Carlo E, Lattanzio G, Tripodi M, Musiani P, Poli V. Induced somatic inactivation of STAT3 in mice triggers the development of a fulminant form of enterocolitis. *Cytokine* 2004; **26**: 45-56
24 **Mizoguchi A**, Mizoguchi E, Bhan AK. Immune networks in animal models of inflammatory bowel disease. *Inflamm Bowel Dis* 2003; **9**: 246-259
25 **Powrie F**. T cells in inflammatory bowel disease: protective and pathogenic roles. *Immunity* 1995; **3**: 171-174
26 **Blumberg RS**, Saubermann LJ, Strober W. Animal models of mucosal inflammation and their relation to human inflammatory bowel disease. *Curr Opin Immunol* 1999; **11**: 648-656
27 **Neurath MF**, Finotto S, Glimcher LH. The role of Th1/Th2 polarization in mucosal immunity. *Nat Med* 2002; **8**: 567-573
28 **Sartor RB**. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. *Gastroenterology* 2004; **126**: 1620-1633
29 **Dalwadi H**, Wei B, Kronenberg M, Sutton CL, Braun J. The Crohn's disease-associated bacterial protein I2 is a novel enteric t cell superantigen. *Immunity* 2001; **15**: 149-158
30 **Lodes MJ**, Cong Y, Elson CO, Mohamath R, Landers CJ, Targan SR, Fort M, Hershberg RM. Bacterial flagellin is a dominant antigen in Crohn disease. *J Clin Invest* 2004; **113**: 1296-1306
31 **Podolsky DK**. Mucosal immunity and inflammation. V. Innate mechanisms of mucosal defense and repair: the best offense is a good defense. *Am J Physiol* 1999; **277**: G495-G499
32 **Hooper LV**, Gordon JI. Commensal host-bacterial relationships in the gut. *Science* 2001; **292**: 1115-1118
33 **Williams KL**, Fuller CR, Dieleman LA, DaCosta CM, Haldeman KM, Sartor RB, Lund PK. Enhanced survival and mucosal repair after dextran sodium sulfate-induced colitis in transgenic mice that overexpress growth hormone. *Gastroenterology* 2001; **120**: 925-937
34 **Kiessling S**, Muller-Newen G, Leeb SN, Hausmann M, Rath HC, Strater J, Spottl T, Schlottmann K, Grossmann J, Montero-Julian FA, Scholmerich J, Andus T, Buschauer A, Heinrich PC, Rogler G. Functional expression of the interleukin-11 receptor alpha-chain and evidence of antiapoptotic effects in human colonic epithelial cells. *J Biol Chem* 2004; **279**: 10304-10315
35 **Peterson RL**, Wang L, Albert L, Keith JC Jr, Dorner AJ. Molecular effects of recombinant human interleukin-11 in the HLA-B27 rat model of inflammatory bowel disease. *Lab Invest* 1998; **78**: 1503-1512
36 **Ohta N**, Hiroi T, Kweon MN, Kinoshita N, Jang MH, Mashimo T, Miyazaki J, Kiyono H. IL-15-dependent activation-induced cell death-resistant Th1 type CD8 alpha beta+NK1.1+ T cells for the development of small intestinal

inflammation. *J Immunol* 2002; **169**: 460-468
37 **Hokama A**, Mizoguchi E, Sugimoto K, Shimomura Y, Tanaka Y, Yoshida M, Rietdijk ST, de Jong YP, Snapper SB, Terhorst C, Blumberg RS, Mizoguchi A. Induced reactivity of intestinal CD4(+) T cells with an epithelial cell lectin, galectin-4, contributes to exacerbation of intestinal inflammation. *Immunity* 2004; **20**: 681-693
38 **Ito H**, Takazoe M, Fukuda Y, Hibi T, Kusugami K, Andoh A, Matsumoto T, Yamamura T, Azuma J, Nishimoto N, Yoshizaki K, Shimoyama T, Kishimoto T. A pilot randomized trial of a human anti-interleukin-6 receptor monoclonal antibody in active Crohn's disease. *Gastroenterology* 2004; **126**: 989-996; discussion 947
39 **Slonim AE**, Bulone L, Damore MB, Goldberg T, Wingertzahn MA, McKinley MJ. A preliminary study of growth hormone therapy for Crohn's disease. *N Engl J Med* 2000; **342**: 1633-1637
40 **Sands BE**, Bank S, Sninsky CA, Robinson M, Katz S, Singleton JW, Miner PB, Safdi MA, Galandiuk S, Hanauer SB, Varilek GW, Buchman AL, Rodgers VD, Salzberg B, Cai B, Loewy J, DeBruin MF, Rogge H, Shapiro M, Schwertschlag US. Preliminary evaluation of safety and activity of recombinant human interleukin 11 in patients with active Crohn's disease. *Gastroenterology* 1999; **117**: 58-64
41 **Liang SC**, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, Fouser LA. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* 2006; **203**: 2271-2279
42 **Zheng Y**, Danilenko DM, Valdez P, Kasman I, Eastham-Anderson J, Wu J, Ouyang W. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature* 2007; **445**: 648-651
43 **Radaeva S**, Sun R, Pan HN, Hong F, Gao B. Interleukin 22 (IL-22) plays a protective role in T cell-mediated murine hepatitis: IL-22 is a survival factor for hepatocytes via STAT3 activation. *Hepatology* 2004; **39**: 1332-1342
44 **Brand S**, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte JM, Diepolder H, Marquardt A, Jagla W, Popp A, Leclair S, Herrmann K, Seiderer J, Ochsenkuhn T, Goke B, Auernhammer CJ, Dambacher J. IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G827-G838
45 **Nagalakshmi ML**, Rascle A, Zurawski S, Menon S, de Waal Malefyt R. Interleukin-22 activates STAT3 and induces IL-10 by colon epithelial cells. *Int Immunopharmacol* 2004; **4**: 679-691
46 **Sugimoto K**, Ogawa A, Mizoguchi E, Shimomura Y, Andoh A, Bhan AK, Blumberg RS, Xavier RJ, Mizoguchi A. IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *J Clin Invest* 2008; **118**: 534-544
47 **Laurence A**, O'Shea JJ, Watford WT. Interleukin-22: a sheep in wolf's clothing. *Nat Med* 2008; **14**: 247-249
48 **Moore KW**, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 2001; **19**: 683-765
49 **Mizoguchi A**, Mizoguchi E, Takedatsu H, Blumberg RS, Bhan AK. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. *Immunity* 2002; **16**: 219-230
50 **Davidson NJ**, Leach MW, Fort MM, Thompson-Snipes L, Kuhn R, Muller W, Berg DJ, Rennick DM. T helper cell 1-type CD4+ T cells, but not B cells, mediate colitis in interleukin 10-deficient mice. *J Exp Med* 1996; **184**: 241-251
51 **Van Montfrans C**, Hooijberg E, Rodriguez Pena MS, De Jong EC, Spits H, Te Velde AA, Van Deventer SJ. Generation of regulatory gut-homing human T lymphocytes using ex vivo interleukin 10 gene transfer. *Gastroenterology* 2002; **123**: 1877-1888
52 **Rogy MA**, Beinhauer BG, Reinisch W, Huang L, Pokieser P. Transfer of interleukin-4 and interleukin-10 in patients with severe inflammatory bowel disease of the rectum. *Hum Gene Ther* 2000; **11**: 1731-1741
53 **De Winter H**, Elewaut D, Turovskaya O, Huflejt M, Shimeld C, Hagenbaugh A, Binder S, Takahashi I, Kronenberg M, Cheroutre H. Regulation of mucosal immune responses by recombinant interleukin 10 produced by intestinal epithelial cells in mice. *Gastroenterology* 2002; **122**: 1829-1841
54 **Shirazi T**, Longman RJ, Corfield AP, Probert CS. Mucins and inflammatory bowel disease. *Postgrad Med J* 2000; **76**: 473-478
55 **Ho SB**, Dvorak LA, Moor RE, Jacobson AC, Frey MR, Corredor J, Polk DB, Shekels LL. Cysteine-rich domains of muc3 intestinal mucin promote cell migration, inhibit apoptosis, and accelerate wound healing. *Gastroenterology* 2006; **131**: 1501-1517
56 **Corfield AP**, Carroll D, Myerscough N, Probert CS. Mucins in the gastrointestinal tract in health and disease. *Front Biosci* 2001; **6**: D1321-D1357
57 **La Cava A**, Matarese G. The weight of leptin in immunity. *Nat Rev Immunol* 2004; **4**: 371-379
58 **Reinecker HC**, MacDermott RP, Mirau S, Dignass A, Podolsky DK. Intestinal epithelial cells both express and respond to interleukin 15. *Gastroenterology* 1996; **111**: 1706-1713
59 **Plevy S**. A STAT need for human immunologic studies to understand inflammatory bowel disease. *Am J Gastroenterol* 2005; **100**: 73-74

S- Editor Li DL **L- Editor** Alpini GD **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5115

World J Gastroenterol 2008 September 7; 14(33): 5115-5124
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Emiko Mizoguchi, MD, PhD, *Series Editor*

New serological biomarkers of inflammatory bowel disease

Xuhang Li, Laurie Conklin, Philip Alex

Xuhang Li, Laurie Conklin, Philip Alex, Division of Gastroenterology, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore MD 21205, United States
Laurie Conklin, Division of Gastroenterology and Nutrition, Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore MD 21205, United States
Author contributions: Li X and Alex P wrote and revised the article; Conklin L helped critical review and provided suggestions; Li X finalized the revision.
Supported by Broad Medical Research Program, No. IBD-0119R; NIH/NIDDK grant, No. 5R21DK77064; NIH/NIDDK, No. KO1-DK62264; NIH Ruth L. Kirschstein National Research Service Awards, Proctor & Gamble Investigator Initiated Grants
Correspondence to: Dr. Xuhang Li, Division of Gastroenterology, Department of Medicine, Johns Hopkins University School of Medicine, 720 Rutland Ave, 918 Ross Research Bldg, Baltimore MD 21205, United States. xuhang@jhmi.edu
Telephone: +1-443-2874804 Fax: +1-410-9559677
Received: June 27, 2008 Revised: August 25, 2008
Accepted: September 2, 2008
Published online: September 7, 2008

Abstract

Serological biomarkers in inflammatory bowel disease (IBD) are a rapidly expanding list of non-invasive tests for objective assessments of disease activity, early diagnosis, prognosis evaluation and surveillance. This review summarizes both old and new biomarkers in IBD, but focuses on the development and characterization of new serological biomarkers (identified since 2007). These include five new anti-glycan antibodies, anti-chitobioside IgA (ACCA), anti-laminaribioside IgG (ALCA), anti-manobioside IgG (AMCA), and antibodies against chemically synthesized (∑) two major oligomannose epitopes, Man α-1,3 Man α-1,2 Man (∑Man3) and Man α-1,3 Man α-1,2 Man α-1,2 Man (∑Man4). These new biomarkers serve as valuable complementary tools to existing biomarkers not only in differentiating Crohn's disease (CD), ulcerative colitis (UC), normal and other non-IBD gut diseases, but also in predicting disease involvement (ileum *vs* colon), IBD risk (as subclinical biomarkers), and disease course (risk of complication and surgery). Interestingly, the prevalence of the antiglycan antibodies, including anti-Saccharomyces cerevisiae antibodies (ASCA), ALCA and AMCA, was found to be associated with single nucleotide polymorphisms (SNPs) of IBD susceptible genes such as NOD2/CARD15, NOD1/CARD4, toll-like receptors (TLR) 2 and 4, and β-defensin-1. Furthermore, a gene dosage effect was observed: anti-glycan positivity became more frequent as the number of NOD2/CARD15 SNPS increased. Other new serum/plasma IBD biomarkers reviewed include ubiquitination factor E4A (UBE4A), CXCL16 (a chemokine), resistin, and apolipoprotein A-IV. This review also discusses the most recent studies in IBD biomarker discovery by the application of new technologies such as proteomics, fourier transform near-infrared spectroscopy, and multiplex enzyme-linked immunosorbent assay (ELISA)'s (with an emphasis on cytokine/chemokine profiling). Finally, the prospects of developing more clinically useful novel diagnostic algorithms by incorporating new technologies in serological biomarker profiling and integrating multiple biomarkers with bioinformatics analysis/modeling are also discussed.

Key words: Serological biomarkers; Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Anti-chitobioside IgA; Anti-laminaribioside IgG; Anti-manobioside IgG; Anti-synthetic mannoside antibodies; Multiplex enzyme-linked immunosorbent assay; Proteomics

Peer reviewer: Emiko Mizoguchi, Professor, Department of Medicine, Gastrointestinal Unit, Massachusetts General Hospital, GRJ 702, 55 Fruit Street, Boston MA 02114, United States

Li X, Conklin L, Alex P. New serological biomarkers of inflammatory bowel disease. *World J Gastroenterol* 2008; 14(33): 5115-5124 Available from: URL: http://www.wjgnet.com/1007-9327/14/5115.asp DOI: http://dx.doi.org/10.3748/wjg.14.5115

INTRODUCTION

Biomarkers of inflammatory bowel disease (IBD) are measurable substances in body fluids [such as blood (serological)], stool, or other parts of the body, as tools for disease diagnosis and/or prognosis. Application of IBD biomarkers is cheaper, less laborious, less invasive, and more objective compared to the endoscopy/biopsy-based approach[1]. Current IBD biomarkers include serological, fecal and genetically predisposed gene polymorphisms[2-5], as well as imaging biomarkers in conjunction

with imaging technologies such as optical, ultrasound, magnetic resonance imaging (MRI), X-ray, computer tomography (CT), position emission tomography (PET), single photon emission computed tomography (SPECT)[6-9]. Among those, fecal[10,11] and serological biomarkers, including systemic level of specific antibodies and other serum proteins[4,12-14], have been most widely explored and/or used in clinical studies. However, none of the current commercially available biomarker tests/assays, including all of those mentioned in this highlight, can be used as stand-alone tools in clinics and, therefore, can only recommended as an adjunct to endoscopy in diagnosis, and prognosis of the disease[1,15]. Considering that endoscopy is a highly resource-intensive process (involving frequent invasive, labor-intensive and expensive colonoscopic procedures), new IBD biomarkers and more comprehensive bioinformatic algorithms with multiple biomarkers are in great need.

The focus of this highlight is on new serological IBD biomarkers. However, the remarkable rapid development of IBD biomarkers in the last decade have made it impossible to separate the "old" from the "new", since multiple biomarkers, both old and new, are being integrated in clinical studies. For example, 5 serum biomarkers, including ASCA, pANCA, anti-OmpC, anti-Cbir and anti-I2 (see below), were the most widely studied in the past decade, but are still being characterized and validated for their clinical utility. Since the status of these biomarkers has been reviewed extensively elsewhere[4,12-14], they will be listed as "old" markers and only briefly reviewed, along with many other serological biomarkers reported before 2007. Anti-glycan antibodies, a newer panel of serum biomarkers, first reported in 2006[16,17] and being validated since 2007, will be one of the major "new" biomarkers in this highlight. Serum cytokines, which are among the earliest inflammatory mediators studied, but are yet to be recognized as useful IBD biomarkers, will be reviewed and discussed. The prospects of developing new serological IBD biomarkers and integrating existing ones will also be discussed, particularly regarding the application of novel molecular approaches and proteomic technologies in biomarker screening and identification, as well as novel bioinformatic analyses of clinical utilities of multiple biomarkers.

"OLD" SEROLOGICAL IBD BIOMARKERS: A BRIEF OVERVIEW

Anti-Saccharomyces cerevisiae antibodies (ASCA) and perinuclear antineutrophil cytoplasmic antibodies (pANCA) were the first extensively characterized serological IBD markers[18,19]. ASCA is more associated with Crohn's disease (CD) while pANCA is more associated with ulcerative colitis (UC)[4,13,20,21]. Three additional serum biomarkers were introduced later, including antibodies against outer membrane porin C (anti-OmpC), Pseudomonas fluorescens bacterial sequence I2 (anti-I2), and bacterial flagellin (anti-CBir 1)[12,13,22,23]. These are five most extensively studied serological biomarkers to date. Although exact data from independent studies vary, combinations of more than one of the 5 serological markers have been shown to have the most clinical value (see reviews[4,12-14,24]). For example, ASCA and pANCA together have a specificity of approximately 90% for both CD and UC[21,25-27]. These markers have been demonstrated as not only being useful for differentiating IBD *vs* healthy control or CD *vs* UC, but also as potential indicators and/or predictors for disease activity/location, disease course/complication, need for surgery, and prognosis of therapy. For example, CD patients who are positive in multiple anti-microbial antibodies (ASCA, anti-OmpC, anti-CBir, and anti-I2) have increased risk of having more complicated disease. Patients who are positive in all four of these biomarkers have 11-fold increased risk to develop penetrating and/or stricturing disease[28-32]. CD patients positive with three markers (anti-OmpC, anti-CBir, and anti-I2) are more likely to have small bowel surgery than those who were negative (72% *vs* 23%). No similar association of serotype was found with disease phenotype of UC[32].

Elevated levels of serological biomarkers were shown to be associated with IBD-susceptible gene variants. Family members of CD patients with NOD2/CARD15 3020insC variant was reported to have increased intestinal permeability, which has been positively associated with elevated serological biomarkers[33,34]. However, reports on this relationship have been inconsistent[35-38], even though more studies presented a positive association between serological biomarkers and susceptible gene variants[32,37,38]. Future studies by independent groups with larger cohorts, well-defined clinical characteristics and patient populations (such as ethnicity) are necessary to resolve this discrepancy.

Other note-worthy aspects of these serological biomarkers include their potential value as subclinical biomarkers and their inherent geographic/ethnic heterogeneity. (1) Independent studies have shown that the prevalence of ASCA positivity is significantly higher (20%-25%) in unaffected first-degree relatives of patients with CD[18,39] compared to general healthy populations (0%-10%), indicating a familial association. A much stronger indication that ASCA may be a potential subclinical biomarkers for CD came in 2005. In a serological analysis of a large serum depository, Israeli *et al* reported that ASCA reactivity was found 38 mo before clinical diagnosis in 32% of the CD patients studied[40]; (2) The diagnostic value of serological biomarkers can vary significantly among different ethnic or geographic populations. For example, both ASCA and pANCA were found to be less sensitive in Chinese and Japanese patients[41,42]. On the other hand, positivity of pANCA was shown to be higher in Mexican-American UC patients: all Mexican-Americans with UC tested had positive pANCA compared to only 40% of Caucasians[42]. These studies suggest that physicians must factor the patients' ethnic background when serological biomarkers are applied in the clinical settings.

At least two dozen non-antibody serum biomarkers have also been reported, including, C-reactive protein,

calprotectin, and PMN-elastase, soluble selectins, adhesion molecules, and procalcitonin (PCT)[4,5,43-45]. However, it is necessary to point out that most of these markers have not been extensively characterized. Many of them are also elevated in a variety of other inflammatory or pathological conditions with a low specificity to IBD. Therefore, their actual clinical value needs to be further investigated or validated.

"NEW" SEROLOGICAL IBD BIOMARKERS

New anti-glycan antibodies: ACCA, ALCA and AMCA

New diagnostic and predicting value: Three new anti-glycan antibodies were first reported as potential novel serological biomarkers in the diagnosis of IBD by Dotan *et al* in 2006 from Glycominds Ltd in Israel[16,17]. Now, as major components of IBDXTM Panel marketed by Glycominds Ltd (http://www.ibdx.net/index.html), this new set of biomarkers contains three anti-glycan antibodies, including anti-chitobioside IgA (ACCA), anti-laminaribioside IgG (ALCA), and anti-mannobioside IgG (AMCA) (Table 1). The fourth component in the IBDXTM Panel is gASCA (ASCA IgG), virtually the same as ASCA, which is the first antiglycan IBD serological biomarker identified. Since 2007, several independent studies on these anti-glycan antibodies have been reported, and their clinical utility has been validated by independent laboratories (see below). Glycan, a generic term for all molecules bearing glycosidic bonds, includes mono-, oligo- and ploy-saccharides or carbohydrates[17]. Glycans are major building blocks of cell surface components and immunogens (erythrocytes, immune cells, and microorganisms) that lead to generation of a variety of anti-glycan antibodies, including IgG, IgA, IgE and IgM, which have been demonstrated in a number of inflammatory and autoimmune diseases[16]. Mannobioside (AMCA) is a dimer of 1,3 linked mannose, and is a component of mannan from pathogenic fungi and yeast[12]. Laminaribioside (ALCA) is the building block of laminarin, a polysaccharide of the β-1-3-glucan family and is found in the cell walls of fungi, yeast, and algae[16,46]. Chitobioside (ACCA) is a component of chitin, found in the insect cuticle and cell walls of infectious pathogens such as bacteria and yeast[16]. Both β-1-3 glucans and chitin modulates the immune system by binding to receptors on neutrophils, macrophages, and natural killer (NK) cells, thereby stimulating cell proliferation, phagocytosis and cytokine secretion[47]. The resultant antibody production specifically against chitin and glucan, and their association with CD also suggests the intrinsic modulation of the adaptive immune system. However, the individual diagnostic differences between ALCA, AMCA, and ACCA have not yet been clearly established.

Using GlycoChip glycan array technology[48,49] and enzyme-linked immunosorbent assay (ELISA), Glycominds Ltd developed the new IBD serological markers (ACCA, ALCA, and AMCA)[16,17]. The initial study of these 3 new markers, which involved a total of 194 patients with CD, 162 with UC and 142 healthy controls, showed that ACCA, ALCA, and AMCA exhibited the highest discriminative capability between CD and UC[16]. Approximately one third of CD patients are positive for each of the 3 new markers[16] (Table 1). More significantly, 44% (12/27) of ASCA-negative CD patients were positive for ALCA or ACCA. Therefore, although the prevalence of each of the individual new biomarker is relatively poor (Table 1), together they are a significant complement to ASCA. In patients that were positive with one of the 3 markers, the sensitivity and specificity for diagnosis of CD were 77.4% and 90.6%, respectively. In patients with 2 or 3 of these antibodies, the specificity increased to 99.1%. Higher levels of ALCA and AMCA were significantly associated with small intestinal disease.

Ferrante *et al*[46] reported a study that involved a larger cohort, including 1225 IBD patients (913 CD, 272 UC, and 40 IC), 200 ethnically matched healthy controls, and 113 patients with non-IBD intestinal inflammation (diverticulitis, infectious colitis, ischemic colitis and pseudomembranous colitis). In this study, IBDX™ Panel (ACCA, ALCA, AMCA, and gASCA) and anti-OmpC were analyzed. 76% of CD patients are positive for at least one of the 5 markers. All antiglycan and anti-OmpC were specific for CD (80.5%-93%). The sensitivity was calculated as: gASCA = 56.4%; ALCA = 17.7%; ACCA = 20.7%; AMCA = 28.1%; and anti-OmpC = 29.1%. Among all 913 CD patients, only 13 (1.4%) were positive for all the 5 CD-associated markers. Fifty percent of CD patients (n = 435) who were either sASCA negative/OmpC positive (n = 93) or gASCA positive/OmpC negative (n = 342), were positive for at least one of the antiglycan markers. Sixty-seven percent of gASCA/OmpC-positive CD patients were also positive for at least one of the other anti-glycan

Table 1 New serological IBD biomarkers

		CD	UC	Control	Ref.
1	ALCA	Approximately 19%-38%	Low (approximately 7%)	Low	16, 17 18, 50
2	ACCA	Approximately 21%-40%	Low	Low	16, 17 18, 50
3	AMCA	Approximately 28%	Low	Low	16, 17 46
4	A∑MA: Anti-∑Man3 Anti-∑Man4	38.60% 22.10% 28.50%	Low	Low	55
5	UBE4A	46.20%	7.10%	3.30%	56
6	CXCL16	Elevated	Elevated		57
7	Apolipoprotein A-IV	Elevated in active CD			58
8	Resistin	Elevated	Elevated		59
9	PF4, MRP8, FIBA and Hpα2				68, 69
10	Cytokines/ chemokines & their receptors				

ACCA: Anti-chitobioside IgA; ALCA: Anti-laminaribioside IgG; AMCA: Anti-mannobioside IgG; A∑MA: Anti-∑Man3 or ∑Man antibodies; ∑Man3: Synthetic Man α-1,3 Man α-1,2 Man; ∑Man4: synthetic Man α-1,3 Man α-1,2 Man α-1,2 Man; UBE4A: Ubiquitination factor E4A; CXCL16: A transmembrane protein functioning as a chemokine and a scavenger receptor.

antibodies. Of 305 sASCA/OmpC-negative CD patients, 7% were ALCA positive, 12% ACCA positive, and 13% SMCA positive. Although addition of ALCA (gASCA/pANCA/ALCA combination) resulted in only minor improvement in differentiating CD from UC compared to the classic gASCA/pANCA combination, it significantly enhanced the accuracy of differentiating IBD from healthy controls and non-IBD intestinal inflammation. Increasing levels of all 5 markers (gASCA, ALCA, ACCA, AMCA, and OmpC) were significantly associated with more complicated disease behavior, including stricture, fistula and need for surgery. However, a recent report by Simondi *et al*[50] found that, while the level of ASCA appeared to be associated with ileal disease and penetrating/structuring disease, level of ALCA has a similar trend, but did not reach statistic significance ($P = 0.07$ and $P = 0.09$, respectively). This discrepancy may arise from the smaller cohort in Simondi D's study, which involved only 265 subjects (116 CD, 53 UC, 51 healthy controls, and 45 other intestinal diseases).

Similar results on antiglycan antibodies were reported by Papp *et al*[22] in another study that involved 557 CD patients, 95 UC and 100 healthy controls. 66.2% of CD patients were positive for at least one of the 5 biomarkers tested, including gASCA, ALCA, ACCA, AMCA, and anti-OmpC, all of which were highly specific for CD (79%-100% sensitivity). The sensitivities for each of the 5 markers are gASCA = 50.4%; ALCA = 15.2%; ACCA = 11.3%; AMCA = 11.5%; and anti-OmpC = 31.2% (the 3 new markers, ALCA, ACCA and AMCA were all lower than the results of most other studies[16,17,46,50]. Overall, increasing levels of these markers were again associated with more complicated disease behavior and incidence of surgery. Among CD patients, gASCA and ALCA were associated with early disease onset (occurring at younger age ($P < 0.0001$ and $P = 0.0012$, respectively, while gASCA was associated with perianal disease ($P < 0.0001$) and azathioprine use ($P = 0.016$). However, no association was found between these serological biomarkers and gender, familial disease, smoking habit and extraintestinal manifestations (EIM). It is interesting to mention that in Simondi D's report[50], (1) among CD patients, AMCA was found to be significantly higher in women and in smokers than in men ($P = 0.02$) and non-smokers ($P = 0.03$); and (2), CD patients with at least one affected first-degree relative exhibited significantly higher levels of ALCA than those without familial cases (59.8% *vs* 34.7%, $P = 0.0005$), suggestive of a familial association of ALCA.

Association of the antiglycan antibodies with variants of IBD susceptible genes: NOD2/CARD15. Like ASCA[32,37,38], the newly identified antiglycan antibodies were also found to be associated with single nucleotide polymorphisms (SNPs) of IBD susceptible genes. The first study, reported by Henckaerts *et al* in 2007[51], examined the influence of mutations in several innate immune receptor genes on the development of anti-glycan and anti-OmpC antibodies in IBD, including NOD2/CARD15, NOD1/CARD4, TUCAN/CARDINAL/CARD8, Toll-like receptor (TLR) 4, TLR2, TLR1 and TLR6. The study involved 1163 unrelated IBD patients (874 CD, 259 UC, and 30 IC) and 312 healthy controls. CD patients with at least one NOD2/CARD15 variant (1) were more frequently gASCA or ALCA positive than those with no mutation (gASCA: 66.1% *vs* 51.5%, $P < 0.0001$; ALCA: 43.3% *vs* 34.9%, $P = 0.018$); and (2) had higher gASCA titers (85.7 *vs* 51.8 ELISA units, $P < 0.0001$). This association was independent of ileal involvement. More interestingly, a gene dosage effect was observed when positivities of antiglycan antibodies in CD patients carrying 0, 1 and 2 NOD2/CARD15 variants were compared. Anti-glycan positivity became more frequent as the number of NOD2/CARD15 mutations increased (Table 2).

Table 2 A gene dosage effect: % positivity of antiglycan antibodies in CD patients carrying zero, one and two NOD2/CARD15 variants

	CD patients with 0 NOD2/CARD15 variant	CD patients with 1 NOD2/CARD15 variant	CD patients with 2 NOD2/CARD15 variants	*P*	Ref.
gASCA	51.50	64.20	72.30	< 0.0001	51
	41.60	64.60	67.50	< 0.0001	22
ALCA	34.90	42.10	46.70	< 0.04	51
AMCA	9.80	14.10	30	< 0.001	22
Any antiglycan	52.40	69.70	80	< 0.0001	22

A remarkably similar gene dosage effect on gASCA was observed by Papp *et al*[22] in 2008 (Table 2) as compared to Henckaerts L's study[51]. They analyzed the association of antiglycan with NOD2/CARD15 variants from studying 557 CD patients, 95 UC and 100 healthy controls. When comparing CD patients with NOD2/CARD15 variants to those with wild type alleles, positivity of gASCA is 65.2% *vs* 41.8% ($P < 0.0001$), AMCA is 18.8% *vs* 9.7% ($P = 0.009$), and any antiglycan is 72.5% *vs* 52.5% ($P < 0.0001$). In addition, Papp M's report also showed the gene dosage effect on AMCA and any antiglycan antibodies (Table 2).

NOD1/CARD4, TLR2, and TLR4: In Henckaerts L's report[51], CD patients carrying one GG-indel allele in NOD1/CARD4 had a higher prevalence of gASCA than those with wild type allele (63.8% *vs* 55.2%, $P = 0.014$). Gene dosage effect of NOD1/CARD4 was also evident as the number of mutant alleles increased, but did not reach statistical significance. Interestingly, CD patients with at least one mutation TLR4 (D299G) had a lower prevalence of ACCA compared with TLR4 wild type (D299A) (23.4% *vs* 35%, $P = 0.013$), and a lower ACCA titer (39 *vs* 49 EU, $P = 0.05$). An inverse gene dosage effect of TLR4 was observed: the prevalence of ACCA is 34.9%, 21.1% and 9.1% in CD patients with 0, 1 and 2 NOD1/CARD4 variants, respectively. A similar inverse gene dosage effect was also seen for anti-OmpC prevalence gene in relation to the number of TLR2 mutations. Of note, other reports did not find any

significant association of TLR4 (25) or NOD1/CARD4 (variant E266K)[52] with the positivity of any antiglycan antibodies.

DEFB1: Lakatos *et al*[52] recently reported an association of the antiglycan antibodies with specific variants of β-defensin-1 (DEFB1). In this study of a sex-matched cohort of 276 CD patients and 100 healthy controls, four IBD susceptible genes were tested, including NOD2/CARD15, NOD1/CARD4, DLG5, and DEFB1. Two DEDB1 variants, G20A and C44G, were found to be inversely associated with the positivity of antiglycan antibodies. For example, 29.6% of DEFEB1 20A carriers were antiglycan positive compared to 46% positivity in non-carriers ($P < 0.038$). However, no association of antiglycan positivity was found with either DEFEB1 G52A variant or DLG5 (R30Q), although both were shown to be associated with increased risk for CD[53,54]. Furthermore, in contrast to other reports[32,51], no gene dosage effect was observed on any of the antiglycan antibodies.

Anti-synthetic mannoside antibodies: Evidence for the existence of a new subset of antiglycan antibodies

Based on the chemical structure of mannose epitopes for ASCA, the antibodies against mannose residues [Man α-1,3 (Man α-1,2 Man) 1-2] that are the most widely recognized biomarkers for CD, Vandewalle-El Khoury *et al* recently[55] chemically synthesized (∑) two major oligomannose epitopes, Man α-1,3 Man α-1,2 Man (∑ Man3) and Man α-1,3 Man α-1,2 Man α-1,2 Man (∑ Man4). Their goals were to test the immuno-reactivity of the ∑Man3 and ∑Man4 with specific serum antibodies (termed "A∑MA" for anti-synthetic mannoside antibodies) and compared A∑MA with ASCA for their values as serological biomarkers for CD. An impressively large cohort was used in the study, including a total of 1365 subjects (772 CD, 261 UC, 43 IC and 289 controls).

Overall, while the specificity of A∑MA for CD was quite similar to that of ASCA (89% *vs* 93%), the specificity was lower (38% *vs* 55%). 16% of CD patients were positive for both ASCA and ∑Man3, and 24% positive for ∑Man4. Interestingly, 11% of ASCA-negative CD patients were A∑MA positive (5% for ∑Man3, 4% for ∑Man4, and 2% for both). Together 24% of CD patients who were negative for ASCA and/or other CD-associated serological biomarkers were positive for A∑ MA, suggesting a previously unrecognized new subset of anti-mannose antibodies are present in patients with CD. Therefore, it is conceivable that a combination of A∑MA with ASCA, ACCA, ALCA and AMCA would significantly increase the diagnostic value of the existing panel of antiglycan biomarkers.

A∑MA was analyzed for its predictive value for the evolution of IC patients as well as the involvement of disease location of CD (small bowl *vs* colon). Twenty IC patients (out of a total of 43) evolved to a final diagnosis of CD ($n = 11$; UC = 7; UC-like CD = 2). Among 11 patients with final diagnosis as CD, one was ASCA-positive/A∑MA-negative, two were ASCA-positive/A ∑MA-positive, and three were ASCA-negative/A∑MA-positive. None of the UC patients were A∑MA-positive. Therefore, A∑MA was more sensitive (45% *vs* 27%) and more specific (100% *vs* 71%) than ASCA for predicting evolution of IC toward CD. As for the predictive value of disease location, although positivity of A∑MA had no association with any particular disease phenotype, among the ASCA-negative CD patients, A∑MA positivity was significantly associated with colonic involvement. This indicates that A∑MA may provide new diagnostic value to colonic CD, for which ASCA is less frequently detected.

Other serum/plasma biomarkers

Ubiquitination factor E4A (UBE4A): By screening a phage library from normal terminal ileum with sera from patients with CD, Sakiyama *et al*[56] identified a strongly immunoreactive cDNA clone encoding the C-terminal subunit of the UBE4A, a U-box-type ubiquitin-protein ligase. To investigate the specificity of the serum anti-UBE4A autoantibodies in CD patients *vs* UC patients *vs* healthy controls, a GST-C-terminal UBE4A fusion protein was made, and used to test the immunoreactivity of sera from 39 patients with CD, 28 with UC, and 60 healthy controls. The prevalence of anti-UBE4A IgG was significantly higher in CD than that in UC or healthy controls (46.2% *vs* 7.1% *vs* 3.3%, respectively; $P < 0.0006$) (Table 1). The levels of anti-UBE4A IgG were correlated well with the disease activity ($P < 0.0001$). More interestingly, higher level of anti-UBE4A IgG was associated with complicated disease behavior (stricturing and penetrating) ($P = 0.0028$), and patients positive with anti-UBE4A IgG were more likely to undergo surgery ($P = 0.0013$). Although UBE4A expression was low in the cytoplasm of enterocytes and goblet cells, immunohistological analysis showed that UBE4A expression was highly elevated only in enteroendocrine cells of ileal, mucosa from CD patients, but not in normal subjects. It was speculated that production of anti-UBE4A autoantibodies might be a result of increased expression of UBE4A in the inflamed ileal mucosa. The exact role of UBE4A elevation and production of anti-UBE4A autoantibodies in the pathogenesis of CD remains to be determined.

CXCL16, apolipoprotein A-IV, and resistin: These newly reported serum/plasma IBD biomarkers were reported by the same working group in Germany[57-59]. It appears that the same cohort of study subjects was used for all three studies, or at least cohorts might have some overlapping subjects. Further validation study of these markers by other independent laboratories is necessary to evaluate their usefulness.

CXCL16 (Table 1), an intriguing transmembrane protein composed of an extracellular chemokine domain fused with a mucin stalk that extends through cell surface, functions as a chemokine and a scavenger receptor and has been implicated in various inflammatory diseases. It becomes soluble after being cleaved by metalloproteinase ADAM 10 and exerts chemokine functions[60].

Lehrke *et al*[57] reported that CXCL16 could potentially be a surrogate IBD biomarker after having examined the serum levels of soluble CXCL16 in a cohort of 239 patients with CD, 114 UC, and 144 healthy controls. Soluble CXCL16 levels were found to be the highest in CD patients ($P < 0.001$) compared with UC and healthy controls. UC patients had a relatively modest, but significant elevation of CXCL16 compared with healthy controls ($P < 0.001$). No significant difference was seen between active and inactive state of CD or UC.

Apolipoprotein A-IV (Table 1), a structural component of intestine derived triacylglycerol-rich chylomicron particles with anti-oxidant, anti-atherogenic, and anti-inflammatory properties, has been recently shown to inhibit DSS-induced mouse colitis[61]. For this reason, Broedl *et al*[58] tested if plasma level of apolipoprotein A-IV was associated with IBD, and found that it was inversely associated with disease activity and CRP levels in patients with CD (but not UC) ($P < 0.005$). However, since the actual difference in the level of apolipoprotein A-IV between active and inactive CD is quite small, the clinical value of this marker remains to be determined.

Resistin (Table 1), also known as adipocyte secreted factor or FIZZ-3, is a peptide hormone that is associated with multiple inflammatory conditions[62]. Studies by Konrad *et al*[59] showed that patients with both CD and UC had significantly higher plasma levels of resistin compared to healthy controls ($P < 0.0001$). The levels of resistin in both CD and UC were significantly associated with white blood cell count ($P < 0.0001$), CRP ($P < 0.0001$), and disease activity ($P < 0.0001$).

Application of proteomic and infrared spectroscopic technologies in serological IBD biomarker profiling

The rapid development of proteomic technologies recently has revolutionized the way and capacity by which biomarker discovery is performed[63-67]. Current proteomic methodologies include three sub-categories: mass spectrometry (MS)-based technologies, array-based technologies and imaging MS (see review[66]). Blood is the most explored source for disease biomarkers by proteomics[63-65,67]. Proteomics of IBD was recently reviewed[80].

SELDI-TOF-MS and MALDL-TOF-MS: Unlike the quests for biomarkers of other major diseases (such as cancers), application of proteomic technologies in IBD biomarker discovery is only in its infancy. So far (as of June 17, 2008) only three full original MS-based reports are available, of which, two used SELDI-TOF-MS by Merville/Louis groups[68,69] and one used MALDI-TOF-MS by Roda's group[70]. Using SELDI-TOF-MS (Surface Enhanced Laser Desorption Ionization-Time of Flight-Mass Spectrometer), Meuwis *et al*[68] analyzed protein profiles of 120 serum samples collected from a cohort of 30 CD, 30 UC, 30 inflammatory controls and 30 healthy controls. Multivariate analysis generated models that could classify samples with minimum 80% sensitivity and specificity in discriminating groups of patients. Four peptides were identified from potential peaks that could best discriminate the four groups, leading to the identification of 4 serum biomarkers, including platelet aggregation factor 4 (PF4), MRP8 (S100A8), FIBA (a peptide released during clotting from fibrinogen precursor) and Hpα2 (haptoglobin α2) (Table 1). The diagnostic value of these markers remains to be further examined. Using similar approaches, the same group piloted a study of sera from 20 CD patients who showed either response or no response to infliximab[69]. PF4 was again identified as a potential marker. The intensity level of SELDI peak in which PF4 was identified was inversely associated with infliximab non-responders. Unfortunately, such association could not be confirmed by ELISA measurement of PF4. Also, PF4 did not exhibit a significant correlation with other disease markers (sCD40L, IL-6, and CRP) or CDAI, casting doubt that PF4 probably would have any clinical diagnostic value.

The third MS-based profiling of serum IBD markers was reported by Nanni *et al*[70] using MALTI-TOF-MS (Matrix-Assisted Laser Desorption/Ionization Time of Flight-Mass Spectrometer). The study, which involved a small cohort (15 CD, 26 UC and 22 healthy controls), found the revered-phase extraction and selection of 20 m/z value gave the best overall predictive value (96.9%). In another study, reported at Digestive Disease Week (DDW) 2008, Subramanian *et al*[71] analyzed sera from a cohort of 62 UC and 48 CD by SELDI-TOF MS. Biostatistical analysis identified 12 discriminative peaks, with specificity and sensitivity approximately 95% (compared to 80.9% of the sensitivity of ASCA for CD and 64.5% of pANCA for UC). Four serum proteins were identified as inter alpha trypsin inhibitor 4, apolipoprotein C1, platelet activated factor 4 variant, which are expected to be further analyzed for their clinical utility.

Protein and antibody arrays/chips: Compared with other proteomic approaches, protein/antibody array (or chip) array technologies offer the advantages of being highly specific with high-throughput nature and capacity. The application of these technologies in IBD biomarkers discovery has just begun, and thus, the data are limited. Since 2006, our laboratory began using proteins that were robotically spotted on array slides as bait to screen serum IBD biomarkers[72]. Since the currently known major serological IBD biomarkers or antibodies are against microbes or protein of human origin, including ASCA, ANCA, OmpC, Cbir, and the new antiglycan antibodies, we hypothesized that disease-specific antibodies to microbial or to human protein (autoantibodies) are present in patients' sera (manuscript in preparation). We found to our surprise that human sera contain antibodies immuno-reactive to hundreds of proteins from *E. coli*, yeast, and even humans. The numbers and immunoreactivity of these antibodies vary greatly among IBD patients and even among healthy individuals. From our experiences, when protein arrays are used, the serum quality must be high, the screening process must be standardized, and sufficient number of subjects (at least approximately 30 per comparing group) should be included. This results in a high cost of experiments due to the expense of the commercial protein- or antibody arrays (unless made using assembled robotic ar-

rayers). One presentation by Vermeulen *at al*[73] at DDW 2008 reported a study of using commercial human protein arrays to profile serum IBD biomarkers from a very small cohort of subjects (10 UC, 15 CD and 5 healthy controls). They found that 75 proteins reacted more strongly with sera from IBD than those from healthy controls, while reactivity of another 88 proteins was just opposite. One identified antigen, described as an autoantigen in IBD was pleckstrin homology-like domain, family A, member 1 (Phla1). Validation experiment using a larger cohort of subjects found that approximately 46% of IBD [UC: 42.8% (27 of 63); CD: 50.0% (33 of 66)] were positive for anti-Phla1 antibodies, compared to 28.7% healthy controls (19 of 66) and 33.3% non-IBD gastrointestinal controls (22 of 66). Therefore, the discriminative power of this anti-Phla1 for CD *vs* UC or IBD *vs* controls is poor.

So far, antibody arrays were used in only one report by Kader *et al*[74] for identifying IBD serum biomarkers. In this study, antibody arrays containing 78 cytokines, growth factors, and soluble receptors were used to screen 65 patients with CD and 23 with UC. Univariate analysis found that the levels 4 cytokines (PLGF, IL-7, IL-12p40, and TGF-β1) were significantly elevated in patients with clinical remission compared to active disease ($P < 0.01$). However, only the difference in IL12p40 reached statistical significance ($P < 0.02$).

Fourier transform near-infrared spectroscopy: Haas *et al*[75] reported at DDW 2008 a new application of Fourier Transform Near-Infrared Spectroscopy (FT-NIR) in serum biomarker profiling. Specific spectra or fingerprints of serum samples from 139 patients with CD and 120 with UC were obtained by FT-NIR, and analyzed by Artificial Neural Networks (ANN) and cluster analyses. ANN-analysis showed that the sensitivity, specificity and accuracy for IBD *vs* healthy controls were 94.5%, 99.1%, and 96.7%, respectively, compared to cluster analysis (71.8%, 66.6%, and 70.1%). Cluster analysis correctly identified 80% of UC and 61.5% of CD, while ANN-analysis was 69.8% and 91.8%, respectively. This proof-of-concept study suggests a potential usefulness of this technology in identifying serum "fingerprints" as serological biomarkers for IBD diagnostics.

Serum cytokines/chemokines and their receptors

Serum cytokines and their soluble forms of receptors have been extensively studied, both as markers for IBD diagnosis and as molecules for IBD pathogenesis, as well as IBD therapeutic targets[4,76-78]. A long list of cytokines/chemokines and their receptors have been shown to up-regulated in active and even inactive IBD, including IL-1α, IL-1β, IL-1R (R: receptor), IL-1Ra (Ra: receptor antagonist), IL-2, sIL-2R (s: soluble), IL-6, IL-6R, IL-7, IL-8, IL-10, IL-12, IL-15, IL-16, IL-17, IL-18R, IL-27, IFN-α, IFN-β, TGF-β, TNF-α, TNF-αR, as well as most chemokines. However, the diagnostic value of cytokines/chemokines has been limited, at least in part due to studies based on the analysis of individual or only a few selected cytokine/chemokines. The ideal situation would be to profile a large number of serum cytokines/chemokines from well characterized cohorts. An example of this is the utility of a more robust and high-throughput multiplex sandwich ELISA (which allows simultaneous analysis of up to 100 analytes), in conjunction with biostatistical analysis tools [such as that of discriminant functional analysis (DFA) and multidimensional scaling (MDS)], as we and others have described[79-83]. As a proof-of-concept in the context of IBD, we recently analyzed 16 serum cytokines by multiplex ELISA from a cohort of 64 mice with or without induced experimental colitis[84]. Distinctive disease-specific cytokine profiles were identified with significant correlations to disease activity and duration of disease. Our data showed that CD-like TNBS colitis exhibits heightened Th1-Th17 response (increased IL-12 and IL-17) as the disease becomes chronic. In contrast, UC-like DSS colitis switches from a Th1-Th17-mediated acute inflammation (increased TNFα, IL6, IL-17 and KC) to a predominant Th2-biased chronic inflammatory response (increase in IL-4 and IL-10 with concomitant decrease in TNFα, IL6, IL-17 and KC). DFA identified 5 discriminatory cytokine biomarkers (IL-6, 12, 4, 17, INF-γ) that can sufficiently distinguish healthy controls from diseases, and one disease type from another[84]. A pilot study profile of 17 human cytokines/chemokines from a small cohort of 33 IBD patients (19 CD and 14 UC) with 33 matched healthy controls using multiplex ELISA also identified patterns of cytokines/chemokines that were correlated with disease phenotypes (CD *vs* UC) and severity[82]. A similar human cytokine profiling study using 24 cytokines/chemokines from a larger cohort of 400 IBD patients (with various levels of disease severity) is near completion. It will be interesting to see if the cytokine profiles can: (1) enable disease subtype stratification; (2) correlate with disease severity; and (3) if the profiles that were identified in murine experimental colitis would be similar to those of human IBD.

CONCLUSION

It is evident that while current serological IBD biomarkers are useful, their clinical utility has been limited. New technologies, such as those described in this highlight, demonstrate the significant potential for identifying previously unrecognized IBD biomarkers. Future direction is predicted to be, in addition to the continuation of ongoing efforts in developing novel biomarkers using conventional and new technologies, the integration of multiple biomarkers with extensive bioinformatics analysis/modeling. This will be the key to eventually developing specific "endpoint-oriented" serological biomarker kits. These may include, but not be limited to, individual biomarker sets that are specific for one or more of the following: (1) differentiating CD *vs* UC *vs* normal *vs* other non-IBD gut diseases that share some similar clinical presentations (such as abdominal pain and diarrhea in infectious colitis or IBS); (2) predicting IBD risk (before disease onset; subclinical biomarkers) and disease course (risk of complication and surgery); (3) predicting therapeutic efficacy even before initiation of specific

medication(s); and (4) monitoring therapeutic efficacy and predicting relapse. One can envision that such kits will rely on "integrated algorithms", rather than absolute differences, to enhance the accuracy of diagnosis and/or prognosis of IBD.

ACKNOWLEDGMENTS

The authors thank the generous support of Mr. & Mrs. Morton Hyatt.

REFERENCES

1 **Vermeire S**, Van Assche G, Rutgeerts P. Laboratory markers in IBD: useful, magic, or unnecessary toys? *Gut* 2006; **55**: 426-431
2 **Nikolaus S**, Schreiber S. Diagnostics of inflammatory bowel disease. *Gastroenterology* 2007; **133**: 1670-1689
3 **Cho JH**. Inflammatory bowel disease: genetic and epidemiologic considerations. *World J Gastroenterol* 2008; **14**: 338-347
4 **Desai D**, Faubion WA, Sandborn WJ. Review article: biological activity markers in inflammatory bowel disease. *Aliment Pharmacol Ther* 2007; **25**: 247-255
5 **Langhorst J**, Elsenbruch S, Koelzer J, Rueffer A, Michalsen A, Dobos GJ. Noninvasive markers in the assessment of intestinal inflammation in inflammatory bowel diseases: performance of fecal lactoferrin, calprotectin, and PMN-elastase, CRP, and clinical indices. *Am J Gastroenterol* 2008; **103**: 162-169
6 **Geenen RW**, Hussain SM, Cademartiri F, Poley JW, Siersema PD, Krestin GP. CT and MR colonography: scanning techniques, postprocessing, and emphasis on polyp detection. *Radiographics* 2004; **24**: e18
7 **Schunk K**. Small bowel magnetic resonance imaging for inflammatory bowel disease. *Top Magn Reson Imaging* 2002; **13**: 409-425
8 **Bruining DH**, Loftus EV. Current and future diagnostic approaches: from serologies to imaging. *Curr Gastroenterol Rep* 2007; **9**: 489-496
9 **Van Limbergen J**, Russell RK, Nimmo ER, Ho GT, Arnott ID, Wilson DC, Satsangi J. Genetics of the innate immune response in inflammatory bowel disease. *Inflamm Bowel Dis* 2007; **13**: 338-355
10 **Schoepfer AM**, Trummler M, Seeholzer P, Criblez DH, Seibold F. Accuracy of four fecal assays in the diagnosis of colitis. *Dis Colon Rectum* 2007; **50**: 1697-1706
11 **Konikoff MR**, Denson LA. Role of fecal calprotectin as a biomarker of intestinal inflammation in inflammatory bowel disease. *Inflamm Bowel Dis* 2006; **12**: 524-534
12 **Peyrin-Biroulet L**, Standaert-Vitse A, Branche J, Chamaillard M. IBD serological panels: facts and perspectives. *Inflamm Bowel Dis* 2007; **13**: 1561-1566
13 **Papp M**, Norman GL, Altorjay I, Lakatos PL. Utility of serological markers in inflammatory bowel diseases: gadget or magic? *World J Gastroenterol* 2007; **13**: 2028-2036
14 **Meuwis MA**, Fillet M, Chapelle JP, Malaise M, Louis E, Merville MP. New biomarkers of Crohn's disease: serum biomarkers and development of diagnostic tools. *Expert Rev Mol Diagn* 2008; **8**: 327-337
15 **Kornbluth A**, Sachar DB. Ulcerative colitis practice guidelines in adults (update): American College of Gastroenterology, Practice Parameters Committee. *Am J Gastroenterol* 2004; **99**: 1371-1385
16 **Dotan I, Fishman S**, Dgani Y, Schwartz M, Karban A, Lerner A, Weishauss O, Spector L, Shtevi A, Altstock RT, Dotan N, Halpern Z. Antibodies against laminaribioside and chitobioside are novel serologic markers in Crohn's disease. *Gastroenterology* 2006; **131**: 366-378
17 **Dotan N**, Altstock RT, Schwarz M, Dukler A. Anti-glycan antibodies as biomarkers for diagnosis and prognosis. *Lupus* 2006; **15**: 442-450
18 **Sendid B**, Quinton JF, Charrier G, Goulet O, Cortot A, Grandbastien B, Poulain D, Colombel JF. Anti-Saccharomyces cerevisiae mannan antibodies in familial Crohn's disease. *Am J Gastroenterol* 1998; **93**: 1306-1310
19 **Satsangi J**, Landers CJ, Welsh KI, Koss K, Targan S, Jewell DP. The presence of anti-neutrophil antibodies reflects clinical and genetic heterogeneity within inflammatory bowel disease. *Inflamm Bowel Dis* 1998; **4**: 18-26
20 **Bossuyt X**. Serologic markers in inflammatory bowel disease. *Clin Chem* 2006; **52**: 171-181
21 **Peeters M**, Joossens S, Vermeire S, Vlietinck R, Bossuyt X, Rutgeerts P. Diagnostic value of anti-Saccharomyces cerevisiae and antineutrophil cytoplasmic autoantibodies in inflammatory bowel disease. *Am J Gastroenterol* 2001; **96**: 730-734
22 **Papp M**, Altorjay I, Dotan N, Palatka K, Foldi I, Tumpek J, Sipka S, Udvardy M, Dinya T, Lakatos L, Kovacs A, Molnar T, Tulassay Z, Miheller P, Norman GL, Szamosi T, Papp J, Lakatos PL. New serological markers for inflammatory bowel disease are associated with earlier age at onset, complicated disease behavior, risk for surgery, and NOD2/CARD15 genotype in a Hungarian IBD cohort. *Am J Gastroenterol* 2008; **103**: 665-681
23 **Joossens S**, Colombel JF, Landers C, Poulain D, Geboes K, Bossuyt X, Targan S, Rutgeerts P, Reinisch W. Anti-outer membrane of porin C and anti-I2 antibodies in indeterminate colitis. *Gut* 2006; **55**: 1667-1669
24 **von Roon AC**, Karamountzos L, Purkayastha S, Reese GE, Darzi AW, Teare JP, Paraskeva P, Tekkis PP. Diagnostic precision of fecal calprotectin for inflammatory bowel disease and colorectal malignancy. *Am J Gastroenterol* 2007; **102**: 803-813
25 **Quinton JF**, Sendid B, Reumaux D, Duthilleul P, Cortot A, Grandbastien B, Charrier G, Targan SR, Colombel JF, Poulain D. Anti-Saccharomyces cerevisiae mannan antibodies combined with antineutrophil cytoplasmic autoantibodies in inflammatory bowel disease: prevalence and diagnostic role. *Gut* 1998; **42**: 788-791
26 **Koutroubakis IE**, Petinaki E, Mouzas IA, Vlachonikolis IG, Anagnostopoulou E, Castanas E, Maniatis AN, Kouroumalis EA. Anti-Saccharomyces cerevisiae mannan antibodies and antineutrophil cytoplasmic autoantibodies in Greek patients with inflammatory bowel disease. *Am J Gastroenterol* 2001; **96**: 449-454
27 **Reese GE**, Constantinides VA, Simillis C, Darzi AW, Orchard TR, Fazio VW, Tekkis PP. Diagnostic precision of anti-Saccharomyces cerevisiae antibodies and perinuclear antineutrophil cytoplasmic antibodies in inflammatory bowel disease. *Am J Gastroenterol* 2006; **101**: 2410-2422
28 **Dubinsky MC**, Lin YC, Dutridge D, Picornell Y, Landers CJ, Farrior S, Wrobel I, Quiros A, Vasiliauskas EA, Grill B, Israel D, Bahar R, Christie D, Wahbeh G, Silber G, Dallazadeh S, Shah P, Thomas D, Kelts D, Hershberg RM, Elson CO, Targan SR, Taylor KD, Rotter JI, Yang H. Serum immune responses predict rapid disease progression among children with Crohn's disease: immune responses predict disease progression. *Am J Gastroenterol* 2006; **101**: 360-367
29 **Mow WS**, Vasiliauskas EA, Lin YC, Fleshner PR, Papadakis KA, Taylor KD, Landers CJ, Abreu-Martin MT, Rotter JI, Yang H, Targan SR. Association of antibody responses to microbial antigens and complications of small bowel Crohn's disease. *Gastroenterology* 2004; **126**: 414-424
30 **Papadakis KA**, Yang H, Ippoliti A, Mei L, Elson CO, Hershberg RM, Vasiliauskas EA, Fleshner PR, Abreu MT, Taylor K, Landers CJ, Rotter JI, Targan SR. Anti-flagellin (CBir1) phenotypic and genetic Crohn's disease associations. *Inflamm Bowel Dis* 2007; **13**: 524-530
31 **Spivak J**, Landers CJ, Vasiliauskas EA, Abreu MT, Dubinsky MC, Papadakis KA, Ippoliti A, Targan SR, Fleshner PR.

Antibodies to I2 predict clinical response to fecal diversion in Crohn's disease. *Inflamm Bowel Dis* 2006; **12**: 1122-1130
32 **Papp M**, Altorjay I, Norman GL, Shums Z, Palatka K, Vitalis Z, Foldi I, Lakos G, Tumpek J, Udvardy ML, Harsfalvi J, Fischer S, Lakatos L, Kovacs A, Bene L, Molnar T, Tulassay Z, Miheller P, Veres G, Papp J, Lakatos PL. Seroreactivity to microbial components in Crohn's disease is associated with ileal involvement, noninflammatory disease behavior and NOD2/CARD15 genotype, but not with risk for surgery in a Hungarian cohort of IBD patients. *Inflamm Bowel Dis* 2007; **13**: 984-992
33 **Buhner S**, Buning C, Genschel J, Kling K, Herrmann D, Dignass A, Kuechler I, Krueger S, Schmidt HH, Lochs H. Genetic basis for increased intestinal permeability in families with Crohn's disease: role of CARD15 3020insC mutation? *Gut* 2006; **55**: 342-347
34 **Harrer M**, Reinisch W, Dejaco C, Kratzer V, Gmeiner M, Miehsler W, Norman GL, Gangl A, Vogelsang H. Do high serum levels of anti-Saccharomyces cerevisiae antibodies result from a leakiness of the gut barrier in Crohn's disease? *Eur J Gastroenterol Hepatol* 2003; **15**: 1281-1285
35 **Arnott ID**, Landers CJ, Nimmo EJ, Drummond HE, Smith BK, Targan SR, Satsangi J. Sero-reactivity to microbial components in Crohn's disease is associated with disease severity and progression, but not NOD2/CARD15 genotype. *Am J Gastroenterol* 2004; **99**: 2376-2384
36 **Abreu MT**, Taylor KD, Lin YC, Hang T, Gaiennie J, Landers CJ, Vasiliauskas EA, Kam LY, Rojany M, Papadakis KA, Rotter JI, Targan SR, Yang H. Mutations in NOD2 are associated with fibrostenosing disease in patients with Crohn's disease. *Gastroenterology* 2002; **123**: 679-688
37 **Devlin SM**, Yang H, Ippoliti A, Taylor KD, Landers CJ, Su X, Abreu MT, Papadakis KA, Vasiliauskas EA, Melmed GY, Fleshner PR, Mei L, Rotter JI, Targan SR. NOD2 variants and antibody response to microbial antigens in Crohn's disease patients and their unaffected relatives. *Gastroenterology* 2007; **132**: 576-586
38 **Dassopoulos T**, Nguyen GC, Bitton A, Bromfield GP, Schumm LP, Wu Y, Elkadri A, Regueiro M, Siemanowski B, Torres EA, Gregory FJ, Kane SV, Harrell LE, Franchimont D, Achkar JP, Griffiths A, Brant SR, Rioux JD, Taylor KD, Duerr RH, Silverberg MS, Cho JH, Steinhart AH. Assessment of reliability and validity of IBD phenotyping within the National Institutes of Diabetes and Digestive and Kidney Diseases (NIDDK) IBD Genetics Consortium (IBDGC). *Inflamm Bowel Dis* 2007; **13**: 975-983
39 **Seibold F**, Stich O, Hufnagl R, Kamil S, Scheurlen M. Anti-Saccharomyces cerevisiae antibodies in inflammatory bowel disease: a family study. *Scand J Gastroenterol* 2001; **36**: 196-201
40 **Israeli E**, Grotto I, Gilburd B, Balicer RD, Goldin E, Wiik A, Shoenfeld Y. Anti-Saccharomyces cerevisiae and antineutrophil cytoplasmic antibodies as predictors of inflammatory bowel disease. *Gut* 2005; **54**: 1232-1236
41 **Hisabe T**, Matsui T, Sakurai T, Murakami Y, Tanabe H, Matake H, Yao T, Kamachi S, Iwashita A. Anti-Saccharomyces cerevisiae antibodies in Japanese patients with inflammatory bowel disease: diagnostic accuracy and clinical value. *J Gastroenterol* 2003; **38**: 121-126
42 **Lawrance IC**, Murray K, Hall A, Sung JJ, Leong R. A prospective comparative study of ASCA and pANCA in Chinese and Caucasian IBD patients. *Am J Gastroenterol* 2004; **99**: 2186-2194
43 **Magro F**, Araujo F, Pereira P, Meireles E, Diniz-Ribeiro M, Velosom FT. Soluble selectins, sICAM, sVCAM, and angiogenic proteins in different activity groups of patients with inflammatory bowel disease. *Dig Dis Sci* 2004; **49**: 1265-1274
44 **Herrlinger K**, Stange EF. [Azathioprine in chronic inflammatory bowel diseases. *Evidence base] Med Klin* (Munich) 2000; **95**: 201-206
45 **Herrlinger KR**, Dittmann R, Weitz G, Wehkamp J, Ludwig D, Schwab M, Stange EF, Fellermann K. Serum procalcitonin differentiates inflammatory bowel disease and self-limited colitis. *Inflamm Bowel Dis* 2004; **10**: 229-233
46 **Ferrante M**, Henckaerts L, Joossens M, Pierik M, Joossens S, Dotan N, Norman GL, Altstock RT, Van Steen K, Rutgeerts P, Van Assche G, Vermeire S. New serological markers in inflammatory bowel disease are associated with complicated disease behaviour. *Gut* 2007; **56**: 1394-1403
47 **Pang Z**, Otaka K, Maoka T, Hidaka K, Ishijima S, Oda M, Ohnishi M. Structure of beta-glucan oligomer from laminarin and its effect on human monocytes to inhibit the proliferation of U937 cells. *Biosci Biotechnol Biochem* 2005; **69**: 553-558
48 **Schwarz M**, Spector L, Gargir A, Shtevi A, Gortler M, Altstock RT, Dukler AA, Dotan N. A new kind of carbohydrate array, its use for profiling antiglycan antibodies, and the discovery of a novel human cellulose-binding antibody. *Glycobiology* 2003; **13**: 749-754
49 **Schwarz M**, Spector L, Gortler M, Weisshaus O, Glass-Marmor L, Karni A, Dotan N, Miller A. Serum anti-Glc(alpha1,4)Glc(alpha) antibodies as a biomarker for relapsing-remitting multiple sclerosis. *J Neurol Sci* 2006; **244**: 59-68
50 **Simondi D**, Mengozzi G, Betteto S, Bonardi R, Ghignone RP, Fagoonee S, Pellicano R, Sguazzini C, Pagni R, Rizzetto M, Astegiano M. Antiglycan antibodies as serological markers in the differential diagnosis of inflammatory bowel disease. *Inflamm Bowel Dis* 2008; **14**: 645-651
51 **Henckaerts L**, Pierik M, Joossens M, Ferrante M, Rutgeerts P, Vermeire S. Mutations in pattern recognition receptor genes modulate seroreactivity to microbial antigens in patients with inflammatory bowel disease. *Gut* 2007; **56**: 1536-1542
52 **Lakatos PL**, Altorjay I, Mandi Y, Lakatos L, Tumpek J, Kovacs A, Molnar T, Tulassay Z, Miheller P, Palatka K, Szamosi T, Fischer S, Papp J, Papp M. Interaction between seroreactivity to microbial antigens and genetics in Crohn's disease: is there a role for defensins? *Tissue Antigens* 2008; **71**: 552-559
53 **Browning BL**, Annese V, Barclay ML, Bingham SA, Brand S, Buning C, Castro M, Cucchiara S, Dallapiccola B, Drummond H, Ferguson LR, Ferraris A, Fisher SA, Gearry RB, Glas J, Henckaerts L, Huebner C, Knafelz D, Lakatos L, Lakatos PL, Latiano A, Liu X, Mathew C, Muller-Myhsok B, Newman WG, Nimmo ER, Noble CL, Palmieri O, Parkes M, Petermann I, Rutgeerts P, Satsangi J, Shelling AN, Siminovitch KA, Torok HP, Tremelling M, Vermeire S, Valvano MR, Witt H. Gender-stratified analysis of DLG5 R30Q in 4707 patients with Crohn disease and 4973 controls from 12 Caucasian cohorts. *J Med Genet* 2008; **45**: 36-42
54 **Molnar T**, Hofner P, Nagy F, Lakatos PL, Fischer S, Lakatos L, Kovacs A, Altorjay I, Papp M, Palatka K, Demeter P, Tulassay Z, Nyari T, Miheller P, Papp J, Mandi Y, Lonovics J. NOD1 gene E266K polymorphism is associated with disease susceptibility but not with disease phenotype or NOD2/CARD15 in Hungarian patients with Crohn's disease. *Dig Liver Dis* 2007; **39**: 1064-1070
55 **Vandewalle-El** Khoury P, Colombel JF, Joossens S, Standaert-Vitse A, Collot M, Halfvarson J, Ayadi A, Landers CJ, Vermeire S, Rutgeerts P, Targan SR, Chamaillard M, Mallet JM, Sendid B, Poulain D. Detection of antisynthetic mannoside antibodies (ASigmaMA) reveals heterogeneity in the ASCA response of Crohn's disease patients and contributes to differential diagnosis, stratification, and prediction. *Am J Gastroenterol* 2008; **103**: 949-957
56 **Sakiyama T**, Fujita H, Tsubouchi H. Autoantibodies against ubiquitination factor E4A (UBE4A) are associated with severity of Crohn's disease. *Inflamm Bowel Dis* 2008; **14**: 310-317
57 **Lehrke M**, Konrad A, Schachinger V, Tillack C, Seibold F, Stark R, Parhofer KG, Broedl UC. CXCL16 is a surrogate marker of inflammatory bowel disease. *Scand J Gastroenterol* 2008; **43:** 283-288

58 **Broedl UC**, Schachinger V, Lingenhel A, Lehrke M, Stark R, Seibold F, Goke B, Kronenberg F, Parhofer KG, Konrad-Zerna A. Apolipoprotein A-IV is an independent predictor of disease activity in patients with inflammatory bowel disease. *Inflamm Bowel Dis* 2007; **13**: 391-397

59 **Konrad A**, Lehrke M, Schachinger V, Seibold F, Stark R, Ochsenkuhn T, Parhofer KG, Goke B, Broedl UC. Resistin is an inflammatory marker of inflammatory bowel disease in humans. *Eur J Gastroenterol Hepatol* 2007; **19**: 1070-1074

60 **Gough PJ**, Garton KJ, Wille PT, Rychlewski M, Dempsey PJ, Raines EW. A disintegrin and metalloproteinase 10-mediated cleavage and shedding regulates the cell surface expression of CXC chemokine ligand 16. *J Immunol* 2004; **172**: 3678-3685

61 **Vowinkel T**, Mori M, Krieglstein CF, Russell J, Saijo F, Bharwani S, Turnage RH, Davidson WS, Tso P, Granger DN, Kalogeris TJ. Apolipoprotein A-IV inhibits experimental colitis. *J Clin Invest* 2004; **114**: 260-269

62 **Bokarewa M**, Nagaev I, Dahlberg L, Smith U, Tarkowski A. Resistin, an adipokine with potent proinflammatory properties. *J Immunol* 2005; **174**: 5789-5795

63 **Kohn EC**, Azad N, Annunziata C, Dhamoon AS, Whiteley G. Proteomics as a tool for biomarker discovery. *Dis Markers* 2007; **23**: 411-417

64 **Hanash SM**, Pitteri SJ, Faca VM. Mining the plasma proteome for cancer biomarkers. *Nature* 2008; **452**: 571-579

65 **Spisak S**, Tulassay Z, Molnar B, Guttman A. Protein microchips in biomedicine and biomarker discovery. *Electrophoresis* 2007; **28**: 4261-4273

66 **Chaerkady R**, Pandey A. Applications of proteomics to lab diagnosis. *Annu Rev Pathol* 2008; **3**: 485-498

67 **deVera IE**, Katz JE, Agus DB. Clinical proteomics: the promises and challenges of mass spectrometry-based biomarker discovery. *Clin Adv Hematol Oncol* 2006; **4**: 541-549

68 **Meuwis MA**, Fillet M, Geurts P, de Seny D, Lutteri L, Chapelle JP, Bours V, Wehenkel L, Belaiche J, Malaise M, Louis E, Merville MP. Biomarker discovery for inflammatory bowel disease, using proteomic serum profiling. *Biochem Pharmacol* 2007; **73**: 1422-1433

69 **Meuwis MA**, Fillet M, Lutteri L, Maree R, Geurts P, de Seny D, Malaise M, Chapelle JP, Wehenkel L, Belaiche J, Merville MP, Louis E. Proteomics for prediction and characterization of response to infliximab in Crohn's disease: a pilot study. *Clin Biochem* 2008; **41**: 960-967

70 **Nanni P**, Parisi D, Roda G, Casale M, Belluzzi A, Roda E, Mayer L, Roda A. Serum protein profiling in patients with inflammatory bowel diseases using selective solid-phase bulk extraction, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and chemometric data analysis. *Rapid Commun Mass Spectrom* 2007; **21**: 4142-4148

71 **Subramanian V**, Subramanian D, Pollok RC. Serum protein signatures determined by mass Spectrometry (SELDI-ToF) accurately distinguishes Crohn's disease (CD) from ulcerative colitis (UC). *Gastroenterology* 2008; **134**: A196

72 **Sullivan S**, Zhu H, Cuffari C, Brant S, Tao S, Chen YP, Li X. Identification and validation of serological IBD biomarkers by a novel high throughput proteomic approach using high density protein chip technology. *Gastroenterology* 2006; **130**: A24

73 **Vermeulen N**, Vermeire S, Michiels G, Joossens M, Rutgeerts PJ, Bosuyt X. Protein Microarray experiments for profiling of the autoimmune response in inflammatory bowel disease; identification of PHLA1. *Gastroenterology* 2008; **134**: A197

74 **Kader HA**, Tchernev VT, Satyaraj E, Lejnine S, Kotler G, Kingsmore SF, Patel DD. Protein microarray analysis of disease activity in pediatric inflammatory bowel disease demonstrates elevated serum PLGF, IL-7, TGF-beta1, and IL-12p40 levels in Crohn's disease and ulcerative colitis patients in remission versus active disease. *Am J Gastroenterol* 2005; **100**: 414-423

75 **Haas SL**, Bocker U, Bugert P, Singer MV, Backhaus JP. Application of Fourier transform near-infrared spectroscopy of serum samples in patients with inflammatory bowel disease-A pilot study. *Gastroenterology* 2008; **134**: A201

76 **Kaser A**, Tilg H. Novel therapeutic targets in the treatment of IBD. *Expert Opin Ther Targets* 2008; **12**: 553-563

77 **Kmiec Z**. Cytokines in inflammatory bowel disease. *Arch Immunol Ther Exp* (Warsz) 1998; **46**: 143-155

78 **Brown SJ**, Mayer L. The immune response in inflammatory bowel disease. *Am J Gastroenterol* 2007; **102**: 2058-2069

79 **Papadakis KA**, Targan SR. Role of cytokines in the pathogenesis of inflammatory bowel disease. *Annu Rev Med* 2000; **51**: 289-298

80 **Alex P**, Gucek M, Li X. Applications of proteomics in the study of inflammatory bowel diseases: Current status and future directions with available technologies. *Inflamm Bowel Dis*; 2009; **15:** 616-629

81 **Szodoray P**, Alex P, Chappell-Woodward CM, Madland TM, Knowlton N, Dozmorov I, Zeher M, Jarvis JN, Nakken B, Brun JG, Centola M. Circulating cytokines in Norwegian patients with psoriatic arthritis determined by a multiplex cytokine array system. *Rheumatology* (Oxford) 2007; **46**: 417-425

82 **Alex P**, Zachos NC, Conklin LS, Kwon JH, Harris ML, Bayless TM, Centola M, Li XH. Distinct cytokine patterns as effective indicators of disease activity and severity in IBD. *Gastroenterology* 2008; **134**: A204-A204

83 **Alex P**, Szodoray P, Knowlton N, Dozmorov IM, Turner M, Frank MB, Arthur RE, Willis L, Flinn D, Hynd RF, Carson C, Kumar A, El-Gabalawy HS, Centola M. Multiplex serum cytokine monitoring as a prognostic tool in rheumatoid arthritis. *Clin Exp Rheumatol* 2007; **25**: 584-592

84 **Alex P**, Zachos NC, Nguyen T, Gonzales L, Chen TE, Conklin LS, Centola M, Li X. Distinct cytokine patterns identified from multiplex profiles of murine DSS and TNBS-induced colitis. *Inflamm Bowel Dis*; 2009; **15:** 341-52

S- Editor Li DL **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5125

World J Gastroenterol 2008 September 7; 14(33): 5125-5132
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Emiko Mizoguchi, MD, PhD, *Series Editor*

Helminth infections and intestinal inflammation

Li Jian Wang, Yue Cao, Hai Ning Shi

Li Jian Wang, Yue Cao, Hai Ning Shi, Mucosal Immunology Laboratory, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts 02129, United States
Supported by Grant DK 074727 (R21) from the National Institutes of Health; An unrestricted educational grant from Wyeth Nutrition; A research fellowship from the Research Center for High Altitude Medicine, Qinghai University Medical School, China
Correspondence to: Hai Ning Shi, DVM, PhD, Mucosal Immunology Laboratory, Massachusetts General Hospital, Building 114 16th Street, Room 3504, Charlestown, Massachusetts 02129, United States. shiha@helix.mgh.harvard.edu
Telephone: +1-617-7264173 Fax: +1-617-7264172
Received: August 5, 2008 Revised: August 14, 2008
Accepted: August 21, 2008
Published online: September 7, 2008

Abstract

Evidence from epidemiological studies indicates an inverse correlation between the incidence of certain immune-mediated diseases, including inflammatory bowel diseases (IBD), and exposure to helminths. Helminth parasites are the classic inducers of Th2 responses. The Th2-polarized T cell response driven by helminth infection has been linked to the attenuation of some damaging Th1 driven inflammatory responses, preventing some Th1-mediated autoimmune diseases in the host, including experimentally induced colitis. Helminth parasites (the porcine whipworm, *Trichuris suis*) have been tested for treating IBD patients, resulting in clinical amelioration of the disease. As a result, there is a great deal of interest in the research community in exploring the therapeutic use of helminth parasites for the control of immune-mediated diseases, including IBD. However, recent studies have provided evidence indicating the exacerbating effects of helminths on bacterial as well as non-infectious colitis in animal models. Therefore, a better understanding of mechanisms by which helminths modulate host immune responses in the gut may reveal novel, more effective and safer approaches to helminth-based therapy of IBD.

Key words: Helminths; Innate and adaptive immunity; Intestinal inflammation

Peer reviewer: Hitoshi Asakura, Director, Emeritus Professor, International Medical Information Center, Shinanomachi Renga Bldg.35, Shinanomachi, Shinjukuku, Tokyo 160-0016, Japan

Wang LJ, Cao Y, Shi HN. Helminth infections and intestinal inflammation. *World J Gastroenterol* 2008; 14(33): 5125-5132 Available from: URL: http://www.wjgnet.com/1007-9327/14/5125.asp DOI: http://dx.doi.org/10.3748/wjg.14.5125

HELMINTH INFECTIONS

Helminths are divided into two major phyla, and all members of each phylum have distinct structural features that separate them from the others. The nematodes (also known as roundworms) include the major intestinal worms and the filarial worms that cause lymphatic filariasis (LF), whereas the platyhelminths include the trematodes (flukes), such as the schistosomes, and the cestodes (tapeworms). Humans and helminths have co-existed throughout our evolution. The history of helminthiases can be tracked back to the earliest record of human beings[1]. Although effective preventive and therapeutic measures have been developed for most parasitic worms, helminth infections are still very common in the developing world today. It has been estimated that one billion people worldwide are infected with one or more helminths. Most of the victims live in regions of sub-Saharan Africa, Asia, and Latin America[2-3]. The most common forms of helminthiasis are infections caused by intestinal helminths, ascariasis, trichuriasis and hookworm, followed by schistosomiasis and LF[4]. Clinical features of helminthiases vary a lot depending on the helminth species, intensity of infection, and host age. *Taenia solium* can cause neurocysticercosis with mass lesions in brain. Ingested eggs of *Echinococcus granulosis* will lead to cysts in the liver and cause life-threatening anaphylaxis if antigens are released from the cysts. Chronic infection with *Schistosoma* causes granulomas, fibrosis, and inflammation of the spleen and liver. Hookworm and schistosomiasis can infect pregnant women, cause neonatal prematurity and increased maternal morbidity and mortality[5]. Children at school-age or younger tend to harbor the greatest numbers of intestinal worms and schistosomes compared with any other age group. As a

result, the young patients suffer from growth retardation and diminished physical fitness, as well as memory and cognition impairment[6]. Most helminth infections, if left untreated, result in multi-year, chronic inflammatory disorders that will eventually cause disability. The chronic, disabling and disfiguring consequences of helminth infections, together with their high prevalence, make them a global problem of significant medical, educational, and economic impact.

HELMINTH INFECTION INDUCES TH2 AND T REG DEVELOPMENT

Since parasitic worms constitute a very heterogenous collection of organisms, a multitude of mechanisms may be expected when they stimulate and modulate host immune responses. However, examination of the immunology of helminth infections reveals a number of characteristics that are generally conserved across all species. Our current understanding of the immune response to helminth infections has largely come from the study of well-defined laboratory models of infection in rodents. Protective immunity to helminths depends on T lymphocytes. It is now well established that the CD4+ subset of T cells plays a major role in the generation of the host protective response that expels the worms, and that CD4+ T cells regulate many of the inflammatory and immune parameters that accompany expulsion of the parasites from the gut. Based on cytokine production profiles, CD4+ T cells can be divided into distinct functional sub-populations, Th1, Th2 and Th17. Th1 cells produce cytokines such as IFN-γ, IL-12 and IL-2 that are involved in controlling of intracellular pathogens. These cells also contribute to inflammatory responses. Recently, a new lineage of Th cells, that selectively produce the cytokine IL-17 (Th17) has been identified, and those cells are thought to be key regulators of inflammation[7,8]. It has also been reported that IL-17 is required for the optimal induction of Th1 and Th2 immune response, although the mechanism has not yet been clarified[9]. IL-17 is a proinflammatory cytokine, which is produced predominately by activated T cells. This cytokine can enhance T cell priming and induces the activation of many cell types including fibroblasts, macrophages and epithelial cells, resulting in the production of multiple proinflammatory mediators by these cells, including IL-1, IL-6, TNF-α and chemokines[8,10]. In the majority of cases, the immune responses of the hosts to worm infections are strikingly dominated by Th2-like T-helper cell responses with a significant production of IL-4, IL-5, IL-10, IL-13, IL-25, and IL-31[7,8,11,12]. Th2 cells contribute to B cell activation and antibody production, eosinophil differentiation and recruitment. Therefore, parasitic worm infection is often associated with high levels of IgE, IgG1 and IgG4, and robust eosinophil and mast cell responses. Th2 immune responses to helminth infections can prevent the survival of invading parasites during a homologous secondary infection[9], expel adult parasites from the gut[10], and allow host survival when the immune responses are not able to clear the parasites. These responses are the basic host protective mechanisms against worms and are highly conserved through evolution. However, Th2 immune responses to helminth infections may also cause host pathology and organ damage. For example, Th2 immunity can mediate pathological fibrotic responses in chronic parasitic infections[13]. This can be understood as the detrimental effects of host immunity to helminths. Cross-regulatory suppression of the Th1 responses by a strong Th2 response has been considered to play a role in modulating diseases that are characterized by a Th1 response. Multiple studies[14-16] have revealed that helminth-induced Th2 responses can attenuate damaging Th1-driven inflammatory responses in the host. However, suppression of Th1 response may also lead to the impairment of host protective Th1 immunity against concurrent infections caused by bacterial, viral and parasitic pathogens, exacerbating the diseases they induce.

In addition to stimulating a vigorous Th2 response, helminth infections are also capable of inducing suppressive T cell populations known as regulatory T cells (Tregs)[17], which may help control morbidity and dampen resistance to re-infection through their potent immune regulatory mechanisms. Several types of Treg cells have been described (natural Tregs, Tr1 and Th3). These regulatory cells constitute 5% to 10% of peripheral CD4+ T cells in naive mice and humans and suppress several potentially pathogenic responses *in vivo*, particularly T cell responses directed to self-antigens. These cells express markers such as Foxp3, CD25, CTLA-4 and GITR, and often secrete IL-10 and/or TGF-β[18-20]. Tregs have been shown to play an important role in regulating immune responses and maintaining homeostasis under various disease conditions including autoimmune disease, inflammation, cancer, and microbial infections. Treg regulate immunity through both cytokine-dependent and independent mechanisms[21]. Th3 cells make TGF-β, which inhibits development of both Th1 and Th2 cells. Tr1 cells regulate immunity through IL-10-dependent mechanisms, which inhibit both Th1 and Th2 responses[22-24]. A recent study has provided a link between the well-established immunoregulatory capacity of Th2 cells and Tregs showing that the Il-4Ra binding cytokines, IL-4 and IL-13 induce FOXP3 expressing Treg from CD4+CD25- precursors[25].

Existing data indicate that helminth infections induce the development of Th2 and/or Treg responses. A polarized Th2 type immune response as well as up-regulated regulatory T cell activity induced by helminths may have a significant impact on the host's ability to cope with concurrent or subsequent viral[26,27], bacterial and parasitic infections[28], by suppressing host protective Th1 responses to microbial pathogens. Consistent with the hygiene hypothesis, helminth-induced responses may also underlie the observed reverse associations between helminthiases and asthma, allergy, IBD, and

other autoimmune diseases[29-31]. A better understanding of immune modulation by helminth infection, therefore, can have significant practical implications for the prevention and treatment of immune-mediated as well as microbial disease.

HELMINTHS AFFECT HOST RESPONSES TO OTHER ANTIGENS AND PATHOGENS

The major importance of helminth infections includes not only the direct pathogenic effect of the worms as described above, but also the modulation of the host immune system, which may alter the response to other pathogens and antigens and cause additional immunopathology. The distribution of several pathogenic helminth infections coincides geographically with many devastating microbial diseases, such as HBV, HCV, HIV/AIDS[26,27,32-34] and malaria[35-37]. Coinfection with helminths increases the transmission of and susceptibility to these infections, and also increases the severity of the associated diseases[27,33-38]. Recently we have demonstrated that an intestinal nematode parasite, *Heligomosomoides polygyrus (H. polygyrus)* infection impairs host Th1 defense against enteric bacterial infection and exacerbates *Citrobacter*-mediated intestinal injury[28,39]. This observation is keeping with a report showing that infection with the helminth *Fasciola hepatica* reduced the protective Th1 response to coinfected *Bordetella pertussis* and exacerbated the bacterial infection[40]. Likewise, infection with *Schistosoma mansoni* downregulated antigen-specific Th1 cytokines and cytotoxic-T-lymphocyte responses, resulting in a delay in vaccinia virus clearance[41]. Moreover, it has been shown that a combined infection with *Trichuris suis* and *Campylobacter jejuni* in immunologically naive, germfree piglets, resulted in an enhanced invasion of the colon by *C. jejuni*, leading to the development of more severe pathology[42]. These observations provide strong evidence to demonstrate that helminth infection can dampen Th1 reactions to other infections and cause impaired immune responses to concurrent viral, bacterial, and parasite infections, as well as to vaccination. The Th2-inducing helminth infection has also been shown to inhibit the development of CD8+ T cell responses[43].

HELEMINTHS MODULATE INFLAMMATORY DISORDERS OF THE HOST

It has been observed that there is an increased incidence of autoimmune disorders and allergic diseases in the developed world. This phenomenon is thought to be the consequence of eradication of major infectious diseases, including helminth infections in this part of the world, a theory termed the hygiene hypothesis. The inverse correlation observed between helminth infection and certain immune-mediated diseases has led to the suggestion that lack of helminth infections favors the induction of Th1 responses, which may, in turn, result in the clinical appearance of gastrointestinal diseases, including inflammatory bowel diseases (IBD)[44-46]. IBD, including Crohn's disease and ulcerative colitis are chronic immune diseases of the gastrointestinal tract with unknown etiology. The current hypothesis indicates that IBD results from an uncontrolled immune response to the normal gut flora[47]. Genetic factors and environmental factors both contribute to the damaging mucosal immune response[48]. The hygiene hypothesis suggests that microbes and worms are important for shaping and tuning the development and function of our immune system[49]. The growing body of epidemiological and experimental data strongly suggest that a reduction in helminth infection is linked to rising rates of autoimmunity and atopy.

The initial work by Elliott *et al*[50] showing a protective response of *Schistosoma mansoni* infection on trinitrobenzene sulphonic acid (TNBS)-induced colitis in mice, a chemically induced Th1-type colitis used as an experimental model of human IBD, have led to several animal studies determining the role of helminth infections in different IBD models. In 2001, Reardon *et al*[51] showed that infection of mice with the tapeworm *Hymenolepis diminuta* ameliorated dextran sodium sulphate (DSS)-induced colitis. Khan *et al*[52] showed that mice that were infected with the nematode, *Trichinella spiralis* are protected from colitis induced by intrarectal challenge with dinitrobenzene sulphate (DNBS). Furthermore, schistosome eggs also provide a protective effect on TNBS-induced colitis in mice[53] and infection with *H. polygyrus* or *T. muris* can prevent or reverse the chronic spontaneous Th1-type colitis in IL-10 deficient mice[46]. A protective effect of infection with *Schistosoma mansoni* on TNBS-colitis in rats was also reported[54]. These observations provide evidence to suggest that helminth parasites (nematode, cestode and trematode) can ameliorate chemically induced colitis in different models. In line with these observations, the Th2 polarized T-cell response driven by helminth infection has also been linked to the amelioration of some Th1-mediated diseases that develop concurrently, such as *Helicobacter*-induced gastritis[14].

Evidence not only suggests that helminths can attenuate experimentally induced IBD in animal models[42,53,54], but also the pioneering work of Weinstock *et al* shows promise using *Trichiuris suis,* a pig helminth, to treat Crohn's patients[16]. Therapeutic potential has also been indicated in CD patients who were infected with the human hookworm *Necator americanus*[55]. The effects of helminth on host intestinal Th1 function could be mediated through several mechanisms, including helminth-induced Th2 response and induction of Tregs. We have shown that infection with the intestinal helminth *H. polygyrus* results in an upregulation of colonic IL-4 expression[28]. Recently Setiawan *et al*[56] have provided evidence to show that *H. polygyrus* infection promotes Th2 cytokine responses (IL-4, IL-5 and IL-13) of colonic lamina propria mononuclear cells. Helminths also induce production of powerful immune modulatory

molecules like IL-10 and TGF-β[57] that can affect both Th1 and Th2 function.

Unfortunately, the anticolitic effect evoked in response to helminths in mice does not extend to all models of colonic inflammation. In a recent study, the ability of the rat tapeworm *Hymenolepis diminuta* to affect the course of oxazolone-induced colitis was determined. A detrimental effect of helminth infection on colitis was detected, as evidenced by the results showing that *H. diminuta* caused a significant exacerbation of oxazolone-induced colitis[58].

Considering the profound and widespread immune activation and dysregulation induced by helminth parasites, the overlapping geographic distributions of helminth and bacterial infections, and the potential to modulate bacteria-associated intestinal inflammation, we have established a co-infection model system to analyze the effect of an intestinal helminth parasite on a concurrent enteric bacterial infection and bacteria-mediated intestinal inflammation[28]. This model involves two murine enteric infectious agents that induce distinct Th-responses: (1) the Th2-inducing helminth *H. polygyrus*, and (2) the bacterial pathogen, *C. rodentium*, which selectively stimulates a Th1-type immune response[59,60]. *C. rodentium* is a mouse pathogen that colonizes the distal colon and causes pathological changes that are similar to those seen in many mouse models of colitis, including transmissible colonic hyperplasia, goblet cell depletion, and mucosal erosion. *C. rodentium* has been used as a model for studying host responses to human pathogens that employ attaching and effacing (A/E) lesion formation for epithelial colonization, such as EPEC, the most important causative agent of severe infantile diarrhea[59,61-64]. We found that mice co-infected with *H. polygyrus* developed a more severe *C. rodentium*-associated colitis. The exacerbation of bacterial colitis induced by helminth co-infection is STAT6 dependent. These results provide evidence to indicate the possibility that helminth infection may have adverse effects on intestinal inflammation (bacteria-mediated as well as inducible colitis). In addition, it has been also reported that *T. suris* ova treatment resulted in infection in the gastrointestinal tract of a pediatric Crohn's patient[65]. These observed deleterious consequences of helminth parasites in intestinal inflammatory responses, therefore, provide a cautionary note for the therapeutic use of helminths in certain forms of IBD. These observations also highlight the need for a more comprehensive understanding of the mechanisms by which helminths modulate host's responses to enteric bacteria and bacteria-associated as well as immune-mediated intestinal inflammation.

IMPACT OF HELMINTHS ON INNATE IMMUNE OF THE HOST

Innate immune cells such as DCs and macrophages are fundamental to directing immune responses along either a tolerating or activating pathway. As master manipulators of the host immune system, helminths have evolved strategies targeting these cells. To effectively control infection, appropriately developed and functionally polarized subsets of Th effector cells are required. DCs, the most competent antigen presenting cells, play an important role in the initiation of both innate and adaptive immune responses to a wide variety of pathogens and Ags, as well as in tolerance[66-69]. DCs can be activated by microbial products through their high-level expression of evolutionarily conserved pattern recognition receptors, such as Toll-like receptors (TLRs)[70,71]. The signals derived from various types of microbial components, such as those from helminth and bacteria, are translated by the DCs into a stimulus for T cells, leading to a productive T cell response. The T cell stimuli derived from DCs include MHC II-peptide (signal 1), the expression or absence of costimulatory molecules (signal 2), and expression or absence of polarizing cytokines (signal 3)[72,73]. Microbial antigens can activate DCs through ligation of TLRs, leading to the up-regulation of functional surface molecules, and the release of cytokines, such as IL-12, IL-18 and IL-27, resulting in the induction of Th1 immunity[70,71,74]. Although it is less clear, there is also evidence indicating that DC function is modulated during helminth infection[75]. Different helminth-derived molecules have been suggested to be able to induce DC activation, and subsequent Th2 and/or T reg responses. Studies have indicated that two *Schistosome* egg-derived components (the glycolipid lysophosphatidylserine and the carbohydrate determinant lacto-N-fucopentaose Ⅲ) can activate TLR4 and TLR2, respectively, in myeloid DCs[76]. Live schistosoma eggs can activate DCs through TLR2 and TLR3[77]. Parasite secreted compounds like ES-62 from filarial nematodes induce the development of DCs and are capable of driving a Th2 response, modulating cytokine production (IL-12 and TNF-α) in macrophages and DCs *via* the TLR4 pathway[78]. A recent report suggests that helminth infection may alter TLR 4 expression in mucosal T cells, and these T cells when stimulated with LPS produce TGF-β[79]. Additionally, murine bone marrow-derived DCs pulsed with the helminth excretory/secretory antigen can, on transfer to naive recipients, prime mice for Th2 responsiveness[80]. Other studies also indicate that the soluble fractions from S. mansoni eggs may alter TLR ligand-induced activation of DCs[81]. These results clearly indicate the possibility that helminth infection may modulate TLR expression of DCs and responsiveness of DCs to TLR ligands.

In addition to Ag-specific MHC-peptide complexes and costimulatory molecules (B7.1 and B7.2, signal 2), other signals derived from Ag-primed DCs have been thought to be required for the differentiation of naive Th cells into Th1 and Th2 cells[72,73,82]. It has been shown recently by us and by others that intestinal helminth infection induces DC activation and up-regulates the DC IL-10 response[39,83], which may provoke a Th2 and/or T reg dominant response.

HELMINTH INFECTION INDUCES THE DEVELOPMENT OF ALTERNATIVELY ACTIVATED MACROPHAGES

The helminth infection induced-Th2 cytokine response has been suggested to affect macrophages[84]. Although macrophages share some functional similarities with DCs, macrophages have distinct functions. As discussed above, DCs play an important role in initiating and regulating host immune responses, whereas macrophages, contribute significantly to the effector phase, i.e. elimination of bacteria, and are also thought to be critical mediators of many chronic inflammatory diseases. Distinct phenotypes of macrophages have also been reported. Activation of macrophages by bacterial products (through TLR engagement) or pro-inflammatory stimuli such as Th1 cytokines leads to the development of the classically-activated macrophages. We and others have shown that helminth-induced Th2 cytokines induce a different phenotype, namely, the alternatively activated macrophages[85-87]. One of the distinctive characteristics of this type of macrophage is the ability to suppress the proliferation of other cells with which they are co-cultured[88,89]. A recent report suggests that alternative activation of macrophages by IL-4 results in impaired nitric oxide (NO) production and increased expression of the transferrin receptor, supporting intracellular growth of bacteria (*Mycobacterium tuberculosis*)[90]. Recently, we observed that the exacerbated *C. rodentium*-mediated colitis that develops in helminth-coinfected mice correlates with the marked accumulation of alternatively activated macrophages in colonic LP *via* a STAT6-dependent mechanism[85]. Functional analysis indicates that these helminth-stimulated macrophages have an impaired ability to effectively control the multiplication of phagocytosed *C. rodentium*. Presumably as a result of the increased bacterial load, these cells also produce increased amounts of TNF-α, a cytokine that has a well-established role in intestinal and other types of inflammation[91,92]. These observations, therefore, provide evidence to suggest an underlying mechanism for the enhanced bacterial infection and exacerbated bacteria-induced intestinal injury in hosts that are coinfected with helminth parasites.

CONCLUSION

In many developing countries, exposure to helminth infections and simultaneous infection with other pathogens, such as enteric bacteria, are quite common. Recent evidence indicates that in the developed world, a complete absence of helminth infection may be a predisposing factor for the development of certain immune-mediated disorders[45]. The emerging evidence indicates the complexity of immune regulation by helminths in host protective immunity and inflammatory diseases. The differences in the effects of helminths on the development and progression of intestinal inflammation observed in various models emphasize the need for a better understanding of the mechanisms by which helminths modulate host mucosal immunity. It is clear that a more thorough understanding of the complex relationship between the human host and parasitic worms will be required to develop safer, novel and more effective treatments for microbial diseases and immune-mediated disorders such as IBD.

ACKNOWLEDGMENTS

We thank Dr. Bobby Cherayil for critical review of the manuscript.

REFERENCES

1 **Cox FE**. History of human parasitology. *Clin Microbiol Rev* 2002; **15**: 595-612
2 **Hotez PJ**, Ferris MT. The antipoverty vaccines. *Vaccine* 2006; **24**: 5787-5799
3 **Hotez PJ**, Molyneux DH, Fenwick A, Kumaresan J, Sachs SE, Sachs JD, Savioli L. Control of neglected tropical diseases. *N Engl J Med* 2007; **357**: 1018-1027
4 **Hotez PJ**, Brindley PJ, Bethony JM, King CH, Pearce EJ, Jacobson J. Helminth infections: the great neglected tropical diseases. *J Clin Invest* 2008; **118**: 1311-1321
5 **Christian P**, Khatry SK, West JP. Antenatal anthelminthic treatment, Birth weight, and infant survival in rural Nepal. *Lancet* 2004; **364**: 981-983
6 **Crompton DW**, Nesheim MC. Nutritional impact of intestinal helminthiasis during the human life cycle. *Annu Rev Nutr* 2002; **22**: 35-59
7 **Fort MM**, Cheung J, Yen D, Li J, Zurawski SM, Lo S, Menon S, Clifford T, Hunte B, Lesley R, Muchamuel T, Hurst SD, Zurawski G, Leach MW, Gorman DM, Rennick DM. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity* 2001; **15**: 985-995
8 **Harnett W**, Harnett MM. Molecular basis of worm-induced immunomodulation. *Parasite Immunol* 2006; **28**: 535-543
9 **Voehringer D**, Reese TA, Huang X, Shinkai K, Locksley RM. Type 2 immunity is controlled by IL-4/IL-13 expression in hematopoietic non-eosinophil cells of the innate immune system. *J Exp Med* 2006; **203**: 1435-1446
10 **Finkelman FD**, Shea-Donohue T, Morris SC, Gildea L, Strait R, Madden KB, Schopf L, Urban JF Jr. Interleukin-4- and interleukin-13-mediated host protection against intestinal nematode parasites. *Immunol Rev* 2004; **201**: 139-155
11 **Dillon SR**, Sprecher C, Hammond A, Bilsborough J, Rosenfeld-Franklin M, Presnell SR, Haugen HS, Maurer M, Harder B, Johnston J, Bort S, Mudri S, Kuijper JL, Bukowski T, Shea P, Dong DL, Dasovich M, Grant FJ, Lockwood L, Levin SD, LeCiel C, Waggie K, Day H, Topouzis S, Kramer J, Kuestner R, Chen Z, Foster D, Parrish-Novak J, Gross JA. Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice. *Nat Immunol* 2004; **5**: 752-760
12 **Finkelman FD**, Shea-Donohue T, Morris SC, Gildea L, Strait R, Madden KB, Schopf L, Urban JF Jr. Interleukin-4- and interleukin-13-mediated host protection against intestinal nematode parasites. *Immunol Rev* 2004; **201**: 139-155
13 **Wynn TA**, Thompson RW, Cheever AW, Mentink-Kane MM. Immunopathogenesis of schistosomiasis. *Immunol Rev* 2004; **201**: 156-167
14 **Fox JG**, Beck P, Dangler CA, Whary MT, Wang TC, Shi HN, Nagler-Anderson C. Concurrent enteric helminth infection modulates inflammation and gastric immune responses and reduces helicobacter-induced gastric atrophy. *Nat Med* 2000; **6**: 536-542
15 **Zaccone P**, Fehervari Z, Jones FM, Sidobre S, Kronenberg M, Dunne DW, Cooke A. Schistosoma mansoni antigens

modulate the activity of the innate immune response and prevent onset of type 1 diabetes. *Eur J Immunol* 2003; **33**: 1439-1449
16 **Summers RW**, Elliott DE, Urban JF Jr, Thompson RA, Weinstock JV. Trichuris suis therapy for active ulcerative colitis: a randomized controlled trial. *Gastroenterology* 2005; **128**: 825-832
17 **Belkaid Y**, Rouse BT. Natural regulatory T cells in infectious disease. *Nat Immunol* 2005; **6**: 353-360
18 **Takahashi T**, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, Mak TW, Sakaguchi S. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* 2000; **192**: 303-310
19 **Shimizu J**, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* 2002; **3**: 135-142
20 **Hori S**, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; **299**: 1057-1061
21 **Bluestone JA**, Abbas AK. Natural versus adaptive regulatory T cells. *Nat Rev Immunol* 2003; **3**: 253-257
22 **Taylor A**, Verhagen J, Blaser K, Akdis M, Akdis CA. Mechanisms of immune suppression by interleukin-10 and transforming growth factor-beta: the role of T regulatory cells. *Immunology* 2006; **117**: 433-442
23 **Doetze A**, Satoguina J, Burchard G, Rau T, Loliger C, Fleischer B, Hoerauf A. Antigen-specific cellular hyporesponsiveness in a chronic human helminth infection is mediated by T(h)3/T(r)1-type cytokines IL-10 and transforming growth factor-beta but not by a T(h)1 to T(h)2 shift. *Int Immunol* 2000; **12**: 623-630
24 **Satoguina J**, Mempel M, Larbi J, Badusche M, Loliger C, Adjei O, Gachelin G, Fleischer B, Hoerauf A. Antigen-specific T regulatory-1 cells are associated with immunosuppression in a chronic helminth infection (onchocerciasis). *Microbes Infect* 2002; **4**: 1291-1300
25 **Skapenko A**, Kalden JR, Lipsky PE, Schulze-Koops H. The IL-4 receptor alpha-chain-binding cytokines, IL-4 and IL-13, induce forkhead box P3-expressing CD25+CD4+ regulatory T cells from CD25-CD4+ precursors. *J Immunol* 2005; **175**: 6107-6116
26 **Kamel MA**, Miller FD, el Masry AG, Zakaria S, Khattab M, Essmat G, Ghaffar YA. The epidemiology of Schistosoma mansoni, hepatitis B and hepatitis C infection in Egypt. *Ann Trop Med Parasitol* 1994; **88**: 501-509
27 **Borkow G**, Bentwich Z. HIV and helminth co-infection: is deworming necessary? *Parasite Immunol* 2006; **28**: 605-612
28 **Chen CC**, Louie S, McCormick B, Walker WA, Shi HN. Concurrent infection with an intestinal helminth parasite impairs host resistance to enteric Citrobacter rodentium and enhances Citrobacter-induced colitis in mice. *Infect Immun* 2005; **73**: 5468-5481
29 **Weinstock JV**. Helminths and mucosal immune modulation. *Ann N Y Acad Sci* 2006; **1072**: 356-364
30 **Wilson MS**, Taylor MD, Balic A, Finney CA, Lamb JR, Maizels RM. Suppression of allergic airway inflammation by helminth-induced regulatory T cells. *J Exp Med* 2005; **202**: 1199-1212
31 **Smits HH**, Yazdanbakhsh M. Chronic helminth infections modulate allergen-specific immune responses: Protection against development of allergic disorders? *Ann Med* 2007; **39**: 428-439
32 **Pereira LM**, Melo MC, Saleh MG, Massarolo P, Koskinas J, Domingues AL, Spinelli V, Mies S, Williams R, McFarlane IG. Hepatitis C virus infection in Schistosomiasis mansoni in Brazil. *J Med Virol* 1995; **45**: 423-428
33 **Secor WE**. Interactions between schistosomiasis and infection with HIV-1. *Parasite Immunol* 2006; **28**: 597-603
34 **Kjetland EF**, Ndhlovu PD, Gomo E, Mduluza T, Midzi N, Gwanzura L, Mason PR, Sandvik L, Friis H, Gundersen SG. Association between genital schistosomiasis and HIV in rural Zimbabwean women. *AIDS* 2006; **20**: 593-600
35 **Gallagher M**, Malhotra I, Mungai PL, Wamachi AN, Kioko JM, Ouma JH, Muchiri E, King CL. The effects of maternal helminth and malaria infections on mother-to-child HIV transmission. *AIDS* 2005; **19**: 1849-1855
36 **Druilhe P**, Tall A, Sokhna C. Worms can worsen malaria: towards a new means to roll back malaria? *Trends Parasitol* 2005; **21**: 359-362
37 **Brooker S**, Clements AC, Hotez PJ, Hay SI, Tatem AJ, Bundy DA, Snow RW. The co-distribution of Plasmodium falciparum and hookworm among African schoolchildren. *Malar J* 2006; **5**: 99
38 **Maruyama T**, Schodel F, Iino S, Koike K, Yasuda K, Peterson D, Milich DR. Distinguishing between acute and symptomatic chronic hepatitis B virus infection. *Gastroenterology* 1994; **106**: 1006-1015
39 **Chen CC**, Louie S, McCormick BA, Walker WA, Shi HN. Helminth-primed dendritic cells alter the host response to enteric bacterial infection. *J Immunol* 2006; **176**: 472-483
40 **Brady MT**, O'Neill SM, Dalton JP, Mills KH. Fasciola hepatica suppresses a protective Th1 response against Bordetella pertussis. *Infect Immun* 1999; **67**: 5372-5378
41 **Actor JK**, Shirai M, Kullberg MC, Buller RM, Sher A, Berzofsky JA. Helminth infection results in decreased virus-specific CD8+ cytotoxic T-cell and Th1 cytokine responses as well as delayed virus clearance. *Proc Natl Acad Sci USA* 1993; **90**: 948-952
42 **Mansfield LS**, Gauthier DT, Abner SR, Jones KM, Wilder SR, Urban JF. Enhancement of disease and pathology by synergy of Trichuris suis and Campylobacter jejuni in the colon of immunologically naive swine. *Am J Trop Med Hyg* 2003; **68**: 70-80
43 **Khan IA**, Hakak R, Eberle K, Sayles P, Weiss LM, Urban JF Jr. Coinfection with Heligmosomoides polygyrus fails to establish CD8+ T-cell immunity against Toxoplasma gondii. *Infect Immun* 2008; **76**: 1305-1313
44 **Raine T**, Zaccone P, Dunne DW, Cooke A. Can helminth antigens be exploited therapeutically to downregulate pathological Th1 responses? *Curr Opin Investig Drugs* 2004; **5**: 1184-1191
45 **Yazdanbakhsh M**, Kremsner PG, van Ree R. Allergy, parasites, and the hygiene hypothesis. *Science* 2002; **296**: 490-494
46 **Elliott DE**, Setiawan T, Metwali A, Blum A, Urban JF Jr, Weinstock JV. Heligmosomoides polygyrus inhibits established colitis in IL-10-deficient mice. *Eur J Immunol* 2004; **34**: 2690-2698
47 **Xavier RJ**, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 2007; **448**: 427-434
48 **Fiocchi C**. The multiple components of inflammatory bowel disease pathogenesis: should we invest in all of them or should we pick and choose? *Curr Opin Gastroenterol* 2005; **21**: 399-400
49 **Dunne DW**, Cooke A. A worm's eye view of the immune system: consequences for evolution of human autoimmune disease. *Nat Rev Immunol* 2005; **5**: 420-426
50 **Elliott DE**, Urban JF JR, Argo CK, Weinstock JV. Does the failure to acquire helminthic parasites predispose to Crohn's disease? *FASEB J* 2000; **14**: 1848-1855
51 **Reardon C**, Sanchez A, Hogaboam CM, McKay DM. Tapeworm infection reduces epithelial ion transport abnormalities in murine dextran sulfate sodium-induced colitis. *Infect Immun* 2001; **69**: 4417-4423
52 **Khan WI**, Blennerhasset PA, Varghese AK, Chowdhury SK, Omsted P, Deng Y, Collins SM. Intestinal nematode infection ameliorates experimental colitis in mice. *Infect Immun* 2002; **70**: 5931-5937
53 **Elliott DE**, Li J, Blum A, Metwali A, Qadir K, Urban JF Jr, Weinstock JV. Exposure to schistosome eggs protects mice

from TNBS-induced colitis. *Am J Physiol Gastrointest Liver Physiol* 2003; **284**: G385-G391
54 **Moreels TG**, Nieuwendijk RJ, De Man JG, De Winter BY, Herman AG, Van Marck EA, Pelckmans PA. Concurrent infection with Schistosoma mansoni attenuates inflammation induced changes in colonic morphology, cytokine levels, and smooth muscle contractility of trinitrobenzene sulphonic acid induced colitis in rats. *Gut* 2004; **53**: 99-107
55 **Croese J**, O'neil J, Masson J, Cooke S, Melrose W, Pritchard D, Speare R. A proof of concept study establishing Necator americanus in Crohn's patients and reservoir donors. *Gut* 2006; **55**: 136-137
56 **Setiawan T**, Metwali A, Blum AM, Ince MN, Urban JF Jr, Elliott DE, Weinstock JV. Heligmosomoides polygyrus promotes regulatory T-cell cytokine production in the murine normal distal intestine. *Infect Immun* 2007; **75**: 4655-4663
57 **Maizels RM**, Yazdanbakhsh M. Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nat Rev Immunol* 2003; **3**: 733-744
58 **Hunter MM**, Wang A, McKay DM. Helminth infection enhances disease in a murine TH2 model of colitis. *Gastroenterology* 2007; **132**: 1320-1330
59 **Goncalves NS**, Ghaem-Maghami M, Monteleone G, Frankel G, Dougan G, Lewis DJ, Simmons CP, MacDonald TT. Critical role for tumor necrosis factor alpha in controlling the number of lumenal pathogenic bacteria and immunopathology in infectious colitis. *Infect Immun* 2001; **69**: 6651-6659
60 **Vallance BA**, Deng W, Knodler LA, Finlay BB. Mice lacking T and B lymphocytes develop transient colitis and crypt hyperplasia yet suffer impaired bacterial clearance during Citrobacter rodentium infection. *Infect Immun* 2002; **70**: 2070-2081
61 **Simmons CP**, Clare S, Ghaem-Maghami M, Uren TK, Rankin J, Huett A, Goldin R, Lewis DJ, MacDonald TT, Strugnell RA, Frankel G, Dougan G. Central role for B lymphocytes and CD4+ T cells in immunity to infection by the attaching and effacing pathogen Citrobacter rodentium. *Infect Immun* 2003; **71**: 5077-5086
62 **Svetic A**, Madden KB, Zhou XD, Lu P, Katona IM, Finkelman FD, Urban JF Jr, Gause WC. A primary intestinal helminthic infection rapidly induces a gut-associated elevation of Th2-associated cytokines and IL-3. *J Immunol* 1993; **150**: 3434-3441
63 **Vallance BA**, Chan C, Robertson ML, Finlay BB. Enteropathogenic and enterohemorrhagic Escherichia coli infections: emerging themes in pathogenesis and prevention. *Can J Gastroenterol* 2002; **16**: 771-778
64 **Luperchio SA**, Newman JV, Dangler CA, Schrenzel MD, Brenner DJ, Steigerwalt AG, Schauer DB. Citrobacter rodentium, the causative agent of transmissible murine colonic hyperplasia, exhibits clonality: synonymy of C. rodentium and mouse-pathogenic Escherichia coli. *J Clin Microbiol* 2000; **38**: 4343-4350
65 **Kradin RL**, Badizadegan K, Auluck P, Korzenik J, Lauwers GY. Iatrogenic Trichuris suis infection in a patient with Crohn disease. *Arch Pathol Lab Med* 2006; **130**: 718-720
66 **Cools N**, Ponsaerts P, Van Tendeloo VF, Berneman ZN. Balancing between immunity and tolerance: an interplay between dendritic cells, regulatory T cells, and effector T cells. *J Leukoc Biol* 2007; **82**: 1365-1374
67 **Diebold SS**, Montoya M, Unger H, Alexopoulou L, Roy P, Haswell LE, Al-Shamkhani A, Flavell R, Borrow P, Reis e Sousa C. Viral infection switches non-plasmacytoid dendritic cells into high interferon producers. *Nature* 2003; **424**: 324-328
68 **Asselin-Paturel C**, Boonstra A, Dalod M, Durand I, Yessaad N, Dezutter-Dambuyant C, Vicari A, O'Garra A, Biron C, Briere F, Trinchieri G. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat Immunol* 2001; **2**: 1144-1150
69 **Jung S**, Unutmaz D, Wong P, Sano G, De los Santos K, Sparwasser T, Wu S, Vuthoori S, Ko K, Zavala F, Pamer EG, Littman DR, Lang RA. In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. *Immunity* 2002; **17**: 211-220
70 **Barton GM**, Medzhitov R. Control of adaptive immune responses by Toll-like receptors. *Curr Opin Immunol* 2002; **14**: 380-383
71 **Agrawal S**, Agrawal A, Doughty B, Gerwitz A, Blenis J, Van Dyke T, Pulendran B. Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. *J Immunol* 2003; **171**: 4984-4989
72 **Kalinski P**, Hilkens CM, Wierenga EA, Kapsenberg ML. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. *Immunol Today* 1999; **20**: 561-567
73 **de Jong EC**, Vieira PL, Kalinski P, Schuitemaker JH, Tanaka Y, Wierenga EA, Yazdanbakhsh M, Kapsenberg ML. Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse th cell-polarizing signals. *J Immunol* 2002; **168**: 1704-1709
74 **Blanco P**, Palucka AK, Pascual V, Banchereau J. Dendritic cells and cytokines in human inflammatory and autoimmune diseases. *Cytokine Growth Factor Rev* 2008; **19**: 41-52
75 **Sher A**, Pearce E, Kaye P. Shaping the immune response to parasites: role of dendritic cells. *Curr Opin Immunol* 2003; **15**: 421-429
76 **Thomas PG**, Carter MR, Atochina O, Da'Dara AA, Piskorska D, McGuire E, Harn DA. Maturation of dendritic cell 2 phenotype by a helminth glycan uses a Toll-like receptor 4-dependent mechanism. *J Immunol* 2003; **171**: 5837-5841
77 **Aksoy E**, Zouain CS, Vanhoutte F, Fontaine J, Pavelka N, Thieblemont N, Willems F, Ricciardi-Castagnoli P, Goldman M, Capron M, Ryffel B, Trottein F. Double-stranded RNAs from the helminth parasite Schistosoma activate TLR3 in dendritic cells. *J Biol Chem* 2005; **280**: 277-283
78 **Goodridge HS**, Marshall FA, Else KJ, Houston KM, Egan C, Al-Riyami L, Liew FY, Harnett W, Harnett MM. Immunomodulation via novel use of TLR4 by the filarial nematode phosphorylcholine-containing secreted product, ES-62. *J Immunol* 2005; **174**: 284-293
79 **Ince MN**, Elliott DE, Setiawan T, Blum A, Metwali A, Wang Y, Urban JF Jr, Weinstock JV. Heligmosomoides polygyrus induces TLR4 on murine mucosal T cells that produce TGFbeta after lipopolysaccharide stimulation. *J Immunol* 2006; **176**: 726-729
80 **Balic A**, Harcus Y, Holland MJ, Maizels RM. Selective maturation of dendritic cells by Nippostrongylus brasiliensis-secreted proteins drives Th2 immune responses. *Eur J Immunol* 2004; **34**: 3047-3059
81 **Kane CM**, Cervi L, Sun J, McKee AS, Masek KS, Shapira S, Hunter CA, Pearce EJ. Helminth antigens modulate TLR-initiated dendritic cell activation. *J Immunol* 2004; **173**: 7454-7461
82 **Abbas AK**, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. *Nature* 1996; **383**: 787-793
83 **Ilic N**, Colic M, Gruden-Movsesijan A, Majstorovic I, Vasilev S, Sofronic-Milosavljevic L. Characterization of rat bone marrow dendritic cells initially primed by Trichinella spiralis antigens. *Parasite Immunol* 2008; **30:** 491-495
84 **Gordon S**. Alternative activation of macrophages. *Nat Rev Immunol* 2003; **3**: 23-35
85 **Weng M**, Huntley D, Huang IF, Foye-Jackson O, Wang L, Sarkissian A, Zhou Q, Walker WA, Cherayil BJ, Shi HN.

Alternatively activated macrophages in intestinal helminth infection: effects on concurrent bacterial colitis. *J Immunol* 2007; **179**: 4721-4731

86 **Goerdt S**, Orfanos CE. Other functions, other genes: alternative activation of antigen-presenting cells. *Immunity* 1999; **10**: 137-142

87 **Anthony RM**, Urban JF Jr, Alem F, Hamed HA, Rozo CT, Boucher JL, Van Rooijen N, Gause WC. Memory T(H)2 cells induce alternatively activated macrophages to mediate protection against nematode parasites. *Nat Med* 2006; **12**: 955-960

88 **Loke P**, MacDonald AS, Allen JE. Antigen-presenting cells recruited by Brugia malayi induce Th2 differentiation of naive CD4(+) T cells. *Eur J Immunol* 2000; **30**: 1127-1135

89 **Allen JE**, Lawrence RA, Maizels RM. APC from mice harbouring the filarial nematode, Brugia malayi, prevent cellular proliferation but not cytokine production. *Int Immunol* 1996; **8**: 143-151

90 **Kahnert A**, Seiler P, Stein M, Bandermann S, Hahnke K, Mollenkopf H, Kaufmann SH. Alternative activation deprives macrophages of a coordinated defense program to Mycobacterium tuberculosis. *Eur J Immunol* 2006; **36**: 631-647

91 **Podolsky DK**. Inflammatory bowel disease. *N Engl J Med* 2002; **347**: 417-429

92 **Moller B**, Villiger PM. Inhibition of IL-1, IL-6, and TNF-alpha in immune-mediated inflammatory diseases. *Springer Semin Immunopathol* 2006; **27**: 391-408

S- Editor Cheng JX **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5133

World J Gastroenterol 2008 September 7; 14(33): 5133-5137
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHTS

Emiko Mizoguchi, MD, PhD, *Series Editor*

Roles of galectins in inflammatory bowel disease

Akira Hokama, Emiko Mizoguchi, Atsushi Mizoguchi

Akira Hokama, Department of Medicine and Therapeutics (First Department of Internal Medicine), Control and Prevention of Infectious Disease, Faculty of Medicine, University of the Ryukyus, Okinawa 903-0215, Japan
Emiko Mizoguchi, Gastrointestinal Unit, Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, United States; Center for the Study of Inflammatory Bowel Disease, Massachusetts General Hospital, Harvard Medical School, Boston MA 02114, United States
Atsushi Mizoguchi, Experimental Pathology Unit, Department of Pathology, Massachusetts General Hospital, Harvard Medical School, Boston MA 02114, United States; Center for the Study of Inflammatory Bowel Disease, Massachusetts General Hospital, Harvard Medical School, Boston MA 02114, United States
Author contributions: Hokama A, Mizoguchi E, and Mizoguchi A contributed in writing and reviewing the paper.
Supported by National Institute of Health Grants, No. DK64289 and DK74454; IBD grants from the Eli and Edythe L. Broad Medical Foundation; National Institute of Health grant, DK64351; IBD grants from the Eli and Edythe L. Broad Medical Foundation
Correspondence to: Akira Hokama, Department of Medicine and Therapeutics (First Department of Internal Medicine), Control and Prevention of Infectious Disease, Faculty of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan. hokama-a@med.u-ryukyu.ac.jp
Telephone: +81-98-8951144 Fax: +81-98-8951414
Received: April 9, 2008 Revised: July 20, 2008
Accepted: July 27, 2008
Published online: September 7, 2008

Abstract

Protein/carbohydrate interactions through specific protein families termed lectin control essential biological processes. Galectins, a family of animal lectins defined by shared amino acid sequence with affinity for β-galactosides, appear to be functionally polyvalent in a wide range of biological activity. Recent studies have identified immunoregulatory roles of galectins in intestinal inflammatory disorders. Galectin-1 and galectin -2 contribute to the suppression of intestinal inflammation by the induction of apoptosis of activated T cells, whereas galectin-4 is involved in the exacerbation of this inflammation by specifically stimulating intestinal CD4+ T cells to produce IL-6. We review how different members of the galectins provide inhibitory or stimulatory signals to control intestinal immune response under intestinal inflammation.

Key words: Galectin; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Pathogenesis; Apoptosis; Glycosylation

Hokama A, Mizoguchi E, Mizoguchi A. Roles of galectins in inflammatory bowel disease. *World J Gastroenterol* 2008; 14(33): 5133-5137 Available from: URL: http://www.wjgnet.com/1007-9327/14/5133.asp DOI: http://dx.doi.org/10.3748/wjg.14.5133

INTRODUCTION

Inflammatory bowel disease (IBD), which is characterized by two forms of intestinal inflammation, Crohn's disease (CD) and ulcerative colitis (UC), is a group of chronic, relapsing, and remitting inflammatory conditions that affect individuals throughout life[1]. Several factors such as immune imbalance, dysregulated host/microbial interaction, and genetic susceptibility are involved in the pathogenesis of IBD[2-4]. Experimental IBD models have provided a useful means to dissect the pathogenesis of this disease[2-4]. Among these models, chronic intestinal inflammation that spontaneously develops in T cell receptor α knockout (TCRα KO) mice shares several features with human UC, e.g. marked increase in autoantibodies such as antineutrophil cytoplasmic antigens and antitropomyosin, predominant Th2 responses and negative association of colitis development with prior appendectomy (resection of cecal patch)[5-7]. Importantly, B cells and autoantibodies in TCRα KO mice are involved in the regulation of this inflammation[8-12]. Therefore, a screening approach utilizing autoantibodies present in TCRα KO mice was proposed to have an ability to provide a useful tool in the identification of molecules, which may have a role in the pathogenesis of UC[13]. Indeed, the screening approach [serological analysis of recombinant cDNA expression libraries (SEREX) for the identification of candidate molecules that are recognized by autoantibodies from TCRα KO mice] has provided us an unexpected opportunity to identify galectin-4 as a potential stimulator of CD4+ T cells under intestinal inflammatory conditions[14,15]. Interestingly, galectin-4 was an unexpectedly discovered carbohydrate-binding protein through our screening approach, emphasizing

the importance of carbohydrate/protein interactions in the pathogenesis of intestinal inflammation. Indeed, a recent study has demonstrated that an alteration of carbohydrate composition (carboxylated glycans) on macrophages and dendritic cells contributes to the early onset of intestinal inflammation[16]. Alternatively, carbohydrate/protein interactions also play a regulatory role in the intestinal inflammation as indicated by a suppressive effect of galectin-1 and galectin-2 on this inflammation[17,18]. We, herein, review recently identified novel roles of galectins in immune responses under intestinal inflammation.

GALECTINS

Several families of glycan-binding proteins or lectins, which include C-type lectins (such as selectin, DC-SIGN, dectin, and serum mannose binding protein)[19-23], S-type lectins (galectins)[24-28] and siglecs[29], have been implicated in a wide variety of immunological functions including first-line defense against pathogens, cell trafficking, cell differentiation and immune regulation. Galectins are a family of 15 members (galectin-1 to galectin-15) characterized by two properties: the ability to bind to lactosamine unit within glycans and the preserved carbohydrate recognition domains (CRD) composed of 130 amino acid residues. The 15 members of galectins are structurally classified into three groups; prototype, chimera-type, and tandem repeat type[30-32]. Prototype (galectins-1, -2, -5, -7, -10, -11, -13, -14, and -15) is non-covalent homodimers that are composed of two identical CRDs. Only galectin-3 is chimera type that is composed of a CRD linked to a proline-, glycine-, and tyrosine-rich N-terminal domain. Tandem repeat type (galectins-4, -6, -8, -9, and -12) possesses two distinct CRDs. The ability of CRDs to cross-link the lactosamine unit within surface glycoreceptors allows galectins to actively participate in several immune responses. A large body of evidence indicates important roles of galectins in the development and progression of cancer[30-33]. Recently, compelling evidence has been accumulated regarding the immunoregulatory effects of galectins in inflammatory disorders[34-36]. We focus on four members of galectins (galectins-1, -2, -3 and -4), which have been studied regarding intestinal inflammation.

REGULATORY ROLE OF GALECTIN-1 IN INTESTINAL INFLAMMATION

Galectin-1 (prototype), which is expressed by a wide variety of cell types, is specifically upregulated on CD4+CD25+ regulatory T cells[37]. Soluble galectin-1 has been demonstrated to interact with a lactosamine unit of mature core 2 *O*-glycan that is assembled within some glycoreceptors including CD7, CD43, and CD45, and its cross-linking ability is crucial for the induction of T cell apoptosis[37-40]. The anti-inflammatory properties of galectin-1 have been evaluated in several models of chronic inflammation and autoimmunity including autoimmune encephalomyelitis[41], arthritis[42], uveitis[43], hepatitis[44], and diabetes[45]. As for the colitis model, galectin-1 expression in the colon is upregulated under an intestinal inflammatory condition that is chemically induced in mouse by rectal administration of 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)[17]. Administration of human recombinant galectin-1 contributes to the reduction of Th1 cytokine (TNF-α, IL-1β, IL-12 and IFN-γ) release and the suppression of this intestinal inflammation by specifically inducing the apoptosis of effector T cells responsible for production of IFN-γ[17]. In addition, a recent study has demonstrated that administration of recombinant galectin-1 also contributes to increase IL-10 production in CD4+ and CD8+ T cells[46]. These data clearly address the therapeutic potential of galectin-1 to skew the balance from a Th1- toward a Th2-polarized immune response that induces a remission state in the evolution of the ongoing inflammatory disorders.

REGULATORY ROLE OF GALECTIN-2 IN INTESTINAL INFLAMMATION

Galectin-2 (prototype) is expressed by various cells including intestinal epithelial cells. Galectin-2, structurally related to galectin-1, has been demonstrated to be an inducer of apoptosis of activated T cells, although it lacks reactivity to CD7 characteristic for galectin-1[47].A recent has study shown that galectin-2 is constitutively expressed mainly in the epithelial compartment of the mouse intestine and binds to lamina propria mononuclear cells[18]. In acute and chronic dextran sodium sulfate (DSS)-induced colitis, and in a Th1-driven model of antigen-specific transfer colitis, galectin-2 expression was reduced, but could be restored to normal levels by immunosuppressive treatment. Administration of human recombinant galectin-2 induced apoptosis of mucosal T cells and, thus, ameliorated. Furthermore, pro-inflammatory cytokine (IL-6, IL-12p70) release was inhibited by administration of galectin-2. Their study provides evidence that galectin-2, as well as galectin-1, induces apoptosis *in vivo* and ameliorates acute and chronic murine colitis.

ROLE OF GALECTIN-3 IN INTESTINAL INFLAMMATION

Galectin-3 (chimera type) is a multifunctional protein detected in the nucleus, cytoplasm and extracellular matrix of a wide variety of cells. Galectin-3 has the dual role of protecting T cells from apoptosis when present intracellularly while promoting apoptosis when acting on T cells from the extracellular space[30-32,36]. Regarding intestinal inflammation, a study showed that the titers of anti-galectin-3 autoantibodies were higher in CD patients with low activity index than with active disease[48]. The pathophysiological significance of the anti-galectin-3 autoantibody in Crohn's disease still remains to be elucidated. The same research group

subsequently showed that expression of galectin-3 was reduced in the intestinal epithelium of CD patients and that colonic epithelial adenocarcinoma cell line HCT-8 cells reduced galectin-3 expression by incubation with TNF-α but not with other cytokines[49]. It was speculated that galectin-3 was consequently downregulated by enhanced TNF-α production in CD. Another research group confirmed the similar findings[50]. More recently, soluble galectin-3, which is secreted by colonic epithelial cells, was identified as an activator of lamina propria fibroblasts[51,52]. The study also indicated that galectin-3 induced NF-κB activation and IL-8 secretion *in vitro*. Its role in pathogenesis of intestinal inflammation, especially involvement in fibrosis formation of CD, has to be clarified in further studies. In a protein expression profile study of Enterococcus faecalis-monoassociated IL-10 KO mice under chronic intestinal inflammation and intestinal epithelial cell lines, galectin-3 expression was reduced in association with the activation of caspase 3, a major executive caspase of apoptosis[53]. Further studies are needed to address whether galectin-3 plays a pro-inflammatory role or an anti-inflammatory role in intestinal inflammation.

PATHOGENIC ROLE OF GALECTIN-4 IN INTESTINAL INFLAMMATION

Galectin-4 (tandem repeat type) is expressed only in the digestive tract[54-56] where epithelial cells are responsible for this production[14,55,56]. Galectin-4 can be secreted from both basolateral and apical sides of the intestinal epithelial cells through a nonclassical secretary pathway. In contrast to galectin-1[17], intestinal inflammatory conditions do not enhance the galectin-4 expression: there is no significant difference in the expression level of galectin-4 in the epithelial cells from control *versus* inflamed colons[14]. Interestingly, through a combined screening approach utilizing humoral (SEREX) and cellular immune responses, we have unexpectedly identified galectin-4 as a potential stimulator of CD4+ T cells to exacerbate intestinal inflammation[14]. Neutralization of galectin-4 activity *in vivo* by administration of the specific antibody suppresses the progression of chronic colitis that spontaneously develops in B cell-deficient TCRα double KO mice[14], whereas pretreatment with this antibody fails to abolish the development of colitis in these mice (A.M., unpublished observation). These data suggest that galectin-4 contributes to the exacerbation, rather than initiation, of chronic intestinal inflammation. Because it could be predicted that both acute (induction of inflammation) and healing (recovery from inflammation) processes are simultaneously involved in the chronic intestinal inflammation, galectin-4-mediated exacerbation of this inflammation may result from a suppression of the healing process. Indeed, treatment with recombinant galectin-4 delays the recovery from an acute intestinal inflammation that is induced by transient administration of DSS, whereas treatment with anti-galectin-4 antibody enhances the recovery from this acute inflammation. In contrast, galectin-1, as mentioned above, contributes to the suppression of acute intestinal inflammation[17]. Galectin-1 (prototype) is structurally characterized by homodimers with identical CRDs, and binds to a lactosamine unit within a mature core 2 *O*-glycan, whereas galectin-4 (tandem repeat type) consists of two distinct CRDs and possesses a unique carbohydrate-binding specificity as indicated by the capability of interacting with an immature core 1 *O*-glycan with 3'-O-sulfation[57]. Therefore, it is highly likely that the binding site (lactosamine unit *versus* core 1) and the structure (prototype *versus* tandem repeat type) are an important determinants of galectin-mediated immune function[30-32,36]. Galectin-4 specifically stimulates CD4+ T cells, but not other immune cells such as B cells or macrophages to produce IL-6[14], a well-known cytokine involved in the pathogenesis of not only intestinal inflammation, but also colon cancer[58-60]. Importantly, only CD4+ T cells that are present in the inflamed, but not non-inflamed, intestine can respond to galectin-4[14]. Splenic CD4+ T cells even from the diseased mice are unable to respond to galectin-4. These findings are consistent with the binding intensity of galectin-4 to the surface of CD4+ T cells; galectin-4 binding is significantly enhanced on the CD4+ T cells from the inflamed colon as compared to noninflamed colon and spleen. In addition, galectin-4 specifically binds to the lipid rafts on the CD4+ T cells to activate the protein kinase C θ-associated signaling cascade[14], a common and fundamental pathway in the different types of intestinal inflammation[61]. Notably, galectin-4 has been demonstrated to interact with lipid rafts of enterocytes as well, and subsequently stabilize the raft formation to generate "superrafts"[62]. A recent study has found that galectin-4 interacts with carcinoembryonic antigen of colon adenocarcinoma[63]. Alternatively, it remains obscure which glycosylated receptor(s) on intestinal CD4+ T cells is crosslinked by galectin-4. Production of galectin-4 by colonic epithelial cells is not enhanced under inflammatory condition, whereas the reactivity of CD4+ T cells to galectin-4 is specifically elicited under these conditions. Therefore, it is possible that a specific receptor that is selectively crosslinked by galectin-4 may be expressed on intestinal CD4+ T cells only under inflammatory conditions. However, galectin-4 can bind to the lipid rafts on both CD4+ T cells from inflamed and normal intestines although the binding intensity is much higher on diseased CD4+ T cells[14]. In addition, expression pattern of the enzymes that are involved in the glycan synthesis is altered by several inflammatory stimuli[27,34,35]. Therefore, it is more likely that an altered enzyme expression pattern by intestinal inflammatory stimuli results in the further exposure of core 1 *O*-glycan (a binding partner of galectin-4) on intestinal CD4+ T cells and consequently allows intensified binding of galectin-4 to them. Indeed, our recent studies have found that some glycosylation-associated enzymes, which are involved in the synthesis of core 2 from core 1, are significantly downregulated in the intestinal CD4+

T cells under inflammatory conditions as compared to a state of health (our unpublished observation). These findings provide an insight into an unexpected role of lectin/carbohydrate interaction in the pathogenesis of T cell-mediated chronic colitis.

CONCLUSION

Glycobiology has an exiting impact to molecular biology and clinical fields, given the multifunctional activities of galectins. In this review, we provide novel insights into the role of carbohydrates crosslinked by galectins in the immune responses involved in the pathogenesis of IBD. Different members of the galectin families provide inhibitory or stimulatory signals to control intestinal immune response under intestinal inflammatory conditions. A more thorough understanding of the molecular mechanisms involved in the immunoregulatory functions of galectins is needed before galectin-based therapeutic strategies for IBD can be realized.

REFERENCES

1 **Podolsky DK**. Inflammatory bowel disease. *N Engl J Med* 2002; **347**: 417-429
2 **Strober W**, Fuss IJ, Blumberg RS. The immunology of mucosal models of inflammation. *Annu Rev Immunol* 2002; **20**: 495-549
3 **Mizoguchi A**, Mizoguchi E, Bhan AK. Immune networks in animal models of inflammatory bowel disease. *Inflamm Bowel Dis* 2003; **9**: 246-259
4 **Mizoguchi A**, Mizoguchi E. Inflammatory bowel disease, past, present and future: lessons from animal models. *J Gastroenterol* 2008; **43**: 1-17
5 **Mizoguchi A**, Mizoguchi E, Chiba C, Spiekermann GM, Tonegawa S, Nagler-Anderson C, Bhan AK. Cytokine imbalance and autoantibody production in T cell receptor-alpha mutant mice with inflammatory bowel disease. *J Exp Med* 1996; **183**: 847-856
6 **Mizoguchi A**, Mizoguchi E, Chiba C, Bhan AK. Role of appendix in the development of inflammatory bowel disease in TCR-alpha mutant mice. *J Exp Med* 1996; **184**: 707-715
7 **Sugimoto K**, Ogawa A, Mizoguchi E, Shimomura Y, Andoh A, Bhan AK, Blumberg RS, Xavier RJ, Mizoguchi A. IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *J Clin Invest* 2008; **118**: 534-544
8 **Mizoguchi A**, Mizoguchi E, Smith RN, Preffer FI, Bhan AK. Suppressive role of B cells in chronic colitis of T cell receptor alpha mutant mice. *J Exp Med* 1997; **186**: 1749-1756
9 **Mizoguchi A**, Mizoguchi E, Takedatsu H, Blumberg RS, Bhan AK. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. *Immunity* 2002; **16**: 219-230
10 **Mizoguchi A**, Bhan AK. A case for regulatory B cells. *J Immunol* 2006; **176**: 705-710
11 **Sugimoto K**, Ogawa A, Shimomura Y, Nagahama K, Mizoguchi A, Bhan AK. Inducible IL-12-producing B cells regulate Th2-mediated intestinal inflammation. *Gastroenterology* 2007; **133**: 124-136
12 **Shimomura Y**, Mizoguchi E, Sugimoto K, Kibe R, Benno Y, Mizoguchi A, Bhan AK. Regulatory role of B-1 B cells in chronic colitis. *Int Immunol* 2008; **20**: 729-737
13 **Targan SR**. The search for pathogenic antigens in ulcerative colitis. *Gastroenterology* 1998; **114**: 1099-1100
14 **Hokama A**, Mizoguchi E, Sugimoto K, Shimomura Y, Tanaka Y, Yoshida M, Rietdijk ST, de Jong YP, Snapper SB, Terhorst C, Blumberg RS, Mizoguchi A. Induced reactivity of intestinal CD4(+) T cells with an epithelial cell lectin, galectin-4, contributes to exacerbation of intestinal inflammation. *Immunity* 2004; **20**: 681-693
15 **Mizoguchi E**, Mizoguchi A. Is the sugar always sweet in intestinal inflammation? *Immunol Res* 2007; **37**: 47-60
16 **Srikrishna G**, Turovskaya O, Shaikh R, Newlin R, Foell D, Murch S, Kronenberg M, Freeze HH. Carboxylated glycans mediate colitis through activation of NF-kappa B. *J Immunol* 2005; **175**: 5412-5422
17 **Santucci L**, Fiorucci S, Rubinstein N, Mencarelli A, Palazzetti B, Federici B, Rabinovich GA, Morelli A. Galectin-1 suppresses experimental colitis in mice. *Gastroenterology* 2003; **124**: 1381-1394
18 **Paclik D**, Berndt U, Guzy C, Dankof A, Danese S, Holzloehner P, Rosewicz S, Wiedenmann B, Wittig BM, Dignass AU, Sturm A. Galectin-2 induces apoptosis of lamina propria T lymphocytes and ameliorates acute and chronic experimental colitis in mice. *J Mol Med* 2008; **86:** 1395-1406
19 **Uchimura K**, Rosen SD. Sulfated L-selectin ligands as a therapeutic target in chronic inflammation. *Trends Immunol* 2006; **27**: 559-565
20 **Gringhuis SI**, den Dunnen J, Litjens M, van Het Hof B, van Kooyk Y, Geijtenbeek TB. C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF-kappaB. *Immunity* 2007; **26**: 605-616
21 **Cunningham AL**, Harman AN, Donaghy H. DC-SIGN 'AIDS' HIV immune evasion and infection. *Nat Immunol* 2007; **8**: 556-558
22 **Hodges A**, Sharrocks K, Edelmann M, Baban D, Moris A, Schwartz O, Drakesmith H, Davies K, Kessler B, McMichael A, Simmons A. Activation of the lectin DC-SIGN induces an immature dendritic cell phenotype triggering Rho-GTPase activity required for HIV-1 replication. *Nat Immunol* 2007; **8**: 569-577
23 **van Vliet SJ**, Gringhuis SI, Geijtenbeek TB, van Kooyk Y. Regulation of effector T cells by antigen-presenting cells via interaction of the C-type lectin MGL with CD45. *Nat Immunol* 2006; **7**: 1200-1208
24 **Perillo NL**, Pace KE, Seilhamer JJ, Baum LG. Apoptosis of T cells mediated by galectin-1. *Nature* 1995; **378**: 736-739
25 **Demetriou M**, Granovsky M, Quaggin S, Dennis JW. Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature* 2001; **409**: 733-739
26 **Zhu C**, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, Zheng XX, Strom TB, Kuchroo VK. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol* 2005; **6**: 1245-1252
27 **Toscano MA**, Bianco GA, Ilarregui JM, Croci DO, Correale J, Hernandez JD, Zwirner NW, Poirier F, Riley EM, Baum LG, Rabinovich GA. Differential glycosylation of TH1, TH2 and TH-17 effector cells selectively regulates susceptibility to cell death. *Nat Immunol* 2007; **8**: 825-834
28 **van Kooyk Y**, Rabinovich GA. Protein-glycan interactions in the control of innate and adaptive immune responses. *Nat Immunol* 2008; **9**: 593-601
29 **Crocker PR**, Paulson JC, Varki A. Siglecs and their roles in the immune system. *Nat Rev Immunol* 2007; **7**: 255-266
30 **Liu FT**, Rabinovich GA. Galectins as modulators of tumour progression. *Nat Rev Cancer* 2005; **5**: 29-41
31 **Rabinovich GA**, Liu FT, Hirashima M, Anderson A. An emerging role for galectins in tuning the immune response: lessons from experimental models of inflammatory disease, autoimmunity and cancer. *Scand J Immunol* 2007; **66**: 143-158
32 **Elola MT**, Wolfenstein-Todel C, Troncoso MF, Vasta GR, Rabinovich GA. Galectins: matricellular glycan-binding proteins linking cell adhesion, migration, and survival. *Cell Mol Life Sci* 2007; **64**: 1679-1700
33 **Demetter P**, Nagy N, Martin B, Mathieu A, Dumont P, Decaestecker C, Salmon I. The galectin family and digestive

disease. *J Pathol* 2008; **215**: 1-12
34 **Lowe JB**. Glycosylation, immunity, and autoimmunity. *Cell* 2001; **104**: 809-812
35 **Baum LG**. Developing a taste for sweets. *Immunity* 2002; **16**: 5-8
36 **Toscano MA**, Ilarregui JM, Bianco GA, Campagna L, Croci DO, Salatino M, Rabinovich GA. Dissecting the pathophysiologic role of endogenous lectins: glycan-binding proteins with cytokine-like activity? *Cytokine Growth Factor Rev* 2007; **18**: 57-71
37 **Camby I**, Le Mercier M, Lefranc F, Kiss R. Galectin-1: a small protein with major functions. *Glycobiology* 2006; **16**: 137R-157R
38 **Pace KE**, Hahn HP, Pang M, Nguyen JT, Baum LG. CD7 delivers a pro-apoptotic signal during galectin-1-induced T cell death. *J Immunol* 2000; **165**: 2331-2334
39 **Nguyen JT**, Evans DP, Galvan M, Pace KE, Leitenberg D, Bui TN, Baum LG. CD45 modulates galectin-1-induced T cell death: regulation by expression of core 2 O-glycans. *J Immunol* 2001; **167**: 5697-5707
40 **Stillman BN**, Hsu DK, Pang M, Brewer CF, Johnson P, Liu FT, Baum LG. Galectin-3 and galectin-1 bind distinct cell surface glycoprotein receptors to induce T cell death. *J Immunol* 2006; **176**: 778-789
41 **Offner H**, Celnik B, Bringman TS, Casentini-Borocz D, Nedwin GE, Vandenbark AA. Recombinant human beta-galactoside binding lectin suppresses clinical and histological signs of experimental autoimmune encephalomyelitis. *J Neuroimmunol* 1990; **28**: 177-184
42 **Rabinovich GA**, Daly G, Dreja H, Tailor H, Riera CM, Hirabayashi J, Chernajovsky Y. Recombinant galectin-1 and its genetic delivery suppress collagen-induced arthritis via T cell apoptosis. *J Exp Med* 1999; **190**: 385-398
43 **Toscano MA**, Commodaro AG, Ilarregui JM, Bianco GA, Liberman A, Serra HM, Hirabayashi J, Rizzo LV, Rabinovich GA. Galectin-1 suppresses autoimmune retinal disease by promoting concomitant Th2- and T regulatory-mediated anti-inflammatory responses. *J Immunol* 2006; **176**: 6323-6332
44 **Santucci L**, Fiorucci S, Cammilleri F, Servillo G, Federici B, Morelli A. Galectin-1 exerts immunomodulatory and protective effects on concanavalin A-induced hepatitis in mice. *Hepatology* 2000; **31**: 399-406
45 **Perone MJ**, Bertera S, Tawadrous ZS, Shufesky WJ, Piganelli JD, Baum LG, Trucco M, Morelli AE. Dendritic cells expressing transgenic galectin-1 delay onset of autoimmune diabetes in mice. *J Immunol* 2006; **177**: 5278-5289
46 **van der Leij J**, van den Berg A, Harms G, Eschbach H, Vos H, Zwiers P, van Weeghel R, Groen H, Poppema S, Visser L. Strongly enhanced IL-10 production using stable galectin-1 homodimers. *Mol Immunol* 2007; **44**: 506-513
47 **Sturm A**, Lensch M, Andre S, Kaltner H, Wiedenmann B, Rosewicz S, Dignass AU, Gabius HJ. Human galectin-2: novel inducer of T cell apoptosis with distinct profile of caspase activation. *J Immunol* 2004; **173**: 3825-3837
48 **Jensen-Jarolim E**, Neumann C, Oberhuber G, Gscheidlinger R, Neuchrist C, Reinisch W, Zuberi RI, Penner E, Liu FT, Boltz-Nitulescu G. Anti-Galectin-3 IgG autoantibodies in patients with Crohn's disease characterized by means of phage display peptide libraries. *J Clin Immunol* 2001; **21**: 348-356
49 **Jensen-Jarolim E**, Gscheidlinger R, Oberhuber G, Neuchrist C, Lucas T, Bises G, Radauer C, Willheim M, Scheiner O, Liu FT, Boltz-Nitulescu G. The constitutive expression of galectin-3 is downregulated in the intestinal epithelia of Crohn's disease patients, and tumour necrosis factor alpha decreases the level of galectin-3-specific mRNA in HCT-8 cells. *Eur J Gastroenterol Hepatol* 2002; **14**: 145-152
50 **Muller S**, Schaffer T, Flogerzi B, Fleetwood A, Weimann R, Schoepfer AM, Seibold F. Galectin-3 modulates T cell activity and is reduced in the inflamed intestinal epithelium in IBD. *Inflamm Bowel Dis* 2006; **12**: 588-597
51 **Lippert E**, Falk W, Bataille F, Kaehne T, Naumann M, Goeke M, Herfarth H, Schoelmerich J, Rogler G. Soluble galectin-3 is a strong, colonic epithelial-cell-derived, lamina propria fibroblast-stimulating factor. *Gut* 2007; **56**: 43-51
52 **Lippert E**, Gunckel M, Brenmoehl J, Bataille F, Falk W, Scholmerich J, Obermeier F, Rogler G. Regulation of galectin-3 function in mucosal fibroblasts: potential role in mucosal inflammation. *Clin Exp Immunol* 2008; **152**: 285-297
53 **Werner T**, Shkoda A, Haller D. Intestinal epithelial cell proteome in IL-10 deficient mice and IL-10 receptor reconstituted epithelial cells: impact on chronic inflammation. *J Proteome Res* 2007; **6**: 3691-3704
54 **Rechreche H**, Mallo GV, Montalto G, Dagorn JC, Iovanna JL. Cloning and expression of the mRNA of human galectin-4, an S-type lectin down-regulated in colorectal cancer. *Eur J Biochem* 1997; **248**: 225-230
55 **Gitt MA**, Colnot C, Poirier F, Nani KJ, Barondes SH, Leffler H. Galectin-4 and galectin-6 are two closely related lectins expressed in mouse gastrointestinal tract. *J Biol Chem* 1998; **273**: 2954-2960
56 **Huflejt ME**, Leffler H. Galectin-4 in normal tissues and cancer. *Glycoconj J* 2004; **20**: 247-255
57 **Ideo H**, Seko A, Ohkura T, Matta KL, Yamashita K. High-affinity binding of recombinant human galectin-4 to SO(3)(-)-->3Galbeta1-->3GalNAc pyranoside. *Glycobiology* 2002; **12**: 199-208
58 **Atreya R**, Mudter J, Finotto S, Mullberg J, Jostock T, Wirtz S, Schutz M, Bartsch B, Holtmann M, Becker C, Strand D, Czaja J, Schlaak JF, Lehr HA, Autschbach F, Schurmann G, Nishimoto N, Yoshizaki K, Ito H, Kishimoto T, Galle PR, Rose-John S, Neurath MF. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. *Nat Med* 2000; **6**: 583-588
59 **Becker C**, Fantini MC, Schramm C, Lehr HA, Wirtz S, Nikolaev A, Burg J, Strand S, Kiesslich R, Huber S, Ito H, Nishimoto N, Yoshizaki K, Kishimoto T, Galle PR, Blessing M, Rose-John S, Neurath MF. TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling. *Immunity* 2004; **21**: 491-501
60 **Mudter J**, Neurath MF. Il-6 signaling in inflammatory bowel disease: pathophysiological role and clinical relevance. *Inflamm Bowel Dis* 2007; **13**: 1016-1023
61 **Nagahama K**, Ogawa A, Shirane K, Shimomura Y, Sugimoto K, Mizoguchi A. Protein kinase C theta plays a fundamental role in different types of chronic colitis. *Gastroenterology* 2008; **134**: 459-469
62 **Braccia A**, Villani M, Immerdal L, Niels-Christiansen LL, Nystrom BT, Hansen GH, Danielsen EM. Microvillar membrane microdomains exist at physiological temperature. Role of galectin-4 as lipid raft stabilizer revealed by "superrafts". *J Biol Chem* 2003; **278**: 15679-15684
63 **Ideo H**, Seko A, Yamashita K. Galectin-4 binds to sulfated glycosphingolipids and carcinoembryonic antigen in patches on the cell surface of human colon adenocarcinoma cells. *J Biol Chem* 2005; **280**: 4730-4737

S- Editor Zhong XY **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5138

World J Gastroenterol 2008 September 7; 14(33): 5138-5148
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHTS

Emiko Mizoguchi, MD, PhD, *Series Editor*

Role of mucosal dendritic cells in inflammatory bowel disease

Jan Hendrik Niess

Jan Hendrik Niess, Department of Internal Medicine I, Ulm University, Ulm 89031, Germany
Author contributions: Niess JH contributed all to this paper.
Supported by The Deutsche Forschungsgemeinschaft, No. Ni 575/4-1
Correspondence to: Jan Hendrik Niess, MD, Department of Internal Medicine I, Ulm University, Ulm University Street, Helmholtzstr, 8/1, Ulm 89031, Germany. jan-hendrik.niess@uniklinik-ulm.de
Telephone: +49-731-50044736 Fax: +49-731-50033654
Received: May 12, 2008 Revised: July 25, 2008
Accepted: August 2, 2008
Published online: September 7, 2008

Abstract

The gastrointestinal innate and adaptive immune system continuously faces the challenge of potent stimuli from the commensal microflora and food constituents. These local immune responses require a tight control, the outcome of which is in most cases the induction of tolerance. Local T cell immunity is an important compartment of the specific intestinal immune system. T cell reactivity is programmed during the initial stage of its activation by professional presenting cells. Mucosal dendritic cells (DCs) are assumed to play key roles in regulating immune responses in the antigen-rich gastrointestinal environment. Mucosal DCs are a heterogeneous population that can either initiate (innate and adaptive) immune responses, or control intestinal inflammation and maintain tolerance. Defects in this regulation are supposed to lead to the two major forms of inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC). This review will discuss the emerging role of mucosal DCs in regulating intestinal inflammation and immune responses.

Key words: Dendritic cells; Commensal; Inflammatory bowel disease; Mucosal immunity; Host defence

Peer reviewer: Bret Lashner, MD, Professor of Medicine, Department of Gastroenterology, Cleveland Clinic/A30, 9500 Euclid Avenue, Cleveland, OH 44195, United States

Niess JH. Role of mucosal dendritic cells in inflammatory bowel disease. *World J Gastroenterol* 2008; 14(33): 5138-5148 Available from: URL: http://www.wjgnet.com/1007-9327/14/5138.asp DOI: http://dx.doi.org/10.3748/wjg.14.5138

INTRODUCTION

The intestinal innate and adaptive immune system has evolved in response to potent stimuli derived from constituents of the commensal microflora. In most cases these local immune responses achieve tolerance to the intestinal microflora and food antigens. Defects of the tightly regulated mucosal immune responses are assumed to result in inflammatory bowel disease (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC)[1,2]. Local T cell immunity is an important compartment of the specific intestinal immune system. T cell reactivity is programmed during the initial stage of its activation by dendritic cells (DCs) that can either initiate (innate and adaptive) immune responses, or control intestinal inflammation and maintain tolerance[3-5]. DCs reside in mucosal tissues or recirculate in the blood and lymphoid tissues[6]. The lamina propria of the small and large intestine are effector sites of mucosal tissues. Inductive sites are Peyer's patches (PP), intestinal lymph follicles (iLFs), DC aggregates and mesenteric lymph nodes (MLNs) (Table 1). The local microenvironment influences the phenotype of DCs, a heterogeneous population that can be divided into conventional DCs ($CD8\alpha^+CD11b^-$, $CD4^+CD11b^+$, $CD4^-CD11b^+$)[7,8] and plasmacytoid DCs ($B220^+CD11c^{low}$) (Table 2) and are characterized by a remarkable plasticity between DCs[9]. In the lamina propria of the small and large intestine, DCs are ideally situated to survey the constituents of the commensal microflora and monitor food antigens[10]. After antigen recognition at peripheral sites DCs migrate to regional draining lymph nodes to initiate (innate and adaptive) immune responses[11]. This review will discuss the emerging role of mucosal DCs in regulating intestinal inflammation and immune responses.

DCs IN THE SMALL INTESTINE

The lamina propria of the small intestine is populated by $CD4^-CD8\alpha^-$ cDCs. Only 2%-5% plasmacytoid DCs reside in the small intestinal lamina propria[12]. Mucosal DCs can be discriminated into DCs that express CX3CR1 (the receptor for fractalkine/

Table 1 Anatomic compartments of GALT

Compartment			Gut segment		
Inductive/effector sites	Structure	Abbreviation	Small intestine		Large intestine[3]
			Upper[1]	Lower[2]	
Effector	Lamina propria	LP	+++	+++	+++
	Intraepithelial lymphocytes	IEL	+++	+++	+++
Inductive	Peyer´s patch	PP	+	+++	-
	Intestinal lymph follicle	iLF	+	+++	-
	Intestinal lymph aggregate	iLA	+	++	++
	Mesenteric lymph node	MLN	-	-	-

[1]Distribution of the described structures in the upper small intestine (duodenum and jejunum); [2]Distribution of the described structures in the upper small intestine (ileum); [3]Distribution of the described structures in the upper small intestine (ileum). "+++": Very frequent; "++": Frequent; "+": Randomly; "-": Not present.

Table 2 Mucosal DCs and their proposed function

DC[1]		Intestinal compartment[2]					Comment[3]	Ref.
Lineage	Phenotype	MLN	siLP	cLP	PP	iLF		
cDC	$CD4^-CD8^-$ $CD11b^+CD11c^+$	+++	+++	+++	+++	+++	Express CD103 or CX3cR1. Mediate intestinal antigen acquisition. Involved in the RA dependent T_{reg} conversation. Permitting homing of conventional T cells to intestinal tissues by inducing CCR9 and $\alpha_4\beta_7$.	12-14, 47, 86, 99
	$CD4^+CD8^-$ $CD11b^+CD11c^+$	+++	-	-	++	+	Prime CD4 T cell responses.	8, 28
	$CD4^-CD8^+$ $CD11b^-CD11c^+$	++	-	-	++	++	Prime CD8 T cell responses.	8, 28
pDC	$B220^+PDCA1^+CD11c^+$	+	+/-	+/-	+	+	Produce type I interferons	10

cDC: Conventional DC; pDC: Plasmacytoid DC; MLN: Mesenteric lymph node; PP: Peyer´s patch; cLP: colonic lamina propria; siLP: small intestinal lamina propria; iLF: intestinal lymph follicle. [1]DC lineage; [2]Phenotype of the described DC lineage; [3]Distribution of the described DC lineage in distinct intestinal compartments.

CX3CL1)[12] and into DCs that express the integrin αE chain CD103[13,14]. $CD103^+$ DCs originate from Ly-$6C^{high}CCR2^{high}$ monocytes. Conversely Ly-$6C^{low}CCR2^{low}$ monocytes repopulate $CX3CR1^+CD11b^+$ mucosal DCs[15]. $CX3CR1^+CD11b^+$ DCs directly access the intestinal lumen by extending transepithelial dendrites in a CX3CR1-dependent manner to survey the intestinal lumen[12], whereas $CD103^+$ DCs induce the expression of CCR9 and $\alpha_4\beta_7$ integrin on cognate CD4 and CD8 T cells to facilitate homing of T cells to small intestinal tissues[13,16] and induce the differentiation of regulatory T cells (T_{regs}) in the absence of exogenous cytokines.

DCs IN THE LARGE INTESTINE

The cLP is populated by $CD4^-CD8^-$ DCs which endocytose and process antigens and induce T cell proliferation. Compared to their splenic counterparts TLR-4, -5 and -9 expression by colonic $CD4^-CD8^-$C DCs is low[17]. Only few $CD8^+$ and plasmacytoid DCs are found in the cLP[17]. In human biopsies from colonic tissues, $CD3^-CD14^-CD16^-CD19^-CD34^-$ DCs with an immature state with low TLR-2 and -4 expressions were observed[18]. In addition the presence of $CD83^+$ and DC^-SIGN^+ DCs was described[19]. Human and mouse mucosal DCs are assumed to be less responsive to microbial-derived TLR-ligands compared to spleen or blood born DCs[17] Despite the functional subspecifications of DCs, DCs are characterized by a remarkable plasticity between DCs which is influenced by the local environment, the antigen itself or the activation state of the DC[9,20].

DCs IN PPs

PP, iLF, CP and DC clusters of the small and large intestine belong to the gut associated lymphoid tissues (GALTs)[6], secondary lymphoid structure, which lack in contrast to lymph nodes the afferent lymph and are located in close proximity to the intestinal epithelium. In PP the subepithelial dome regions beneath the follicle-associated epithelium can be discriminated from follicular and interfollicular regions, which serve as inductive sites where immune responses are primed. Specialized epithelial cells, the M cells, deliver luminal antigens to DCs located in the subepithelial dome regions beneath the follicle associated epithelium[21]. The subepithelial dome regions are populated with $CD8\alpha^-$ $CD11b^+B220^-$ DCs and with $CD8\alpha^-CD11b^-B220^-$ DCs[22,23]. By expressing high concentrations of the chemokines CCL20, CXCL16 and CCL9, the follicle-associated epithelium creates a specific micromilleu that allows DCs to selectively migrate towards the follicle associated epithelium[24,25]. Upon pathogen challenge

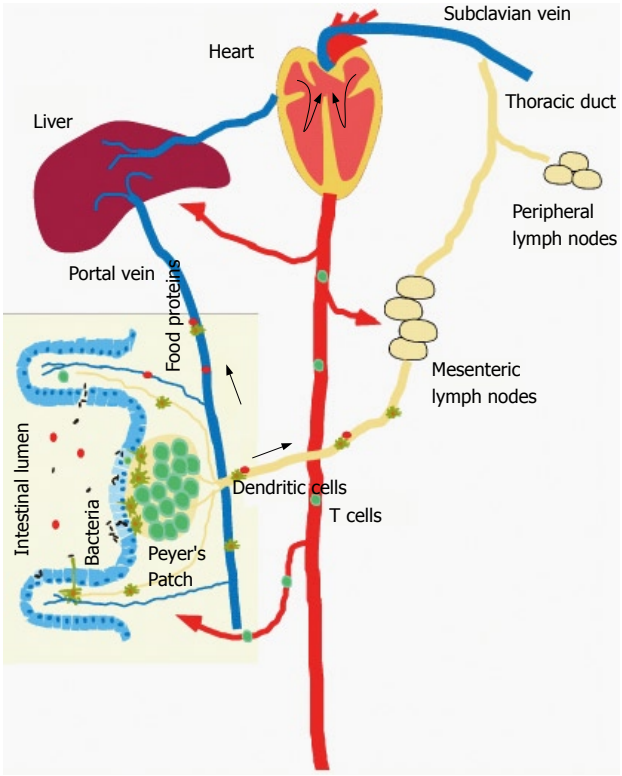


Figure 1 Bacterial and food antigens are continuously surveyed by the mucosal immune system, processed and transported *via* the lymph to mesenteric lymph nodes or *via* the portal vein to the liver. DCs present processed luminal antigens to naïve T cells to achieve tolerance or to initiate host defence to pathogens.

CCR6$^+$ DCs which are located in the interfollicular regions are recruited towards the follicle-associated epithelium to process microbial antigens, and to facilitate rapid local adaptive immune responses[23]. In the absence of CCR6 in CCR6$^{GFP/GFP}$ mice CX3CR1$^+$ DCs that lack CCR6 expression reside in the follicle-associated epithelium[23,26]. In the interfollicular regions CD11b$^-$ CD8α^- and CD11b$^-$ CD8α^+ DCs as well as plasmacytoid DCs are found that produce IL-12 and IL-10, and induce the differentiation of IFN-γ secreting TH1 cells, whereas the CD11b$^+$ subsets produce low IL-12 and high IL-10 levels and prime IL-10 producing T cells[22,27]. CD8$^-$33D1$^+$ DCs, but not CD8α^+DEC205$^+$ DCs are specialized to present processed antigens in a MHC class Ⅱ dependent manner to CD4 T cells[28]. Reoviral antigens are sampled and processed by CD11b$^-$CD8α^- DCs to initiate TH1 adaptive immune responses likely resulting in protective host defence to viral antigens[29].

DCs IN INTESTINAL LYMPHOID TISSUES

Intestinal lymphoid aggregates (iLAs) can be discriminated from PP, which comprises iLF and CP that are closely associated with the epithelial lining in the small and large intestine[30]. CP were defined as tiny aggregates of c-Kit$^+$IL-7R$^+$ cells[31], whereas iLFs are constituted by solitary B follicles that are localized in the anti-mesenteric regions of the intestinal wall, and contain small numbers of mature T lymphocytes, but also c-Kit$^+$ and IL-7R$^+$ cells[32-34]. By analyzing large numbers of such aggregates, the classification of iLA into CP and iLF was recently challenged showing that most of them display properties intermediate between CP and iLF and were termed small intestinal lymphoid tissues (siLT)[35]. In the large intestine, subepithelial DC aggregates locate in the regions of cLP where inflammation develops[36]. DC aggregates are formed by CD8α^+, CD8α^- and B220^{lo} DCs. DCs with high MHC Ⅱ expression, but low expression of the costimulatory molecules CD40, CD80 and CD86 extend transepithelial dendrites into the intestinal lumen[37]. During colitis induced by adoptive transfer of CD45RB^{hi} CD4 T cells into immunocompromised RAG mice, FoxP3 regulatory T cells accumulate in colonic DC aggregates producing TGF-β suggesting that DC aggregates are important structures to regulate inflammation[37].

DCs IN MLN

Luminal antigens are sampled by lamina propria DCs and by DCs located in PP, CP, iLF and DC clusters at the base of intestinal villi. The DCs transport the sampled antigens *via* the lymph to MLN, where naïve T cells are primed and adaptive immune responses are induced[38] (Figure 1). In MLN, blood-born DCs and DCs that originate from PP or the lamina propria are present[6]. In MLN, blood-born DCs are assumed to express CD8α^{hi}CD11b$^-$αL^{int}β7^{int} and CD8α^- CD11b^{hi}αL^{lo}β7^{lo} DCs, whereas DCs derived from mucosal sites are characterized by CD8α^{int}CD11b$^-$ αL^{lo}β7^{hi} and CD8α^-CD11b^{hi}αL^{lo}β7^{hi} expression[39], allowing the discrimination of DCs of distinct origins. CCR7 expression guides lamina propria DCs to MLN[40]. Blood-born DCs enter the MLN *via* interactions with mucosal addressin cellular adhesion molecule (MAdCAM-1) and peripheral node adressin (PNAd) expressed by high endothelial venules (HEVs)[41]. Apoptotic cell bodies derived from intestinal epithelial cells (iECs) are phagocytosed and transported to MLN to induce tolerance against self-antigens[42]. The depletion of MLN results in defective oral tolerance. Lamina propria DCs are continuously carrying commensal bacteria to the MLN[43], which can be further potentate by stimulating DCs with TLR-7/8 agonists[44]. IgA production is induced that is supposed to limit the dissemination of commensal bacteria further than the MLN preventing effectively systemic infections[45].

DCs IN INTESTINAL ANTIGEN ACQUISITION PATHWAYS

Intestinal DCs play a key role in monitoring the intestinal lumen by continuously sampling and processing luminal antigens[46,47]. There are different pathways by which luminal antigens can gain access in the lamina propria[48]. In all pathways, mucosal DCs are supposed to play key roles. Restricted to the ileum DCs extend transepithelial dendrites in a CX3CR1 mediated manner to survey the

intestinal lumen to continuously survey the commensal microflora and to monitor food antigens[12,49]. M cells located in the follicle associated epithelium of PP are specialized epithelial cells which gained the ability to translocate luminal antigens *via* the intestinal barrier and deliver luminal antigens to DCs located in the subepithelial dome regions of PP[21]. Upon pathogen challenge, $CCR6^+$ DCs are recruited to the dome regions to process pathogens, and to trigger rapid local adaptive immune responses[23]. As well $CD11b^-CD8\alpha^-$ $CD11c^+$ interfollicular DCs process antigens derived from luminal reovirus[29]. M cells within the small intestinal villous epithelium are able to translocate pathogens *via* the epithelial barrier to DCs located in the lamina propria[50]. The intestinal epithelium by itself plays an important role in sensing the luminal microenvironment. The intestinal epithelium express Toll-like receptors, such as TLR2[51], TLR-4[52], TLR-5[53] and TLR-9[54] to recognize microbial derived pathogen associated molecular patterns (PAMPs) for the regulation of protective innate immune responses. As well upon LPS-stimulation iECs express the intracellular pathogen recognition pattern NOD1[55] and NOD2[56,57], in which mutations are associated with an increased susceptibility for CD[58,59]. IECs directly participate in intestinal antigen uptake pathways by delivering antigens or exosomes to lamina propria DCs. Secreted IgG binds to cognate luminal antigens to form IgG/antigen complexes that are recycled by neonatal FcRn receptors and delivered to lamina propria DCs[60,61]. IECs also express MHC Ⅱ and may present luminal antigens to CD4 T cells. After luminal exposure with ovalbumin (OVA), OVA is taken up at apical and basolateral surfaces of the epithelial cells. Then, it enters the early endosomes and may be delivered to late endosomes where it is processed in the presence of MHC Ⅱ, and presented in the context of class Ⅱ to T cells[62]. It needs to be dissected in future work whether all or specific luminal antigen acquisition routes are linked to the induction and constant renewal of tolerance and to the development of protective immune responses. DCs are assumed to play key roles in all luminal antigen acquisition routes, and are supposed to represent a major factor for the processing and presentation of orally delivered antigens and the induction of tolerance.

DC MEDIATE INNATE AND ADAPTIVE IMMUNE RESPONSES TO THE COMMENSAL FLORA

The gastrointestinal immune system faces the tremendous challenge to deal with potent stimuli for the innate and adaptive immune system derived from the commensal microflora and food antigens. DCs are sentinels of the mucosal immune system to survey the constituents of the luminal commensals and to trigger host responses to pathogens[63]. It is thought that gastrointestinal diseases, such as IBDs are results of deregulated immune responses to the commensal microflora[1]. Although germ-free (GF) mice and rats generate alloreactive T cell responses[64] and cellular immunity to certain bacteria (after monocolonization)[65,66], the cellularity of their immune system is greatly reduced[67-69]. In this regard, the numbers of IL-17A producing TH17 cells is reduced in the lamina propria of GF animals[70]. Because commensal flora is a major driving force of the homeostatic proliferation of naïve T cells in the periphery, the reduced cellularity in the immune system of GF mice may be the result of the deficient peripheral expansion of recent thymic T cell emigrants[71,72]. When GF mice are fed bacterial carbohydrate polysaccharide A (PSA) CD4 T cells are activated resulting in a TH1 response imprinted by DCs[72]. After conventionalization of GF rats $OX62^+$ DCs increased, whereas $CD4^+$ DCs located in the follicle associated epithelium decreased indicating that $CD4^+$ DCs phagocytose microbial derived antigens, relocate to the follicular regions, and elicit rapid immune responses to the microflora to achieve tolerance[68]. Furthermore, it has been described that the inducible isoform of nitric oxide synthase (iNOS) expressing DCs is markedly reduced in GF or MyD88-deficient animals indicating that the commensal flora is required for the accumulation of iNOS expressing DCs at mucosal sites[73]. In $iNOS^{-/-}$ animals serum IgA levels are reduced suggesting that iNOS expressing DCs play a role in IgA class switching required to prevent the uncontrolled dissemination of the commensal flora into the host.

HEMATOGENOUS DISSEMINATION OF INTESTINAL ANTIGENS

Although the priming of T cells mainly occurs in draining lymph nodes, antigen-rich blood from the small and large intestine is transported to the liver *via* the portal vein, and pressed through a network of sinusoids and scanned by antigen-presenting cells and lymphocytes in the liver[74,75] (Figure 1). One third of liver cells are constituted by non-parenchymal cells (NPCs), which include liver sinusoidal endothelial cells (LSECs), Kupffer cells, biliary cells, stellate cells (Ito or fat-storing) cells and lymphocytes[74,75]. Resident APCs in the liver are Kupffer cells, LSEC[76], Ito cells[77] and DCs[75]. Plasmacytoid $B220^+CD11c^+$ DCs as well as $B220^-CD11c^+$ DCs are found. The latter can be further divided into major immature ($CD40^{lo}$ $CD80^{lo}$ $CD86^{lo}$ MHC class $Ⅱ^{lo}$) $CD11c^{int}$ and minor mature ($CD40^{hi}$ $CD80^{hi}$ $CD86^{hi}$ MHC class $Ⅱ^{hi}$) $CD11c^{hi}$ DCs[78]. Further, the presence of $CD8\alpha^+CD11b^-$ and $CD8\alpha^-CD11b^+$ DCs has been reported[78]. The continuous exposure of resident APCs with the bacterial cell wall derived LPS promotes the induction of CD4 regulatory T cells and may explain the dominance of IL-10 in the liver[79,80]. If Kupffer cells are depleted by gadolonium chloride treatment, liver tolerance becomes impaired raising the possibility that the liver is increasingly recognized as an innate and adaptive immune organ[81].

DCs IN ORAL TOLERANCE INDUCTION

Mucosal DCs play a key role in the development of oral tolerance, a phenomenon, in which systemic immune responses to a defined peptide/protein are blunted, when the same protein has been orally fed before the rodents were systemically challenged[82]. The triggering of oral tolerance in patients with IBD is impaired[83]; however, the exact mechanism has not been determined. Mucosal DCs are critical for the induction of oral tolerance as shown by studies, in which flt-3 ligand was injected in mice leading to the expansion of DC subsets[84]. After the expansion of the intestinal DC pool, enhanced oral tolerance was observed. Because oral tolerance can be transferred by adoptive transfer of T cells into recipient mice, interactions between DCs and T cells seems to be essential for the development of oral tolerance, in which CD4 CD25 FoxP3 regulatory and IL-10 and TGF-β secreting TH3 cells that suppress systemic immune responses are primed[85]. When spleen DCs are compared to mucosal DCs, the mucosal DCs are more efficient in inducing FoxP3 expression than spleen DCs in the presence of TGF-β[86,87]. Further analyses demonstrated that $CD103^+$ DCs are able to induce the differentiation of T_{reg} cells *via* the production of the Vitamin A metabolite, retinoic acid, in presence of TGF-β, which in addition results in the recruitment of T cells and B cells to intestinal tissues[88-90]. Blocking of TGF-β abrogates the ability of $CD103^+$ DCs to induce T_{reg} cells[91,92]. However, parallel studies have indicated that exogenous TGF-β has not to been added to this system in order to obtain FoxP3 expressing T_{reg} cells[86,87] raising the possibility that gut DCs activates latent TGF-β present in mucosal tissues. In this regard, the integrin $\alpha_v\beta_8$ expressed by DCs is required to activate TGF-β *in vivo*. Mice, in which DCs lack $\alpha_v\beta_8$, develop IBD and autoimmunity[93].

Oral tolerance is not impaired in PP-deficient mice demonstrating that lamina propria DCs play a key role in the development of oral tolerance by sampling the luminal content, and transporting antigens to the MLN[94]. After depletion of the MLN, the development of oral tolerance is reduced indicating that the MLN are a major site for priming tolerance[95]. Interestingly, it has been suggested, that intestinal self antigens can be presented by lymph node stroma cells beyond the MLNs[96]. It also may depend on the presence of certain pathogens within the lumen, because *Heligmosomoides polygyus* infection correlates with DC activation and IL-10 expression[97]. This provides evidence that the studies of intestinal microbial responses not only require the recognition of region- and compartment-specific immune responses, but the consideration of the interplay of different commensals and pathogens in modulating mucosal immune responses.

DC DEPENDENT T_{reg} CELL CONVERSION

T_{reg} cells play a key role for the development of oral tolerance, and the regulation of intestinal inflammation triggered by the intestinal microflora. $CD103^+$ but not $CD103^-$ DCs purified from GALT-induced generation of FoxP3 cells *in vitro*[98]. T_{reg} cells can be distinguished from other T cell populations by the surface expression of the α chain of the high affinity IL-2 receptor (CD25), and the transcription factor FoxP3 that is essential for T_{reg} development. Deadly autoimmunity is prevented by T_{reg} cells as exemplified in X-linked immunodeficiency, polyendocrinopathy, enteropathy (IPEX) syndrome in humans[98]. The conversion of T_{reg} cells in the GALT can be prevented by inhibitors of retinal dehydrogenases, an enzyme that is highly expressed by $CD103^+$ DCs, which converts retinol (vitamine A) into retinoic acid[86,87,91,99]. Retinoic acid binds to nuclear retinoic acid receptors that upon ligation can inhibit the activity of activating protein-1 (AP-1), a transcription factor, which can interfere with nuclear factor of activated T cells (NFAT)-FoxP3 complex[100,101]. However, the exact retinoic acid dependent signal transduction pathways required for the conversion of T_{reg} cells remains to be elucidated. Retinoic acid production by $CD103^+$ DCs also induce the up-regulation of the integrin $\alpha_4\beta_7$, and the CC chemokine receptor CCR9 which binds thymus-expressed chemokine (TECK) permitting the accumulation of T_{reg} cells intestinal tissues[13]. The retinoic acid dependent conversation of T_{reg} cells depends on the presence of TGF-β, a cytokine that, in the presence of IL-6 and IL-23, can also induce the generation of TH17 cells[102] (Figure 2). A small subset of naïve T cells in the small intestine co-express the transcription factors FoxP3 and RORγt[103]. High expression of TGF-β represses IL-23 receptor expression and favours the generation of FoxP3 T_{reg} cells, whereas low TGF-β expression in concert with IL-6, IL-21 and IL-23 relieves FoxP3 mediated RORγT inhibition promoting TH17 cells[103]. TH17 cells accumulate in the lamina propria of patients with IBD indicating that TGF-β can induce regulatory and pro-inflammatory T cell subsets[104].

DCs IN IBD

Various animal models have provided insights that mucosal DCs play a key role in IBD. However, the specific function of certain DCs are unknown and needs to be determined in future work, which will provide information on mechanisms leading to IBD and limiting intestinal inflammation to achieve protective mucosal immune responses. In agreement with animal models DCs accumulate at sites of inflammation in patients with IBD. It was found that the pathogen recognition receptors TLR-2 and -4 as well as the activation/maturation marker CD40 are upregulated by intestinal DCs derived from patients with CD[18]. Furthermore, increased numbers of TNF-α producing $MDC8^+$ monocytes, which may be precursors of mucosal DC populations, were found in patients with IBD and, hence, the treatment CD patients with anti-TNF-α antibodies resulted in reduced DC activation[105,106]. In inflamed tissues DCs are matured and increased in

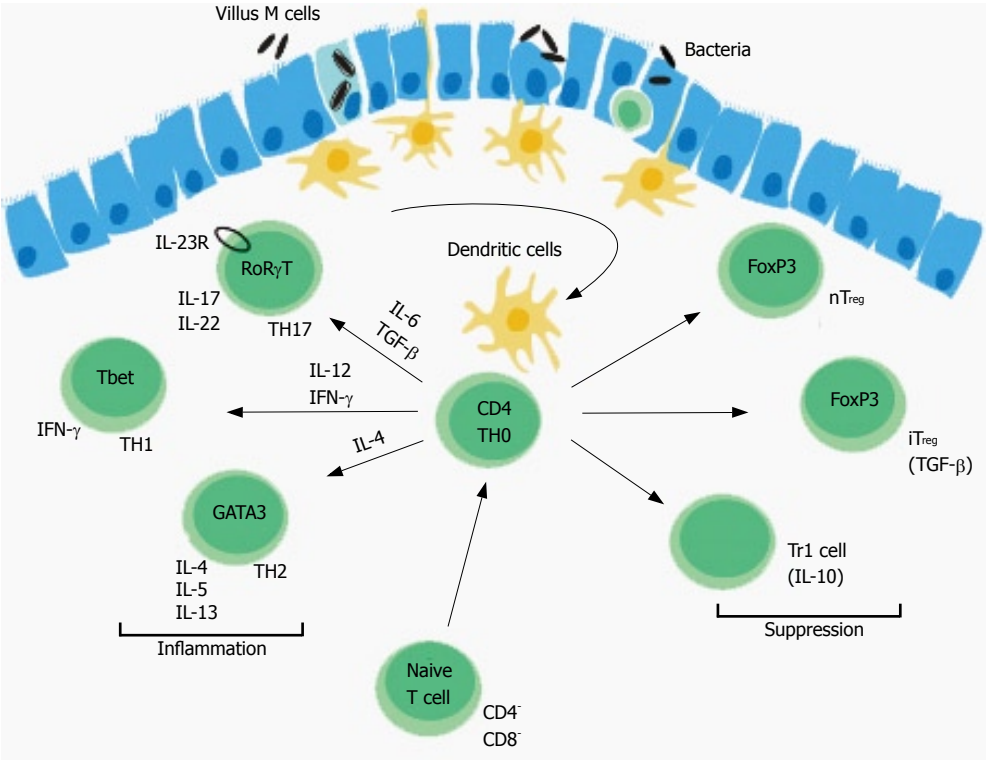


Figure 2 DCs continuously survey the intestinal lumen, phagocytose and process luminal antigens and present them to T cells, which will differentiate in presence of IL-6 and TGF-β to IL-17A and IL-17F producing TH17 cells, in presence of IL-12 and IFN-γ into TH1 cells or in presence of IL-4 into TH2 cells. nTreg, natural occurring regulatory T cell; iTreg, inducible regulatory T cells; Tr1, regulating T cell.

numbers. The $CD83^{-}CD80^{+}DCSIGN^{+}$ DC subsets produce the cytokine IL-12 and IL-18, which promote TH1 development[19]. Also, in the peripheral blood and in the lamina propria of patients with CD or UC the numbers of $CD86^{+}CD40^{+}$ DCs are increased. In addition, DCs generated *ex vitro* from peripheral blood monocytes of IBD patients show increased abilities to stimulate immune responses[107-109]. Mice studies indicated that DCs isolated from inflamed colonic tissues and DCs located in the terminal ileum, which continuously sample commensal bacteria produce IL-23, but little IL-12[110,111]. It was discovered that IL-10 KO mice that spontaneously develop colitis are protected from colitis when bred on IL12p19, but not on IL-12 p35-deficient mice. In similar studies, *Helicobacter hepaticus* infected immunocompromised $RAG^{-/-}$ bred on IL-12 p19 deficient mice, but not on IL-12 p35 deficient background mice were protected from colitis indicating that DC derived IL-23 plays a major role in intestinal pathology[112,113]. The formation of granulomas, histological characteristics of CD, depends on the release of IL-23 by DC-like cell types that are characterized by CD11c and F4/80 expression[114]. Genome-wide association studies showed associations between CD and UC patients, and a gene encoding a subunit for the IL-23 receptor suggested a major role of IL-23 in the pathogenesis of IBD[115,116]. IL-23 seems to be essential for the expansion and maintenance, but not for the initial induction of IL-17 producing CD4 T cells (TH17) cells[117]. Studies in which TH1 and TH17 cells were generated co-cultures, in which naïve T cells were cultured with fecal extracts pulsed DCs, and in the presence of TH1 or TH17 promoting cytokines indicated that TH17 cells are more pathogenic than TH1 cells[118]. Recent published observations report that colitis induced by transfer of IFNγ-deficient T cells in $RAG^{-/-}$ mice is associated with elevated numbers of TH17 cells in the lamina propria[70]. The adoptive transfer of IL-17F, but not IL-17A deficient CD4 T cells ameliorated the IBD in the transfer model[119,120]. Interesting findings implicated that the IL-1 and IL-23 dependent priming of TH17 effector cells required a NOD2 dependent pathway, and that monocyte derived DCs from CD patients with mutated NOD2 failed to efficiently activate TH17 effector cells[121]. In this regard the TH17 cytokine IL-22 mediates mucosal defence to bacterial pathogens, and ameliorates chronic colitis in the TCRαKO model by stimulating mucus production and globlet cell restitution under inflammatory conditions[122]. In addition IL-22, which is released by T and DCs, act together with IL-17 to clear bacterial infections at mucosal sites[123,124]. When conventional DCs are depleted in a CD11c DTR transgenic animal system by diphtheria toxin applications, the severity of colitis is suppressed in the dextran sodium sulfate (DSS) colitis model[125]. Furthermore, in mice with an iEC specific deletion of IKKβ failed to clear *Trichuris muris* infection characterized by severe intestinal pathology[126]. In these mice an increased accumulation of DCs at mucosal sites was observed that produce IL-12/23 p40 and TNF-α. In addition, an accumulation of IFN-γ producing TH1 and IL-17 producing TH17 cells in the MLN was found. Specific depletion of NEMO (IKKγ) or of both IKKα and IKKβ is essential for the activation of NF-κB activation induce IBD[127]. Constitutive NF-κB activation in IECs by commensal flora may condition DCs to prevent tissue inflammation. Thymic stromal lymphopoietin (TSLP) produced by epithelial cells is involved in the

conditioning of DCs to prime less harmful TH2 and T_{reg} responses[126,128]. Together, these data suggest that DCs are conditioned by iECs to promote immunosuppressive T cell responses. However, DCs and their precursors are sensitive to proinflammatory activation signals, which could help to participate into the long persistence of local T cell activation patterns promoting IBD.

CONCLUSION

Mucosal DCs may have several functions in the mucosal immune system to accomplish tolerance and to maintain homeostasis. Tolerance to intestinal self antigens, oral antigens and the commensal flora is achieved by interactions of DCs with regulatory and effector T cells. DCs are also involved in triggering deleterious T cell responses to the endogenous microflora being the basis of IBD. Mucosal DCs express the integrin alpha E (CD103) or the receptor for fractalkine/CX3CR1. CX3CR1 expressing DCs are involved in luminal antigen recognition pathways, whereas CD103 DCs metabolize vitamin A to retinoic acid and are involved in the conversion of T cells to regulatory T cells. Genetically engineered mouse models, and cellular approaches will be increasingly available to study the biology of CD103 and CX3CR1 DCs in immune responses to the commensal flora, and their role in initiating and regulating intestinal inflammation. DCs maintain intestinal homeostasis allowing the peaceful coexistence with the endogenous microflora. The discovery of specific DCs associated with luminal antigen acquisition and oral tolerance will allow developing strategies for targeting defined antigen acquisition routes to design therapeutic treatments for patients with IBD.

ACKNOWLEDGMENT

The author is indebted to Mitsue Shimada for continuous support.

REFERENCES

1 **Xavier RJ**, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 2007; **448**: 427-434
2 **Strober W**. Immunology. Unraveling gut inflammation. *Science* 2006; **313**: 1052-1054
3 **Nagler-Anderson C**. Man the barrier! Strategic defences in the intestinal mucosa. *Nat Rev Immunol* 2001; **1**: 59-67
4 **Niess JH**, Reinecker HC. Dendritic cells in the recognition of intestinal microbiota. *Cell Microbiol* 2006; **8**: 558-564
5 **Steinman RM**, Nussenzweig MC. Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. *Proc Natl Acad Sci USA* 2002; **99**: 351-358
6 **Iwasaki A**. Mucosal dendritic cells. *Annu Rev Immunol* 2007; **25**: 381-418
7 **Vremec D**, Shortman K. Dendritic cell subtypes in mouse lymphoid organs: cross-correlation of surface markers, changes with incubation, and differences among thymus, spleen, and lymph nodes. *J Immunol* 1997; **159**: 565-573
8 **Hochrein H**, Shortman K, Vremec D, Scott B, Hertzog P, O'Keeffe M. Differential production of IL-12, IFN-alpha, and IFN-gamma by mouse dendritic cell subsets. *J Immunol* 2001; **166**: 5448-5455
9 **Kelsall BL**, Rescigno M. Mucosal dendritic cells in immunity and inflammation. *Nat Immunol* 2004; **5**: 1091-1095
10 **Bjorck P**. Isolation and characterization of plasmacytoid dendritic cells from Flt3 ligand and granulocyte-macrophage colony-stimulating factor-treated mice. *Blood* 2001; **98**: 3520-3526
11 **Shortman K**, Naik SH. Steady-state and inflammatory dendritic-cell development. *Nat Rev Immunol* 2007; **7**: 19-30
12 **Niess JH**, Brand S, Gu X, Landsman L, Jung S, McCormick BA, Vyas JM, Boes M, Ploegh HL, Fox JG, Littman DR, Reinecker HC. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* 2005; **307**: 254-258
13 **Johansson-Lindbom B**, Svensson M, Pabst O, Palmqvist C, Marquez G, Forster R, Agace WW. Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing. *J Exp Med* 2005; **202**: 1063-1073
14 **Annacker O**, Coombes JL, Malmstrom V, Uhlig HH, Bourne T, Johansson-Lindbom B, Agace WW, Parker CM, Powrie F. Essential role for CD103 in the T cell-mediated regulation of experimental colitis. *J Exp Med* 2005; **202**: 1051-1061
15 **Jakubzick C**, Tacke F, Ginhoux F, Wagers AJ, van Rooijen N, Mack M, Merad M, Randolph GJ. Blood monocyte subsets differentially give rise to CD103+ and CD103- pulmonary dendritic cell populations. *J Immunol* 2008; **180**: 3019-3027
16 **Stenstad H**, Ericsson A, Johansson-Lindbom B, Svensson M, Marsal J, Mack M, Picarella D, Soler D, Marquez G, Briskin M, Agace WW. Gut-associated lymphoid tissue-primed CD4+ T cells display CCR9-dependent and -independent homing to the small intestine. *Blood* 2006; **107**: 3447-3454
17 **Takenaka S**, Safroneeva E, Xing Z, Gauldie J. Dendritic cells derived from murine colonic mucosa have unique functional and phenotypic characteristics. *J Immunol* 2007; **178**: 7984-7993
18 **Hart AL**, Al-Hassi HO, Rigby RJ, Bell SJ, Emmanuel AV, Knight SC, Kamm MA, Stagg AJ. Characteristics of intestinal dendritic cells in inflammatory bowel diseases. *Gastroenterology* 2005; **129**: 50-65
19 **te Velde AA**, van Kooyk Y, Braat H, Hommes DW, Dellemijn TA, Slors JF, van Deventer SJ, Vyth-Dreese FA. Increased expression of DC-SIGN+IL-12+IL-18+ and CD83+IL-12-IL-18- dendritic cell populations in the colonic mucosa of patients with Crohn's disease. *Eur J Immunol* 2003; **33**: 143-151
20 **Kelsall BL**. Innate and adaptive mechanisms to control of pathological intestinal inflammation. *J Pathol* 2008; **214**: 242-259
21 **Neutra MR**, Mantis NJ, Kraehenbuhl JP. Collaboration of epithelial cells with organized mucosal lymphoid tissues. *Nat Immunol* 2001; **2**: 1004-1009
22 **Iwasaki A**, Kelsall BL. Unique functions of CD11b+, CD8 alpha+, and double-negative Peyer's patch dendritic cells. *J Immunol* 2001; **166**: 4884-4890
23 **Salazar-Gonzalez RM**, Niess JH, Zammit DJ, Ravindran R, Srinivasan A, Maxwell JR, Stoklasek T, Yadav R, Williams IR, Gu X, McCormick BA, Pazos MA, Vella AT, Lefrancois L, Reinecker HC, McSorley SJ. CCR6-mediated dendritic cell activation of pathogen-specific T cells in Peyer's patches. *Immunity* 2006; **24**: 623-632
24 **Iwasaki A**, Kelsall BL. Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3alpha, MIP-3beta, and secondary lymphoid organ chemokine. *J Exp Med* 2000; **191**: 1381-1394
25 **Zhao X**, Sato A, Dela Cruz CS, Linehan M, Luegering A, Kucharzik T, Shirakawa AK, Marquez G, Farber JM, Williams I, Iwasaki A. CCL9 is secreted by the follicle-associated epithelium and recruits dome region Peyer's patch CD11b+ dendritic cells. *J Immunol* 2003; **171**: 2797-2803
26 **Cook DN**, Prosser DM, Forster R, Zhang J, Kuklin NA,

Abbondanzo SJ, Niu XD, Chen SC, Manfra DJ, Wiekowski MT, Sullivan LM, Smith SR, Greenberg HB, Narula SK, Lipp M, Lira SA. CCR6 mediates dendritic cell localization, lymphocyte homeostasis, and immune responses in mucosal tissue. *Immunity* 2000; **12**: 495-503

27 **Iwasaki A**, Kelsall BL. Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells. *J Exp Med* 1999; **190**: 229-239

28 **Dudziak D**, Kamphorst AO, Heidkamp GF, Buchholz VR, Trumpfheller C, Yamazaki S, Cheong C, Liu K, Lee HW, Park CG, Steinman RM, Nussenzweig MC. Differential antigen processing by dendritic cell subsets in vivo. *Science* 2007; **315**: 107-111

29 **Fleeton MN**, Contractor N, Leon F, Wetzel JD, Dermody TS, Kelsall BL. Peyer's patch dendritic cells process viral antigen from apoptotic epithelial cells in the intestine of reovirus-infected mice. *J Exp Med* 2004; **200**: 235-245

30 **Blum KS**, Pabst R. Keystones in lymph node development. *J Anat* 2006; **209**: 585-595

31 **Kanamori Y**, Ishimaru K, Nanno M, Maki K, Ikuta K, Nariuchi H, Ishikawa H. Identification of novel lymphoid tissues in murine intestinal mucosa where clusters of c-kit+ IL-7R+ Thy1+ lympho-hemopoietic progenitors develop. *J Exp Med* 1996; **184**: 1449-1459

32 **Lorenz RG**, Chaplin DD, McDonald KG, McDonough JS, Newberry RD. Isolated lymphoid follicle formation is inducible and dependent upon lymphotoxin-sufficient B lymphocytes, lymphotoxin beta receptor, and TNF receptor I function. *J Immunol* 2003; **170**: 5475-5482

33 **Hamada H**, Hiroi T, Nishiyama Y, Takahashi H, Masunaga Y, Hachimura S, Kaminogawa S, Takahashi-Iwanaga H, Iwanaga T, Kiyono H, Yamamoto H, Ishikawa H. Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. *J Immunol* 2002; **168**: 57-64

34 **Pabst O**, Herbrand H, Worbs T, Friedrichsen M, Yan S, Hoffmann MW, Korner H, Bernhardt G, Pabst R, Forster R. Cryptopatches and isolated lymphoid follicles: dynamic lymphoid tissues dispensable for the generation of intraepithelial lymphocytes. *Eur J Immunol* 2005; **35**: 98-107

35 **McKenna DJ**, Guan XM, Shulgin AT. 3,4-Methylenedioxyamphetamine (MDA) analogues exhibit differential effects on synaptosomal release of 3H-dopamine and 3H-5-hydroxytryptamine. *Pharmacol Biochem Behav* 1991; **38**: 505-512

36 **Leithauser F**, Trobonjaca Z, Moller P, Reimann J. Clustering of colonic lamina propria CD4(+) T cells to subepithelial dendritic cell aggregates precedes the development of colitis in a murine adoptive transfer model. *Lab Invest* 2001; **81**: 1339-1349

37 **Leithauser F**, Meinhardt-Krajina T, Fink K, Wotschke B, Moller P, Reimann J. Foxp3-expressing CD103+ regulatory T cells accumulate in dendritic cell aggregates of the colonic mucosa in murine transfer colitis. *Am J Pathol* 2006; **168**: 1898-1909

38 **Mowat AM**. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol* 2003; **3**: 331-341

39 **Jang MH**, Sougawa N, Tanaka T, Hirata T, Hiroi T, Tohya K, Guo Z, Umemoto E, Ebisuno Y, Yang BG, Seoh JY, Lipp M, Kiyono H, Miyasaka M. CCR7 is critically important for migration of dendritic cells in intestinal lamina propria to mesenteric lymph nodes. *J Immunol* 2006; **176**: 803-810

40 **Sallusto F**, Palermo B, Lenig D, Miettinen M, Matikainen S, Julkunen I, Forster R, Burgstahler R, Lipp M, Lanzavecchia A. Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *Eur J Immunol* 1999; **29**: 1617-1625

41 **Soderberg KA**, Linehan MM, Ruddle NH, Iwasaki A. MAdCAM-1 expressing sacral lymph node in the lymphotoxin beta-deficient mouse provides a site for immune generation following vaginal herpes simplex virus-2 infection. *J Immunol* 2004; **173**: 1908-1913

42 **Huang FP**, Platt N, Wykes M, Major JR, Powell TJ, Jenkins CD, MacPherson GG. A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. *J Exp Med* 2000; **191**: 435-444

43 **Macpherson AJ**, Harris NL. Interactions between commensal intestinal bacteria and the immune system. *Nat Rev Immunol* 2004; **4**: 478-485

44 **Yrlid U**, Cerovic V, Milling S, Jenkins CD, Klavinskis LS, MacPherson GG. A distinct subset of intestinal dendritic cells responds selectively to oral TLR7/8 stimulation. *Eur J Immunol* 2006; **36**: 2639-2648

45 **Macpherson AJ**, Uhr T. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science* 2004; **303**: 1662-1665

46 **Niess JH**, Reinecker HC. Dendritic cells: the commanders-in-chief of mucosal immune defenses. *Curr Opin Gastroenterol* 2006; **22**: 354-360

47 **Rescigno M**, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, Granucci F, Kraehenbuhl JP, Ricciardi-Castagnoli P. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol* 2001; **2**: 361-367

48 **Corthesy B**. Roundtrip ticket for secretory IgA: role in mucosal homeostasis? *J Immunol* 2007; **178**: 27-32

49 **Chieppa M**, Rescigno M, Huang AY, Germain RN. Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. *J Exp Med* 2006; **203**: 2841-2852

50 **Jang MH**, Kweon MN, Iwatani K, Yamamoto M, Terahara K, Sasakawa C, Suzuki T, Nochi T, Yokota Y, Rennert PD, Hiroi T, Tamagawa H, Iijima H, Kunisawa J, Yuki Y, Kiyono H. Intestinal villous M cells: an antigen entry site in the mucosal epithelium. *Proc Natl Acad Sci USA* 2004; **101**: 6110-6115

51 **Cario E**, Rosenberg IM, Brandwein SL, Beck PL, Reinecker HC, Podolsky DK. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. *J Immunol* 2000; **164**: 966-972

52 **Hornef MW**, Frisan T, Vandewalle A, Normark S, Richter-Dahlfors A. Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. *J Exp Med* 2002; **195**: 559-570

53 **Hershberg RM**. The epithelial cell cytoskeleton and intracellular trafficking. V. Polarized compartmentalization of antigen processing and Toll-like receptor signaling in intestinal epithelial cells. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G833-G839

54 **Lee J**, Mo JH, Katakura K, Alkalay I, Rucker AN, Liu YT, Lee HK, Shen C, Cojocaru G, Shenouda S, Kagnoff M, Eckmann L, Ben-Neriah Y, Raz E. Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. *Nat Cell Biol* 2006; **8**: 1327-1336

55 **Girardin SE**, Tournebize R, Mavris M, Page AL, Li X, Stark GR, Bertin J, DiStefano PS, Yaniv M, Sansonetti PJ, Philpott DJ. CARD4/Nod1 mediates NF-kappaB and JNK activation by invasive Shigella flexneri. *EMBO Rep* 2001; **2**: 736-742

56 **Barnich N**, Aguirre JE, Reinecker HC, Xavier R, Podolsky DK. Membrane recruitment of NOD2 in intestinal epithelial cells is essential for nuclear factor-{kappa}B activation in muramyl dipeptide recognition. *J Cell Biol* 2005; **170**: 21-26

57 **Hisamatsu T**, Suzuki M, Reinecker HC, Nadeau WJ, McCormick BA, Podolsky DK. CARD15/NOD2 functions as an antibacterial factor in human intestinal epithelial cells. *Gastroenterology* 2003; **124**: 993-1000

58 **Ogura Y**, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, Britton H, Moran T, Karaliuskas R, Duerr RH, Achkar JP, Brant SR, Bayless TM, Kirschner BS, Hanauer

SB, Nunez G, Cho JH. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 2001; **411**: 603-606

59 **Hugot JP**, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, Almer S, Tysk C, O'Morain CA, Gassull M, Binder V, Finkel Y, Cortot A, Modigliani R, Laurent-Puig P, Gower-Rousseau C, Macry J, Colombel JF, Sahbatou M, Thomas G. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 2001; **411**: 599-603

60 **Yoshida M**, Kobayashi K, Kuo TT, Bry L, Glickman JN, Claypool SM, Kaser A, Nagaishi T, Higgins DE, Mizoguchi E, Wakatsuki Y, Roopenian DC, Mizoguchi A, Lencer WI, Blumberg RS. Neonatal Fc receptor for IgG regulates mucosal immune responses to luminal bacteria. *J Clin Invest* 2006; **116**: 2142-2151

61 **Yoshida M**, Claypool SM, Wagner JS, Mizoguchi E, Mizoguchi A, Roopenian DC, Lencer WI, Blumberg RS. Human neonatal Fc receptor mediates transport of IgG into luminal secretions for delivery of antigens to mucosal dendritic cells. *Immunity* 2004; **20**: 769-783

62 **Buning J**, Hundorfean G, Schmitz M, Zimmer KP, Strobel S, Gebert A, Ludwig D. Antigen targeting to MHC class II-enriched late endosomes in colonic epithelial cells: trafficking of luminal antigens studied in vivo in Crohn's colitis patients. *FASEB J* 2006; **20**: 359-361

63 **Hapfelmeier S**, Muller AJ, Stecher B, Kaiser P, Barthel M, Endt K, Eberhard M, Robbiani R, Jacobi CA, Heikenwalder M, Kirschning C, Jung S, Stallmach T, Kremer M, Hardt WD. Microbe sampling by mucosal dendritic cells is a discrete, MyD88-independent step in DeltainvG S. Typhimurium colitis. *J Exp Med* 2008; **205**: 437-450

64 **Nielsen HE**. Reactivity of lymphocytes from germfree rats in mixed leukocyte culture and in graft-versus-host reaction. *J Exp Med* 1972; **136**: 417-425

65 **Butterton JR**, Ryan ET, Shahin RA, Calderwood SB. Development of a germfree mouse model of Vibrio cholerae infection. *Infect Immun* 1996; **64**: 4373-4377

66 **Yrios JW**, Balish E. Immune response of athymic and euthymic germfree mice to Campylobacter spp. *Infect Immun* 1986; **54**: 339-346

67 **Helgeland L**, Vaage JT, Rolstad B, Midtvedt T, Brandtzaeg P. Microbial colonization influences composition and T-cell receptor V beta repertoire of intraepithelial lymphocytes in rat intestine. *Immunology* 1996; **89**: 494-501

68 **Yamanaka T**, Helgeland L, Farstad IN, Fukushima H, Midtvedt T, Brandtzaeg P. Microbial colonization drives lymphocyte accumulation and differentiation in the follicle-associated epithelium of Peyer's patches. *J Immunol* 2003; **170**: 816-822

69 **Helgeland L**, Dissen E, Dai KZ, Midtvedt T, Brandtzaeg P, Vaage JT. Microbial colonization induces oligoclonal expansions of intraepithelial CD8 T cells in the gut. *Eur J Immunol* 2004; **34**: 3389-3400

70 **Niess JH**, Leithauser F, Adler G, Reimann J. Commensal gut flora drives the expansion of proinflammatory CD4 T cells in the colonic lamina propria under normal and inflammatory conditions. *J Immunol* 2008; **180**: 559-568

71 **Mazmanian SK**, Liu CH, Tzianabos AO, Kasper DL. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* 2005; **122**: 107-118

72 **Wang Q**, McLoughlin RM, Cobb BA, Charrel-Dennis M, Zaleski KJ, Golenbock D, Tzianabos AO, Kasper DL. A bacterial carbohydrate links innate and adaptive responses through Toll-like receptor 2. *J Exp Med* 2006; **203**: 2853-2863

73 **Tezuka H**, Abe Y, Iwata M, Takeuchi H, Ishikawa H, Matsushita M, Shiohara T, Akira S, Ohteki T. Regulation of IgA production by naturally occurring TNF/iNOS-producing dendritic cells. *Nature* 2007; **448**: 929-933

74 **Racanelli V**, Rehermann B. The liver as an immunological organ. *Hepatology* 2006; **43**: S54-S62

75 **Wick MJ**, Leithauser F, Reimann J. The hepatic immune system. *Crit Rev Immunol* 2002; **22**: 47-103

76 **Rubinstein D**, Roska AK, Lipsky PE. Antigen presentation by liver sinusoidal lining cells after antigen exposure in vivo. *J Immunol* 1987; **138**: 1377-1382

77 **Winau F**, Hegasy G, Weiskirchen R, Weber S, Cassan C, Sieling PA, Modlin RL, Liblau RS, Gressner AM, Kaufmann SH. Ito cells are liver-resident antigen-presenting cells for activating T cell responses. *Immunity* 2007; **26**: 117-129

78 **Jomantaite I**, Dikopoulos N, Kroger A, Leithauser F, Hauser H, Schirmbeck R, Reimann J. Hepatic dendritic cell subsets in the mouse. *Eur J Immunol* 2004; **34**: 355-365

79 **Knolle PA**, Uhrig A, Hegenbarth S, Loser E, Schmitt E, Gerken G, Lohse AW. IL-10 down-regulates T cell activation by antigen-presenting liver sinusoidal endothelial cells through decreased antigen uptake via the mannose receptor and lowered surface expression of accessory molecules. *Clin Exp Immunol* 1998; **114**: 427-433

80 **Knolle PA**, Germann T, Treichel U, Uhrig A, Schmitt E, Hegenbarth S, Lohse AW, Gerken G. Endotoxin down-regulates T cell activation by antigen-presenting liver sinusoidal endothelial cells. *J Immunol* 1999; **162**: 1401-1407

81 **Roland CR**, Mangino MJ, Duffy BF, Flye MW. Lymphocyte suppression by Kupffer cells prevents portal venous tolerance induction: a study of macrophage function after intravenous gadolinium. *Transplantation* 1993; **55**: 1151-1158

82 **Macpherson AJ**, Smith K. Mesenteric lymph nodes at the center of immune anatomy. *J Exp Med* 2006; **203**: 497-500

83 **Kraus TA**, Toy L, Chan L, Childs J, Mayer L. Failure to induce oral tolerance to a soluble protein in patients with inflammatory bowel disease. *Gastroenterology* 2004; **126**: 1771-1778

84 **Viney JL**, Mowat AM, O'Malley JM, Williamson E, Fanger NA. Expanding dendritic cells in vivo enhances the induction of oral tolerance. *J Immunol* 1998; **160**: 5815-5825

85 **Nagatani K**, Sagawa K, Komagata Y, Yamamoto K. Peyer's patch dendritic cells capturing oral antigen interact with antigen-specific T cells and induce gut-homing CD4(+)CD25(+) regulatory T cells in Peyer's patches. *Ann N Y Acad Sci* 2004; **1029**: 366-370

86 **Sun CM**, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, Belkaid Y. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* 2007; **204**: 1775-1785

87 **Mucida D**, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M, Cheroutre H. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* 2007; **317**: 256-260

88 **Mora JR**, Bono MR, Manjunath N, Weninger W, Cavanagh LL, Rosemblatt M, Von Andrian UH. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature* 2003; **424**: 88-93

89 **Mora JR**, Iwata M, Eksteen B, Song SY, Junt T, Senman B, Otipoby KL, Yokota A, Takeuchi H, Ricciardi-Castagnoli P, Rajewsky K, Adams DH, von Andrian UH. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. *Science* 2006; **314**: 1157-1160

90 **Iwata M**, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY. Retinoic acid imprints gut-homing specificity on T cells. *Immunity* 2004; **21**: 527-538

91 **Coombes JL**, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, Powrie F. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* 2007; **204**: 1757-1764

92 **Denning TL**, Wang YC, Patel SR, Williams IR, Pulendran B. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. *Nat Immunol* 2007; **8**: 1086-1094

93 **Travis MA**, Reizis B, Melton AC, Masteller E, Tang Q,

Proctor JM, Wang Y, Bernstein X, Huang X, Reichardt LF, Bluestone JA, Sheppard D. Loss of integrin alpha(v)beta8 on dendritic cells causes autoimmunity and colitis in mice. *Nature* 2007; **449**: 361-365

94 **Kraus TA**, Brimnes J, Muong C, Liu JH, Moran TM, Tappenden KA, Boros P, Mayer L. Induction of mucosal tolerance in Peyer's patch-deficient, ligated small bowel loops. *J Clin Invest* 2005; **115**: 2234-2243

95 **Worbs T**, Bode U, Yan S, Hoffmann MW, Hintzen G, Bernhardt G, Forster R, Pabst O. Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. *J Exp Med* 2006; **203**: 519-527

96 **Lee JW**, Epardaud M, Sun J, Becker JE, Cheng AC, Yonekura AR, Heath JK, Turley SJ. Peripheral antigen display by lymph node stroma promotes T cell tolerance to intestinal self. *Nat Immunol* 2007; **8**: 181-190

97 **Chen CC**, Louie S, McCormick BA, Walker WA, Shi HN. Helminth-primed dendritic cells alter the host response to enteric bacterial infection. *J Immunol* 2006; **176**: 472-483

98 **von Boehmer H**. Oral tolerance: is it all retinoic acid? *J Exp Med* 2007; **204**: 1737-1739

99 **Benson MJ**, Pino-Lagos K, Rosemblatt M, Noelle RJ. All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. *J Exp Med* 2007; **204**: 1765-1774

100 **Li Y**, Hashimoto Y, Agadir A, Kagechika H, Zhang X. Identification of a novel class of retinoic acid receptor beta-selective retinoid antagonists and their inhibitory effects on AP-1 activity and retinoic acid-induced apoptosis in human breast cancer cells. *J Biol Chem* 1999; **274**: 15360-15366

101 **Wu Y**, Borde M, Heissmeyer V, Feuerer M, Lapan AD, Stroud JC, Bates DL, Guo L, Han A, Ziegler SF, Mathis D, Benoist C, Chen L, Rao A. FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell* 2006; **126**: 375-387

102 **Veldhoen M**, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 2006; **24**: 179-189

103 **Zhou L**, Lopes JE, Chong MM, Ivanov II, Min R, Victora GD, Shen Y, Du J, Rubtsov YP, Rudensky AY, Ziegler SF, Littman DR. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature* 2008; **453**: 236-240

104 **Fujino S**, Andoh A, Bamba S, Ogawa A, Hata K, Araki Y, Bamba T, Fujiyama Y. Increased expression of interleukin 17 in inflammatory bowel disease. *Gut* 2003; **52**: 65-70

105 **de Baey A**, Mende I, Riethmueller G, Baeuerle PA. Phenotype and function of human dendritic cells derived from M-DC8(+) monocytes. *Eur J Immunol* 2001; **31**: 1646-1655

106 **de Baey A**, Mende I, Baretton G, Greiner A, Hartl WH, Baeuerle PA, Diepolder HM. A subset of human dendritic cells in the T cell area of mucosa-associated lymphoid tissue with a high potential to produce TNF-alpha. *J Immunol* 2003; **170**: 5089-5094

107 **Ikeda Y**, Akbar F, Matsui H, Onji M. Characterization of antigen-presenting dendritic cells in the peripheral blood and colonic mucosa of patients with ulcerative colitis. *Eur J Gastroenterol Hepatol* 2001; **13**: 841-850

108 **Murakami H**, Akbar SM, Matsui H, Horiike N, Onji M. Macrophage migration inhibitory factor activates antigen-presenting dendritic cells and induces inflammatory cytokines in ulcerative colitis. *Clin Exp Immunol* 2002; **128**: 504-510

109 **Yeung MM**, Melgar S, Baranov V, Oberg A, Danielsson A, Hammarstrom S, Hammarstrom ML. Characterisation of mucosal lymphoid aggregates in ulcerative colitis: immune cell phenotype and TcR-gammadelta expression. *Gut* 2000; **47**: 215-227

110 **Krajina T**, Leithauser F, Moller P, Trobonjaca Z, Reimann J. Colonic lamina propria dendritic cells in mice with CD4+ T cell-induced colitis. *Eur J Immunol* 2003; **33**: 1073-1083

111 **Becker C**, Wirtz S, Blessing M, Pirhonen J, Strand D, Bechthold O, Frick J, Galle PR, Autenrieth I, Neurath MF. Constitutive p40 promoter activation and IL-23 production in the terminal ileum mediated by dendritic cells. *J Clin Invest* 2003; **112**: 693-706

112 **Hue S**, Ahern P, Buonocore S, Kullberg MC, Cua DJ, McKenzie BS, Powrie F, Maloy KJ. Interleukin-23 drives innate and T cell-mediated intestinal inflammation. *J Exp Med* 2006; **203**: 2473-2483

113 **Kullberg MC**, Jankovic D, Feng CG, Hue S, Gorelick PL, McKenzie BS, Cua DJ, Powrie F, Cheever AW, Maloy KJ, Sher A. IL-23 plays a key role in Helicobacter hepaticus-induced T cell-dependent colitis. *J Exp Med* 2006; **203**: 2485-2494

114 **Mizoguchi A**, Ogawa A, Takedatsu H, Sugimoto K, Shimomura Y, Shirane K, Nagahama K, Nagaishi T, Mizoguchi E, Blumberg RS, Bhan AK. Dependence of intestinal granuloma formation on unique myeloid DC-like cells. *J Clin Invest* 2007; **117**: 605-615

115 **Duerr RH**, Taylor KD, Brant SR, Rioux JD, Silverberg MS, Daly MJ, Steinhart AH, Abraham C, Regueiro M, Griffiths A, Dassopoulos T, Bitton A, Yang H, Targan S, Datta LW, Kistner EO, Schumm LP, Lee AT, Gregersen PK, Barmada MM, Rotter JI, Nicolae DL, Cho JH. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* 2006; **314**: 1461-1463

116 **Glas J**, Seiderer J, Wetzke M, Konrad A, Torok HP, Schmechel S, Tonenchi L, Grassl C, Dambacher J, Pfennig S, Maier K, Griga T, Klein W, Epplen JT, Schiemann U, Folwaczny C, Lohse P, Goke B, Ochsenkuhn T, Muller-Myhsok B, Folwaczny M, Mussack T, Brand S. rs1004819 is the main disease-associated IL23R variant in German Crohn's disease patients: combined analysis of IL23R, CARD15, and OCTN1/2 variants. *PLoS ONE* 2007; **2**: e819

117 **Steinman L**. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. *Nat Med* 2007; **13**: 139-145

118 **Elson CO**, Cong Y, Weaver CT, Schoeb TR, McClanahan TK, Fick RB, Kastelein RA. Monoclonal anti-interleukin 23 reverses active colitis in a T cell-mediated model in mice. *Gastroenterology* 2007; **132**: 2359-2370

119 **Izcue A**, Hue S, Buonocore S, Arancibia-Carcamo CV, Ahern PP, Iwakura Y, Maloy KJ, Powrie F. Interleukin-23 restrains regulatory T cell activity to drive T cell-dependent colitis. *Immunity* 2008; **28**: 559-570

120 **Yang XO**, Chang SH, Park H, Nurieva R, Shah B, Acero L, Wang YH, Schluns KS, Broaddus RR, Zhu Z, Dong C. Regulation of inflammatory responses by IL-17F. *J Exp Med* 2008; **205**: 1063-1075

121 **van Beelen AJ**, Zelinkova Z, Taanman-Kueter EW, Muller FJ, Hommes DW, Zaat SA, Kapsenberg ML, de Jong EC. Stimulation of the intracellular bacterial sensor NOD2 programs dendritic cells to promote interleukin-17 production in human memory T cells. *Immunity* 2007; **27**: 660-669

122 **Sugimoto K**, Ogawa A, Mizoguchi E, Shimomura Y, Andoh A, Bhan AK, Blumberg RS, Xavier RJ, Mizoguchi A. IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *J Clin Invest* 2008; **118**: 534-544

123 **Zheng Y**, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, Abbas AR, Modrusan Z, Ghilardi N, de Sauvage FJ, Ouyang W. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med* 2008; **14**: 282-289

124 **Aujla SJ**, Chan YR, Zheng M, Fei M, Askew DJ, Pociask DA, Reinhart TA, McAllister F, Edeal J, Gaus K, Husain S, Kreindler JL, Dubin PJ, Pilewski JM, Myerburg MM, Mason CA, Iwakura Y, Kolls JK. IL-22 mediates mucosal host

defense against Gram-negative bacterial pneumonia. *Nat Med* 2008; **14**: 275-281

125 **Abe K**, Nguyen KP, Fine SD, Mo JH, Shen C, Shenouda S, Corr M, Jung S, Lee J, Eckmann L, Raz E. Conventional dendritic cells regulate the outcome of colonic inflammation independently of T cells. *Proc Natl Acad Sci USA* 2007; **104**: 17022-17027

126 **Zaph C**, Troy AE, Taylor BC, Berman-Booty LD, Guild KJ, Du Y, Yost EA, Gruber AD, May MJ, Greten FR, Eckmann L, Karin M, Artis D. Epithelial-cell-intrinsic IKK-beta expression regulates intestinal immune homeostasis. *Nature* 2007; **446**: 552-556

127 **Nenci A**, Becker C, Wullaert A, Gareus R, van Loo G, Danese S, Huth M, Nikolaev A, Neufert C, Madison B, Gumucio D, Neurath MF, Pasparakis M. Epithelial NEMO links innate immunity to chronic intestinal inflammation. *Nature* 2007; **446**: 557-561

128 **Rimoldi M**, Chieppa M, Salucci V, Avogadri F, Sonzogni A, Sampietro GM, Nespoli A, Viale G, Allavena P, Rescigno M. Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. *Nat Immunol* 2005; **6**: 507-514

S- Editor Zhong XY **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5149

World J Gastroenterol 2008 September 7; 14(33): 5149-5153
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Emiko Mizoguchi, MD, PhD, *Series Editor*

Toll-like receptors in inflammatory bowel disease-stepping into uncharted territory

Avi Levin, Oren Shibolet

Avi Levin, Oren Shibolet, Liver and Gastroenterology Units, Division of Medicine, Hadassah-Hebrew University Medical Center, Jerusalem 91120, Israel
Correspondence to: Oren Shibolet, MD, Liver and Gastroenterology Units, Division of Medicine, Division of Medicine, POB 12000, Hadassah-Hebrew University Medical Center, Jerusalem 91120, Israel. shibolet@hadassah.org.il
Telephone: +972-2-6778253 Fax: +972-2-6420338
Received: June 11, 2008 Revised: August 19, 2008
Accepted: August 26, 2008
Published online: September 7, 2008

Abstract

Ulcerative colitis and Crohn's disease are chronic relapsing-remitting inflammatory processes of the intestinal tract. The etiology of these diseases is currently unknown. However, inflammation is hypothesized to result from inappropriate activation of mucosal immunity by luminal antigens in genetically susceptible individuals. Toll-like receptors (TLRs) are a family of transmembrane proteins that act as microbial pattern recognition receptors. They are crucial initiators of innate immune responses. The role of TLRs in the pathogenesis of inflammatory bowel disease (IBD) has not been fully elucidated. In this review, we aim to analyze the available data connecting individual TLRs to intestinal inflammation and IBD.

Key words: Toll-like receptors; Inflammatory bowel disease; Intestinal inflammation

Peer reviewers: Didier Merlin, PhD, Associate Professor, Department of Medicine Division of Digestive Diseases, Emory University, 615 Michael Street, Atlanta, GA 30322, United States; Shanthi V Sitaraman, MD, Division of Digestive Diseases, Room 201-F, 615, Michael Street, Whitehead Research Building, Emory University, Atlanta, Georgia 30322, United States

Levin A, Shibolet O. Toll-like receptors in inflammatory bowel disease-stepping into uncharted territory. *World J Gastroenterol* 2008; 14(33): 5149-5153 Available from: URL: http://www.wjgnet.com/1007-9327/14/5149.asp DOI: http://dx.doi.org/10.3748/wjg.14.5149

INTRODUCTION

Inflammatory bowel disease (IBD) is comprised of two major forms of chronic inflammation of the gastrointestinal tract, ulcerative colitis (UC) and Crohn's disease (CD). These two entities differ in their location (colon only *vs* the whole length of the intestinal tract), pattern of distribution (continuous *vs* patchy), depth of involvement (mucosal *vs* transmural) and histology (crypt abscesses *vs* granulomas). The onset of IBD typically occurs in the second and third decades of life, and a majority of affected individuals progress to relapsing and chronic disease[1].

The etiology of IBD is currently unknown. Inflammation is hypothesized to result from inappropriate activation of mucosal immunity by environmental factors in genetically susceptible individuals[2]. There is strong evidence to support the role of intestinal microflora in the pathogenesis of IBD. Mice raised under germ-free conditions do not develop spontaneous colitis in several experimental models[3]. Additionally, antibiotic treatment and probiotic bacteria were shown to induce remission in IBD patients[4-6]. Inappropriate activation of innate immunity is the other arm involved in the pathogenesis of IBD[7,8]. Activation of innate immunity relies at least partially on recognition of conserved microbial motifs known as pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs)[9]. There are two major families of PRRs known as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) receptors.

TLRs

There are currently 11 known mammalian TLRs. They are transmembrane receptors that are found either on the cell membrane (TLR1, 2, 4, 5 and 9) or on intracellular organelles (TLR3, 7 and 8)[10,11]. TLRs are expressed throughout the gastrointestinal (GI) tract on intestinal epithelial cells (IECs), myofibroblasts, enteroendocrine cells, and on immune cells within the lamina propria, such as T cells, and dendritic cells (DCs)[12-16]. Extracellular domains of TLRs consist of leucine-rich repeats (LRRs), whereas their intracellular component contains a TIR (Toll/IL-1 receptor) domain, exhibiting homology with the interleukin-1 receptor (IL-1R) superfamily. Most TLR signal *via* the MyD88

adaptor (TLR1, 2, 4, 5, 6, 7, 8 and 9), whereas TLR3 signaling activates an alternative "MyD88-independent" pathway. TLR4 is the only receptor known to activate both MyD88-dependent and -independent pathways[17]. Ligand binding to TLRs initiates signaling cascades that activate NF-κB, MAPK, and interferon response factors. In this review, we will address the role of the TLR family in the pathogenesis of IBD.

TLR4

The Toll-like receptor 4 (TLR4) gene is located on the long arm of human chromosome 9. Its ligand is lipopolysaccharide (LPS), and signal initiation requires the presence of CD14. In mouse models, TLR4 is involved in regeneration of IECs. TLR4 KO and MyD88 KO mice show impaired mucosal healing, and disturbed barrier function in response to administration of the colitis inducing dextran sulfate sodium (DSS) which leads to an increase in intestinal bleeding, colonic damage, bacterial translocation and to increased mortality. A similar aggravating effect of DSS was observed when natural ligands of TLR4 were eliminated either by broad spectrum antibiotic treatment or by raising the mice in a germ-free environment[18]. TLR4 signaling was shown to play a role in the initiation of intestinal inflammation. Treatment with CRX-526, a TLR4 antagonist, inhibited the development of moderate-to-severe colitis in MDR 1a-deficient mice, and TLR signal abrogation by MyD88 KO prevented development of spontaneous colitis in IL-10 KO mice[19]. Human IECs normally express TLR3 and TLR5, whereas TLR2 and TLR4 are only minimally expressed[20]. However, TLR4 expression is upregulated in both CD and UC. In pediatric IBD patients, higher levels of TLR4 mRNA and protein were found in the inflamed colonic mucosa, but not in non-inflamed controls[21].

Epidemiological studies show an association between TLR4 polymorphism and susceptibility to IBD. In a German cohort, the CD14 promoter 1-260C>T single-nucleotide polymorphism (SNP) was associated with UC, but not CD, while the opposite was found in a Hungarian cohort. No association with IBD of the TLR4 896A>G SNP was found in either cohort[14]. In a Belgian study, the allele frequency of the TLR4 A299G polymorphism, affecting the extracellular domain of TLR4 that is associated with an abrogated response, was significantly higher in CD (11% *vs* 5%, $P = 0.004$ in one cohort and 12% *vs* 5%, $P = 0.007$ in another cohort) and in UC patients (10% *vs* 5%, $P = 0.027$) compared with controls[22]. The same SNP was exclusively related to CD in another study[23] and a third study found no association of this SNP with IBD[24]. Another TLR4 polymorphism, T399IL, was exclusively associated with UC, and not with CD[25].

TLR5

TLR5 is highly expressed in colonic epithelial cells (CECs). The bacterial ligand of TLR5 is flagellin, which is present on most motile bacteria. Expression of TLR5 appears to be basolateral in healthy individuals, and CECs in the intact colon do not respond to flagellin. It is suggested that the response of CECs to flagellin is specifically elicited under inflammatory conditions with epithelial barrier disruption[26,27]. Consistent with these findings, rectal administration of flagellin to control mice did not elicit an inflammatory response, but was able to aggravate DSS-induced colitis[26]. Interestingly, flagellin derived from *Salmonella* species, but not commensal bacteria was able to stimulate proinflammatory chemokines secretion by IECs[28]. In CD patients, tolerance to commensal-derived flagellin is lost and serum reactivity to flagellin can be demonstrated[29]. Additionally, CD patients carrying a susceptibility NOD2 mutation exhibit an enhanced flagellin reactivity which was independently associated with distinct CD phenotypes[30]. TLR5 KO mice develop spontaneous colitis, and a marked elevation in pro-inflammatory cytokine levels. This colitis is mediated *via* TLR4 signaling as TLR5 and TLR4 double KO fail to develop spontaneous colitis[31].

Data connecting TLR5 polymorphism to IBD is limited. Recently a TLR5-stop polymorphism in which a point mutation at nucleotide 1174 generates a stop codon rendering TLR5 inactive was described[32]. It is a relatively common polymorphism with a 5% allele frequency. Healthy subjects carrying the TLR5-stop SNP have significantly lower levels of flagellin-specific IgG and IgA, but similar levels of total and LPS-specific immunoglobulins. Moreover, it was observed that the carriage rate of the TLR5-stop SNP was significantly lower in CD patients as compared with unaffected relatives and unrelated controls[33].

TLR9

Unmethylated cytosine-guanosine dinucleotides (CpG), which are frequently recognized in the DNA of bacteria and viruses, are the ligands for TLR9[34,35]. The role of TLR9 signaling in pathogenesis of IBD is under intense investigation.

It was reported that intragastric and subcutaneous administration of CpG oligodeoxynucleotides (CpG-ODNs) reduced the severity of DSS and TNBS-induced acute colitis and of chronic colitis in IL-10 KO mice[36]. It was further shown that the beneficial therapeutic effect of probiotic bacteria in murine colitis models was mediated *via* the effect of CpG on intact TLR9 signaling[37]. Later, it was suggested that type I interferons may act as immunomodulatory effectors of the TLR9 pathway[38]. Further support for the immunosuppressive role of CpG-ODNs came from studies in which CD4+CD62L+ T lymphocytes from CpG-ODN pretreated donor mice were unable to induce colitis in recipient SCID mice in a transfer model, and were able to suppress CD4+ T cell-mediated colitis when co-transferred. Furthermore, CD4+CD62L+ cells from TLR9 deficient mice induced a significantly more severe colitis in SCID recipients than cells from controls[39]. These data would suggest that TLR9 signaling suppresses intestinal inflammation.

In contrast, a pro-inflammatory effect of TLR9 signaling in colitis models has also been demonstrated.

Intraperitoneal administration of CpG-ODN increased the severity of DSS-induced acute and chronic colitis[40]. Induction of DSS colitis in TLR9-deficient mice resulted in markedly reduced intestinal inflammation and proinflammatory cytokine production. Additionally, treatment with adenoviral ODN, known to block CpG effects, resulted in a significant amelioration of colitis[41].

Recently, a novel mode of TLR9 pathway regulation was described which could, at least in part, explain some of the above mentioned discrepancies. Basolateral TLR9 signaling was shown to activate the NF-κB pathway in CECs, whereas apical TLR9 signaling inhibited its activation by inducing accumulation of ubiquitinated I κB in the cytoplasm. Moreover, apical stimulation of TLR9 prevented activation of NF-κB signaling by other TLRs[8]. These data suggest that different routes of CpG-ODN delivery (intraluminal *versus* systemic) may result in anti- or pro-inflammatory effects, respectively. Additionally, this report provides a possible explanation of the divergent effects of CpGs on colitis depending on the time-point of application. Administration of CpG-ODNs when the epithelial barrier is intact results in a protective effect, while the same administration, when the epithelium, is disrupted leads to aggravation of inflammation[42].

The effect of CpG-ODN stimulation in IBD was assessed using *ex vivo* colonic mucosal biopsies from active UC patients and healthy controls. CpG-ODNs significantly inhibited colonic TNF-α and IL-1β generation in a TLR9-dependent manner in UC, and not in controls[43].

In epidemiological studies, the frequency of the -T1237C SNP of the TLR9 promoter region was significantly increased in patients with Crohn's disease as compared with controls[44].

TLR 1, 2, 6

TLR2 is required for recognition of Gram positive and mycobacterial PAMPs including bacterial lipopeptide, lipotechoic acid (LTA) and peptidoglycan (PGN). Following ligand association, TLR2 and TLR6 ± TLR1 form hetero-dimers that may control signal specificity and enhance signal transduction[45, 46].

TLR2 KO mice display increased susceptibility to DSS-induced colitis. In mice, TLR2 stimulation effectively augments tight junction barrier assembly against stress-induced damage through the activation of PI3K/Akt pathway. Oral treatment of mice with the TLR2 ligand Pam3CSK prior to DSS colitis induction significantly suppressed mucosal inflammation and apoptosis and restored epithelial integrity[47]. In contrast to its anti-inflammatory effect, another report suggested that Pam3CSK administration may actually augment colitis. Suppression of colitis by regulatory T lymphocytes in the CD45RB^{high} T cell transfer model was significantly delayed when the cells were pretreated with Pam3CSK[48].

Baseline expression level of TLR2 on enterocytes was reported to be low, but its levels increased with inflammation. Immunohistochemical analysis showed either a significant increase or no change in TLR2 expression in the terminal ileum of patients with inactive and active IBD compared to controls[20,49]. Another study showed that monocytes isolated from patients with active IBD had higher expression levels of TLR2 on their cell surfaces, and a significantly increased TNF-α production in response to TLR2 agonist stimulation as compared to inactive patients and healthy controls[50]. It was recently suggested that NOD2, which is mutated in 15% of CD patients, and was the first CD susceptibility gene to be discovered, is involved in TLR2 signal regulation. Specifically, it was shown that muramyl-dipeptide (MDP), which is the ligand for NOD2 activation, negatively regulates TLR2 signaling. NOD2 deficient mice adoptively transferred with OVA-specific CD4+ T cells, and stimulated with OVA expressing *E. coli* (ECOVA) developed colitis, whereas wild type controls did not. Importantly, this colitis was TLR2 dependent, since inflammation was suppressed in NOD2-TLR2 double deficient mice[51]. It was later reported that administration of MDP protects mice from the development of experimental colitis by downregulating multiple TLR responses, not just TLR2[52].

Epidemiological data assessing the role of TLR2 in the pathogenesis of IBD are scarce. None of the nonsynonymous SNPs of TLR1, 2 or 6 were involved with IBD susceptibility. However, a number of variants were found to be associated with disease phenotypes. The TLR2 R753G and TLR1 R80T SNPs were found to be associated with pancolitis in UC. The relative risks for heterozygous patients to develop pancolitis were 5.8 and 3.3 for R80T and R753G, respectively[53]. There was a negative association between TLR6 S249P SNP and proctitis in UC patients. In CD there was a negative association between ileal disease involvement and TLR1 S602I SNP.

TLR3

This less studied TLR in the pathogenesis of IBD, signals upon activation by double stranded RNA through a Myd88-independent pathway *via* the adaptor TRIF. Double stranded RNA is produced during viral replication as an intermediate of the replication cycle or as part of the viral RNA genome, and is also produced during apoptosis.

In wild type mice, subcutaneous administration of poly (I:C), a synthetic TLR3 agonist, protected against DSS-induced colitis. In contrast, intragastric administration of poly (I:C) offered no protection in this colitis model nor did its administration activate the innate immune system as assessed by serologic parameters[54].

Activation of TLR3 signaling induced by poly(I:C) was shown to cause an increase in IL-15 secretion leading to mucosal damage in the small intestine. IL-15 is a key regulatory cytokine involved in mucosal homeostasis. IL-15 secretion increased the percentage and number of CD3+NK1.1+ intestinal intraepithelial lymphocytes (IELs) and caused their enhanced cytotoxicity[55]. TLR3 signaling was also shown to induce IEC expression of

Rae1 (a ligand for NKG2D), which mediates epithelial destruction and mucosal injury by interacting with NKG2D expressed on intestinal intraepithelial lymphocytes [56].

In humans, TLR3 expression by IECs of UC patients is comparable to that of healthy controls, while TLR3 expression was significantly downregulated in CD patients, both in inflamed and non-inflamed tissue[20].

CONCLUSION

Inflammatory bowel disease is a chronic relapsing disease of the gastrointestinal tract. Although the etiology is unknown, both innate immunity and the commensal bacterial flora are hypothesized to play a major role in its pathogenesis. The gastrointestinal innate immune system has to recognize, sort and respond to a vast array of microbial products present in the intestinal lumen. TLRs have evolved as the major innate immune surveillance, recognition and response receptors central to efficient host defense and homeostasis of the intestinal mucosa.

The factors directing TLR-regulated immune response in IBD remain poorly understood. TLRs may either enhance or suppress intestinal inflammation. Membrane localization (either basolateral or apical), expression pattern (IECs *versus* regulatory T lymphocytes), parallel signaling by additional TLRs, cytokine combinations and interactions with specific intestinal flora all determine the type and balance of the immune response.

In mouse models, knockout of a single TLR usually does not result in spontaneous colitis. Only after additional genetic and pharmacological interventions does a clear gastrointestinal phenotype emerge. These data suggest that mutations in a single TLR are insufficient to explain the complex pathogenesis of IBD. However, they do suggest that TLRs are crucial for initiation and progression of IBD and play a major role in its pathogenesis.

REFERENCES

1 **Podolsky DK**. Inflammatory bowel disease. *N Engl J Med* 2002; **347**: 417-429
2 **Xavier RJ**, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 2007; **448**: 427-434
3 **Elson CO**, Cong Y, Brandwein S, Weaver CT, McCabe RP, Mahler M, Sundberg JP, Leiter EH. Experimental models to study molecular mechanisms underlying intestinal inflammation. *Ann N Y Acad Sci* 1998; **859**: 85-95
4 **Ewaschuk JB**, Tejpar QZ, Soo I, Madsen K, Fedorak RN. The role of antibiotic and probiotic therapies in current and future management of inflammatory bowel disease. *Curr Gastroenterol Rep* 2006; **8**: 486-498
5 **Gionchetti P**, Rizzello F, Helwig U, Venturi A, Lammers KM, Brigidi P, Vitali B, Poggioli G, Miglioli M, Campieri M. Prophylaxis of pouchitis onset with probiotic therapy: a double-blind, placebo-controlled trial. *Gastroenterology* 2003; **124**: 1202-1209
6 **Sutherland L**, Singleton J, Sessions J, Hanauer S, Krawitt E, Rankin G, Summers R, Mekhjian H, Greenberger N, Kelly M. Double blind, placebo controlled trial of metronidazole in Crohn's disease. *Gut* 1991; **32**: 1071-1075
7 **Rakoff-Nahoum S**, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 2004; **118**: 229-241
8 **Lee J**, Mo JH, Katakura K, Alkalay I, Rucker AN, Liu YT, Lee HK, Shen C, Cojocaru G, Shenouda S, Kagnoff M, Eckmann L, Ben-Neriah Y, Raz E. Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. *Nat Cell Biol* 2006; **8**: 1327-1336
9 **Akira S**, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006; **124**: 783-801
10 **Takeda K**, Akira S. Toll-like receptors in innate immunity. *Int Immunol* 2005; **17**: 1-14
11 **Zhang D**, Zhang G, Hayden MS, Greenblatt MB, Bussey C, Flavell RA, Ghosh S. A toll-like receptor that prevents infection by uropathogenic bacteria. *Science* 2004; **303**: 1522-1526
12 **Cario E**, Rosenberg IM, Brandwein SL, Beck PL, Reinecker HC, Podolsky DK. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. *J Immunol* 2000; **164**: 966-972
13 **Otte JM**, Rosenberg IM, Podolsky DK. Intestinal myofibroblasts in innate immune responses of the intestine. *Gastroenterology* 2003; **124**: 1866-1878
14 **Baumgart DC**, Buning C, Geerdts L, Schmidt HH, Genschel J, Fiedler T, Gentz E, Molnar T, Nagy F, Lonovics J, Lochs H, Wiedenmann B, Nickel R, Witt H, Dignass A. The c.1-260C>T promoter variant of CD14 but not the c.896A>G (p.D299G) variant of toll-like receptor 4 (TLR4) genes is associated with inflammatory bowel disease. *Digestion* 2007; **76**: 196-202
15 **Ince MN**, Elliott DE, Setiawan T, Blum A, Metwali A, Wang Y, Urban JF Jr, Weinstock JV. Heligmosomoides polygyrus induces TLR4 on murine mucosal T cells that produce TGFbeta after lipopolysaccharide stimulation. *J Immunol* 2006; **176**: 726-729
16 **Hart AL**, Al-Hassi HO, Rigby RJ, Bell SJ, Emmanuel AV, Knight SC, Kamm MA, Stagg AJ. Characteristics of intestinal dendritic cells in inflammatory bowel diseases. *Gastroenterology* 2005; **129**: 50-65
17 **Takeda K**, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol* 2003; **21**: 335-376
18 **Rakoff-Nahoum S**, Hao L, Medzhitov R. Role of toll-like receptors in spontaneous commensal-dependent colitis. *Immunity* 2006; **25**: 319-329
19 **Fort MM**, Mozaffarian A, Stover AG, Correia Jda S, Johnson DA, Crane RT, Ulevitch RJ, Persing DH, Bielefeldt-Ohmann H, Probst P, Jeffery E, Fling SP, Hershberg RM. A synthetic TLR4 antagonist has anti-inflammatory effects in two murine models of inflammatory bowel disease. *J Immunol* 2005; **174**: 6416-6423
20 **Cario E**, Podolsky DK. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect Immun* 2000; **68**: 7010-7017
21 **Szebeni B**, Veres G, Dezsofi A, Rusai K, Vannay A, Mraz M, Majorova E, Arato A. Increased expression of Toll-like receptor (TLR) 2 and TLR4 in the colonic mucosa of children with inflammatory bowel disease. *Clin Exp Immunol* 2008; **151**: 34-41
22 **Franchimont D**, Vermeire S, El Housni H, Pierik M, Van Steen K, Gustot T, Quertinmont E, Abramowicz M, Van Gossum A, Deviere J, Rutgeerts P. Deficient host-bacteria interactions in inflammatory bowel disease? The toll-like receptor (TLR)-4 Asp299gly polymorphism is associated with Crohn's disease and ulcerative colitis. *Gut* 2004; **53**: 987-992
23 **Browning BL**, Huebner C, Petermann I, Gearry RB, Barclay ML, Shelling AN, Ferguson LR. Has toll-like receptor 4 been prematurely dismissed as an inflammatory bowel disease gene? Association study combined with meta-analysis shows strong evidence for association. *Am J Gastroenterol* 2007; **102**: 2504-2512

24 **Okayama N**, Fujimura K, Suehiro Y, Hamanaka Y, Fujiwara M, Matsubara T, Maekawa T, Hazama S, Oka M, Nohara H, Kayano K, Okita K, Hinoda Y. Simple genotype analysis of the Asp299Gly polymorphism of the Toll-like receptor-4 gene that is associated with lipopolysaccharide hyporesponsiveness. *J Clin Lab Anal* 2002; **16**: 56-58

25 **Torok HP**, Glas J, Tonenchi L, Mussack T, Folwaczny C. Polymorphisms of the lipopolysaccharide-signaling complex in inflammatory bowel disease: association of a mutation in the Toll-like receptor 4 gene with ulcerative colitis. *Clin Immunol* 2004; **112**: 85-91

26 **Rhee SH**, Im E, Riegler M, Kokkotou E, O'brien M, Pothoulakis C. Pathophysiological role of Toll-like receptor 5 engagement by bacterial flagellin in colonic inflammation. *Proc Natl Acad Sci USA* 2005; **102**: 13610-13615

27 **Gewirtz AT**, Navas TA, Lyons S, Godowski PJ, Madara JL. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol* 2001; **167**: 1882-1885

28 **Sierro F**, Dubois B, Coste A, Kaiserlian D, Kraehenbuhl JP, Sirard JC. Flagellin stimulation of intestinal epithelial cells triggers CCL20-mediated migration of dendritic cells. *Proc Natl Acad Sci USA* 2001; **98**: 13722-13727

29 **Lodes MJ**, Cong Y, Elson CO, Mohamath R, Landers CJ, Targan SR, Fort M, Hershberg RM. Bacterial flagellin is a dominant antigen in Crohn disease. *J Clin Invest* 2004; **113**: 1296-1306

30 **Papadakis KA**, Yang H, Ippoliti A, Mei L, Elson CO, Hershberg RM, Vasiliauskas EA, Fleshner PR, Abreu MT, Taylor K, Landers CJ, Rotter JI, Targan SR. Anti-flagellin (CBir1) phenotypic and genetic Crohn's disease associations. *Inflamm Bowel Dis* 2007; **13**: 524-530

31 **Vijay-Kumar M**, Sanders CJ, Taylor RT, Kumar A, Aitken JD, Sitaraman SV, Neish AS, Uematsu S, Akira S, Williams IR, Gewirtz AT. Deletion of TLR5 results in spontaneous colitis in mice. *J Clin Invest* 2007; **117**: 3909-3921

32 **Hawn TR**, Verbon A, Lettinga KD, Zhao LP, Li SS, Laws RJ, Skerrett SJ, Beutler B, Schroeder L, Nachman A, Ozinsky A, Smith KD, Aderem A. A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to legionnaires' disease. *J Exp Med* 2003; **198**: 1563-1572

33 **Gewirtz AT**, Vijay-Kumar M, Brant SR, Duerr RH, Nicolae DL, Cho JH. Dominant-negative TLR5 polymorphism reduces adaptive immune response to flagellin and negatively associates with Crohn's disease. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G1157-G1163

34 **Hemmi H**, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S. A Toll-like receptor recognizes bacterial DNA. *Nature* 2000; **408**: 740-745

35 **Krieg AM**, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, Koretzky GA, Klinman DM. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 1995; **374**: 546-549

36 **Rachmilewitz D**, Karmeli F, Takabayashi K, Hayashi T, Leider-Trejo L, Lee J, Leoni LM, Raz E. Immunostimulatory DNA ameliorates experimental and spontaneous murine colitis. *Gastroenterology* 2002; **122**: 1428-1441

37 **Rachmilewitz D**, Katakura K, Karmeli F, Hayashi T, Reinus C, Rudensky B, Akira S, Takeda K, Lee J, Takabayashi K, Raz E. Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. *Gastroenterology* 2004; **126**: 520-528

38 **Katakura K**, Lee J, Rachmilewitz D, Li G, Eckmann L, Raz E. Toll-like receptor 9-induced type I IFN protects mice from experimental colitis. *J Clin Invest* 2005; **115**: 695-702

39 **Obermeier F**, Strauch UG, Dunger N, Grunwald N, Rath HC, Herfarth H, Scholmerich J, Falk W. In vivo CpG DNA/toll-like receptor 9 interaction induces regulatory properties in CD4+CD62L+ T cells which prevent intestinal inflammation in the SCID transfer model of colitis. *Gut* 2005; **54**: 1428-1436

40 **Obermeier F**, Dunger N, Deml L, Herfarth H, Scholmerich J, Falk W. CpG motifs of bacterial DNA exacerbate colitis of dextran sulfate sodium-treated mice. *Eur J Immunol* 2002; **32**: 2084-2092

41 **Obermeier F**, Dunger N, Strauch UG, Hofmann C, Bleich A, Grunwald N, Hedrich HJ, Aschenbrenner E, Schlegelberger B, Rogler G, Scholmerich J, Falk W. CpG motifs of bacterial DNA essentially contribute to the perpetuation of chronic intestinal inflammation. *Gastroenterology* 2005; **129**: 913-927

42 **Obermeier F**, Dunger N, Strauch UG, Grunwald N, Herfarth H, Scholmerich J, Falk W. Contrasting activity of cytosin-guanosin dinucleotide oligonucleotides in mice with experimental colitis. *Clin Exp Immunol* 2003; **134**: 217-224

43 **Rachmilewitz D**, Karmeli F, Shteingart S, Lee J, Takabayashi K, Raz E. Immunostimulatory oligonucleotides inhibit colonic proinflammatory cytokine production in ulcerative colitis. *Inflamm Bowel Dis* 2006; **12**: 339-345

44 **Torok HP**, Glas J, Tonenchi L, Bruennler G, Folwaczny M, Folwaczny C. Crohn's disease is associated with a toll-like receptor-9 polymorphism. *Gastroenterology* 2004; **127**: 365-366

45 **Hajjar AM**, O'Mahony DS, Ozinsky A, Underhill DM, Aderem A, Klebanoff SJ, Wilson CB. Cutting edge: functional interactions between toll-like receptor (TLR) 2 and TLR1 or TLR6 in response to phenol-soluble modulin. *J Immunol* 2001; **166**: 15-19

46 **Ozinsky A**, Smith KD, Hume D, Underhill DM. Co-operative induction of pro-inflammatory signaling by Toll-like receptors. *J Endotoxin Res* 2000; **6**: 393-396

47 **Cario E**, Gerken G, Podolsky DK. Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function. *Gastroenterology* 2007; **132**: 1359-1374

48 **Liu H**, Komai-Koma M, Xu D, Liew FY. Toll-like receptor 2 signaling modulates the functions of CD4+ CD25+ regulatory T cells. *Proc Natl Acad Sci USA* 2006; **103**: 7048-7053

49 **Frolova L**, Drastich P, Rossmann P, Klimesova K, Tlaskalova-Hogenova H. Expression of Toll-like receptor 2 (TLR2), TLR4, and CD14 in biopsy samples of patients with inflammatory bowel diseases: upregulated expression of TLR2 in terminal ileum of patients with ulcerative colitis. *J Histochem Cytochem* 2008; **56**: 267-274

50 **Canto E**, Ricart E, Monfort D, Gonzalez-Juan D, Balanzo J, Rodriguez-Sanchez JL, Vidal S. TNF alpha production to TLR2 ligands in active IBD patients. *Clin Immunol* 2006; **119**: 156-165

51 **Watanabe T**, Kitani A, Murray PJ, Wakatsuki Y, Fuss IJ, Strober W. Nucleotide binding oligomerization domain 2 deficiency leads to dysregulated TLR2 signaling and induction of antigen-specific colitis. *Immunity* 2006; **25**: 473-485

52 **Watanabe T**, Asano N, Murray PJ, Ozato K, Tailor P, Fuss IJ, Kitani A, Strober W. Muramyl dipeptide activation of nucleotide-binding oligomerization domain 2 protects mice from experimental colitis. *J Clin Invest* 2008; **118**: 545-559

53 **Pierik M**, Joossens S, Van Steen K, Van Schuerbeek N, Vlietinck R, Rutgeerts P, Vermeire S. Toll-like receptor-1, -2, and -6 polymorphisms influence disease extension in inflammatory bowel diseases. *Inflamm Bowel Dis* 2006; **12**: 1-8

54 **Vijay-Kumar M**, Wu H, Aitken J, Kolachala VL, Neish AS, Sitaraman SV, Gewirtz AT. Activation of toll-like receptor 3 protects against DSS-induced acute colitis. *Inflamm Bowel Dis* 2007; **13**: 856-864

55 **Zhou R**, Wei H, Sun R, Tian Z. Recognition of double-stranded RNA by TLR3 induces severe small intestinal injury in mice. *J Immunol* 2007; **178**: 4548-4556

56 **Zhou R**, Wei H, Sun R, Zhang J, Tian Z. NKG2D recognition mediates Toll-like receptor 3 signaling-induced breakdown of epithelial homeostasis in the small intestines of mice. *Proc Natl Acad Sci USA* 2007; **104**: 7512-7515

S- Editor Cheng JX **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5154

World J Gastroenterol 2008 September 7; 14(33): 5154-5161
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Emiko Mizoguchi, MD, PhD, *Series Editor*

Mucosal cytokine network in inflammatory bowel disease

Akira Andoh, Yuhki Yagi, Makoto Shioya, Atsushi Nishida, Tomoyuki Tsujikawa, Yoshihide Fujiyama

Akira Andoh, Yuhki Yagi, Makoto Shioya, Atsushi Nishida, Tomoyuki Tsujikawa, Yoshihide Fujiyama, Department of Medicine, Shiga University of Medical Science, Seta-Tukinowa, Otsu 520-2192, Japan
Author contributions: Andoh A and Fujiyama Y designed the research; Yagi Y, Shioya M, Nishida A and Tsujikawa T performed the research; and Andoh A wrote the paper.
Correspondence to: Akira Andoh, MD, PhD, Department of Internal Medicine, Shiga University of Medical Science, Seta-Tukinowa, Otsu 520-2192, Japan. andoh@belle.shiga-med.ac.jp
Telephone: +81-77-5482217 Fax: +81-77-5482219
Received: April 10, 2008 Revised: May 23, 2008
Accepted: May 30, 2008
Published online: September 7, 2008

Abstract

Inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's disease (CD) are characterized by ongoing mucosal inflammation in which dysfunction of the host immunologic response against dietary factors and commensal bacteria is involved. The chronic inflammatory process leads to disruption of the epithelial barrier, and the formation of epithelial ulceration. This permits easy access for the luminal microbiota and dietary antigens to cells resident in the lamina propria, and stimulates further pathological immune cell responses. Cytokines are essential mediators of the interactions between activated immune cells and non-immune cells, including epithelial and mesenchymal cells. The clinical efficacy of targeting TNF-α clearly indicates that cytokines are the therapeutic targets in IBD patients. In this manuscript, we focus on the biological activities of recently-reported cytokines [Interleukin (IL)-17 cytokine family, IL-31 and IL-32], which might play a role through interaction with TNF-α in the pathophysiology of IBD.

Key words: Cytokine; Inflammatory bowel disease; Interleukin-17; Interleukin-31; Interleukin-32

Peer reviewers: Dr. Francesco Costa, Dipartimento di Medicina Interna-U.O. di Gastroenterologia Università di Pisa, Via Roma, Pisa 67-56122, Italy; Kiron M Das, MD, PhD, FACP, FRCP, Chief of Gastroenterology and Hepatology, Professor of Medicine, Director of Crohn's and Colitis Center of NJ, 1 Robert Wood Johnson Place, MEB Rm. 478, New Brunswick, NJ 08903, United States

Andoh A, Yagi Y, Shioya M, Nishida A, Tsujikawa T, Fujiyama Y. Mucosal cytokine network in inflammatory bowel disease. *World J Gastroenterol* 2008; 14(33): 5154-5161 Available from: URL: http://www.wjgnet.com/1007-9327/14/5154.asp
DOI: http://dx.doi.org/10.3748/wjg.14.5154

INTRODUCTION

Inflammatory bowel diseases (IBD), ulcerative colitis (UC) and Crohn's disease (CD) are chronic intestinal disorders of unknown etiology[1,2]. The most widely accepted hypothesis on the pathogenesis of IBD is that the mucosal immune system shows an aberrant response towards luminal antigens such as dietary factors and/or commensal bacteria in genetically susceptible individuals[1,3,4]. Environmental factors may also precipitate the onset or reactivation of this disease[1,3,4]. The chronic inflammatory process leads to disruption of the epithelial barrier, and the formation of epithelial ulceration. Abnormal bacterial killing based on genetic factors such as the *NOD2* gene mutation also induces mucosal damage. The easy access for the luminal microbiota and dietary antigens into cells resident in the lamina propria thus stimulates pathological immune cell responses.

Cytokines are essential mediators of the interactions between activated immune cells and non-immune cells, including epithelial and mesenchymal cells[5]. The clinical efficacy of targeting TNF-α clearly indicates that cytokines are one of the therapeutic targets of chronic inflammatory disorders such as IBD. Infliximab, a mouse/human chimeric mAb that binds to TNF-α, has shown efficacy in the treatment of moderate-to severe CD and a number of CD-related complications[6-8]. Although the precise mechanism of the action of infliximab is not clear, it neutralizes soluble TNF-α, alters cell signaling, and induces the apoptosis of activated inflammatory cells expressing surface TNF-α *via* multiple pathways. On the other hand, some patients are resistant to anti-TNF-α therapy. In such patients, the disease might be driven by molecular mechanisms dependent on other cytokines distinct from TNF-α. In this manuscript, we focused on recently-reported cytokines [Interleukin (IL)-17 cytokine family, IL-31 and IL-32] which might play a role through interactions with TNF-α in the pathophysiology of IBD.

IL-17 CYTOKINE FAMILY

IL-17 family and Th17 cells

The interleukin (IL)-17 cytokine family is a group of T cell derived cytokines. IL-17A was originally cloned by Rouvier *et al*[9] and named CTLA8. It was subsequently renamed IL-17, and was more recently termed IL-17A. IL-17A stimulates various cell types to secrete various cytokines and chemokines, resulting in the induction of inflammation[10-15]. The IL-17 family may play a role in a number of diseases mediated by abnormal immune responses, such as rheumatoid arthritis[16,17], pulmonary disease[15,18], lupus[19], multiple sclerosis[20], and IBD[21]. Homology-based cloning recently revealed five additional members of the IL-17 family, termed IL-17B to IL-17F[10]. Among the IL-17 family members, IL-17F has the highest degree of homology with IL-17A (40% to 55%), followed by IL-17B (29%), IL-17D (25%), IL-17C (23%), and IL-17E (also named IL-25) is the most distantly related (17%)[10]. The major cellular source of IL-17A was initially described as activated CD4+ memory T lymphocytes. But, it was subsequently demonstrated that CD8+ memory T lymphocytes, eosinophils, neutrophils and monocytes can also produce IL-17A[10,22]. The cellular sources of IL-17B and IL-17C have not been identified. IL-17D is derived from resting CD4+ T cells and CD19+ B cells[15]. But, IL-17E (IL-25) expression is restricted to Th2 cells and mast cells[15]. The cellular source of the last member, IL-17F, has been reported to be activated CD4+ T cells, basophils and mast cells[15].

One recent topic in immunology is the newly identified Th17 lineage of CD4+ T cells[23]. Th17 cells are characterized by the production of a distinct profile of effector cytokines, including IL-17A, IL-17F, IL-6, IL-22 and IL-26, and have probably evolved to enhance immune and host defense responses distinct from those targeted by Th1 and Th2 cells[24-26]. Th17 cells develop from naïve CD4 T cell precursors in the presence of IL-6 and TGF-β, and full differentiation to Th17 cells is dependent on IL-23[23]. Recent studies demonstrated a role for IL-21 in Th17 development[24,27]. IL-21 serves as an autocrine factor secreted by Th17 cells that promotes or sustains the Th17 lineage commitment. On the other hand, Th1 cells develop from naïve CD4 T cell precursors in the presence of IFN-γ, whereas Th2 cells develop under the control of IL-4. Both IFN-γ and IL-4 inhibit Th17 cell proliferation[28]. A recent study showed that the proliferation of Th17 cells is also inhibited by IL-27, an IL-12-related cytokine frequently present at sites of inflammation[29]. Th17 cells are characterized as a source of IL-17A and IL-17F, and much attention has been focused on their functions in normal and pathological immune responses.

We previously demonstrated that IL-17A-positive cells were increased in the inflamed mucosa of IBD patients[21], and a recent study showed that IL-17F mRNA expression in the mucosa was elevated in CD patients[30]. These observations suggest that IL-17F as well as IL-17A might play a role in the inflammatory responses involved in the pathophysiology of IBD.

Table 1 IL-17A-induced genes in human colonic myofibroblasts

Gene name	Ref seq ID.	Fold increase
CXCL1 (Gro-α)	NM_001511.1	× 26.11
IL-20 receptor, alpha	NM_014432.1	× 15.22
CCR5	NM_000579.1	× 12.75
CXCL6 (GCP-2)	NM_002993.1	× 12.45
CXCL3 (Gro-γ)	NM_002090.1	× 11.70
MMP-16	NM_005941.2	× 10.41
CXCL2 (Gro-β)	NM_002089.1	× 9.75
IRAK3	NM_007199.1	× 9.06
IL-8	NM_000584.2	× 8.91
IL-22 receptor 1	NM_021258.1	× 8.22
MMP-1	NM_004142.1	× 6.35
Complement component 3	NM_000064.1	× 5.56
IL-6	NM_000600.1	× 4.63
Carbonic anhydrase XII	NM_001218.1	× 4.42
Superoxide dismutase 2	NM_000636.1	× 4.25
CCL19 (MIP-3β)	NM_006274.2	× 3.74
CCL7 (MCP-3)	NM_006273.2	× 2.77
CCL2 (MCP-1)	NM_002982.2	× 2.24

Human colonic subepithelial myofibroblasts were stimulated with IL-17A (200 ng/mL) for 12 h, and the changes in gene expression were assessed by IntelliGene HS Human Expression Chip (Takara-Bio, Kyoto, Japan). The fold change values were determined as a ratio of Cy5 signal intensity (IL-17A stimulated values)/Cy3 signal intensity (non-stimulated values). The data were average of three independent analysis. The NCBI reference sequence code was presented following the gene name.

Inflammatory responses induced by IL-17A and IL-17F

IL-17A promotes the expansion and recruitment of innate immune cells such as neutrophils, and also cooperates with TLR ligands, IL-1β, and TNF-α to enhance inflammatory reactions, and to stimulate the production of beta-defensins and other antimicrobial peptides[25,27]. Its receptor, IL-17RA, is ubiquitously expressed and shares many features with classical innate immune receptors such as shared intracellular tail motifs and convergence onto common inflammatory transcription pathways[31]. To investigate the genes altered in response to an IL-17A stimulus, we performed a cDNA microarray analysis in human colonic subepithelial myofibroblasts (SEMFs)[32]. Human colonic SEMFs are located immediately subjacent to the basement membrane in the normal intestinal mucosa, juxtaposed against the bottom of the epithelial cells[33,34], and play a role in inflammation and wound healing in the intestine[33-36]. As shown in Table 1, IL-17A up-regulated several genes which have been reported to exert pro-inflammatory actions in the pathophysiology of acute and/or chronic inflammation. In particular, the induction of CXC-chemokines mRNA expression suggests that IL-17A is a potent inducer of innate immune responses *via* the accumulation and activation of neutrophils in the mucosa.

Recently, we found that among the IL-17 family members, IL-17F also strongly induced the secretion of inflammatory cytokines (IL-6, IL-8 and LIF) and matrix metalloproteinases (MMP-1 and MMP-3) in human colonic SEMFs[32]. Like IL-17A[37], IL-17F stimulated IL-6, IL-8 and MCP-1 secretion *via* NF-κB and MAP kinase activation in human colonic SEMFs. The IL-6,

Table 2 IL-17A augmented TNF-α-induced genes in human colonic myofibroblasts

Gene name	Ref seq ID.	Fold increase
CXCL2 (Gro-β)	NM_00002089.1	× 34.42
CXCL1 (Gro-α)	NM_001511.1	× 23.69
IL-6	NM_000600.1	× 16.92
CXCL3 (Gro-γ)	NM_002090.1	× 15.20
IL-1β	NM_000576.2	× 11.59
GM-CSF	NM_000758.2	× 9.11
IL-1α	NM_000575.3	× 8.45
Amphiregulin	NM_001657.2	× 6.61
CCL20	NM_004591.1	× 5.21
MMP-3	NM_002422.2	× 4.12
CCL7 (MCP-3)	NM_006273.2	× 3.96
IL-13Rα2	NM_000640.2	× 3.76
CXCL6 (GCP-2)	NM_002993.1	× 3.74
IL-11	NM_000641.2	× 3.70
IL-8 (CXCL8)	NM_000584.2	× 3.65
FGF2	NM_002006.2	× 3.12
MMP-1	NM_004142.1	× 2.49
CCL5 (RANTES)	NM_002985.2	× 0.43

Human colonic subepithelial myofibroblasts were stimulated with TNF-α (50 ng/mL) or TNF-α (50 ng/mL) plus IL-17A (200 ng/mL) for 12 h, and the changes in gene expression were assessed by IntelliGene HS Human Expression Chip. The fold change values were determined as a ratio of Cy5 signal intensity (TNF-α plus IL-17A stimulated values)/Cy3 signal intensity (TNF-α stimulated values). The data were average of three independent analysis. The NCBI reference sequence code was presented following the gene name.

IL-8 and LIF secretion by human SEMFs in response to IL-17F as well as IL-17A emphasizes the importance of Th17 products in the induction of intestinal inflammation. Furthermore, the induction of IL-6 secretion from colonic SEMFs has a particular significance in the regulation of immune responses, and in the pathophysiology of IBD; IL-6 has recently been identified as an indispensable factor for the development of Th17 cells[23]. IL-6 commits naïve CD4 T cell precursors to differentiate into Th17 cells[23]. IL-17A and IL-17F might function as potent stimulators for IL-6 production, suggesting an amplification loop for the local development and maturation of Th17 cells. Therefore, the IL-17-colonic SEMFs-IL-6 axis may be important for local Th17 development in the intestinal mucosa.

IL-17A/IL-17F augments TNF-α-induced inflammatory responses

As more important observations, IL-17A and IL-17F augment the TNF-α-induced IL-6 secretion in human colonic SEMFs[32,37]. This augmentation of TNF-α-induced IL-6 production by IL-17A is mediated by enhanced stability of the IL-6 mRNA[37,38]. A similar molecular mechanism can be postulated for the augmentation by IL-17F. IL-17A/IL-17F also augments the TNF-α-induced expression of granulocyte-colony stimulating factor (G-CSF) and granulocyte/macrophage (GM)-CSF in human colonic SEMFs[39].

To further investigate the effects of IL-17A/IL-17F on TNF-α-induced genes, the alterations in gene expression were analyzed by cDNA microarrays in human colonic myofibroblasts. As shown in Table 2, IL-17A further enhanced the expression of various TNF-α-induced genes, such as IL-6, CXC-chemokines and CSFs. These observations suggest that the interactions between TNF-α and IL-17A/IL-17F potently mobilized neutrophils, partially through granulopoiesis and CXC chemokine induction, as well as through increased survival locally. This interaction also potently stimulated Th17 development through the stimulation of IL-6 secretion. Thus, a modulation of the immunological functions of colonic SEMFs by Th17-derived cytokines may be critical for the development of Th17 cells and the mucosal innate immune responses (Figure 1).

Interactions between IL-17A and IL-22

IL-22 was originally described as an IL-9-induced gene, and was termed "IL-10-related T cell-derived-inducible factor" (IL-TIF)[40,41]. IL-22 has 22% amino acid identity with IL-10, and belongs to a family of cytokines with limited homology to IL-10, namely IL-19, IL-20, IL-22, IL-24 and IL-26. The major sources of IL-22 are activated T cells, and IL-22 expression in other leukocyte populations such as monocytes, dendritic cells, NK cells and neutrophils is negligible. Recent studies have shown that Th17 cells are a source of IL-22[42,43]. We recently found that IL-22 expressing cells were increased in the inflamed mucosa of IBD patients[44]. In SEMFs, IL-22 upregulates the expression of inflammatory genes such as IL-6, IL-8, IL-11 and LIF *via* NF-κB, AP-1 and MAP-kinase dependent pathways[44]. Furthermore, the combination of IL-17A plus IL-22 showed an additive effect on transcription factor activation. These concerted responses were also observed as additive effects on cytokine mRNA expression and protein secretion. Thus, the cooperation between Th17 derived cytokines such as IL-17A and IL-22 may play an important role in the pathophysiology of IBD.

IL-31

IL-31 has a Th2 cell origin

IL-31 was cloned, and then found to be mainly produced by CD4+ T cells[45], in particular by skin-homing CD45RO+ (memory) T cells. Transgenic mice overexpressing IL-31 either with a lymphocyte-specific promoter or a ubiquitous promoter exhibit a skin phenotype closely resembling atopic dermatitis in human subjects[45]. In these mice, IL-31 seems to be preferentially produced by T cells skewed towards a Th2 phenotype; however, these Th1-skewed T cells also produce substantial amounts of IL-31[45]. IL-31 mRNA expression is widely detected in various organs, including the gastrointestinal tract[45].

IL-31 is most closely related to the family of IL-6-type cytokines known to be involved in many immunomodulatory functions, particularly the acute-phase response, but also in the proliferation of B and T cells[46]. A recent study indicated that IL-31 sustains the survival of hematopoietic stem cells, and contributes to effects on the cycling and numbers of hematopoietic stem cells

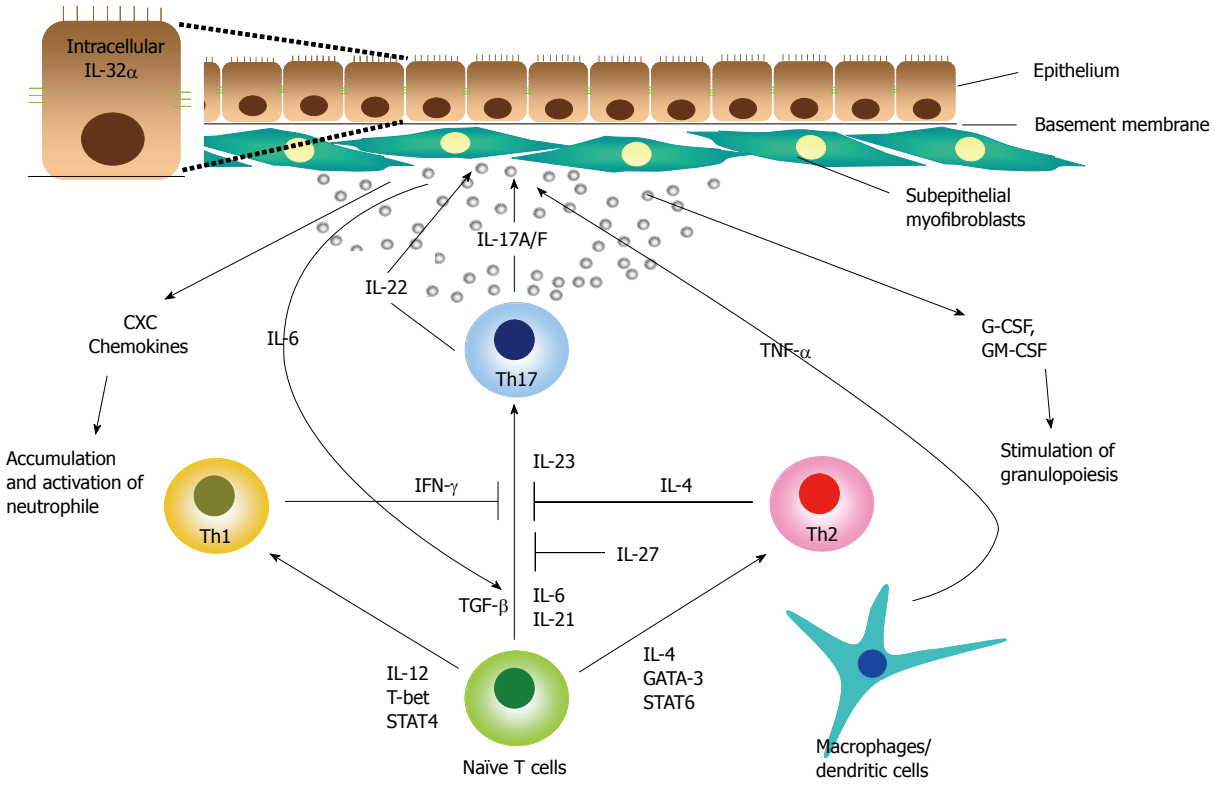


Figure 1 Mucosal cytokine network mediated by IL-17A/F and TNF-α. TGF-β induces the differentiation of Th17cells from naive CD4+ T cells in the presence of IL-6, and full differentiation to Th17 cells is dependent on IL-23. The transcriptional factors required for the development of Th1 (STAT4 and T-bet) and Th2 (STAT6) cells are not required for the induction of Th17/ThIL-17 cells. IFN-γ also suppresses the differentiation of Th17 cells. IL-4 inhibits the development of Th17 cells. One of the important roles of IL-17A/F is the augmentation of the TNF-α-induced secretion of IL-6, a crucial factor for Th17 cell development.

in vivo[47]. However, IL-31 is clearly distinct from the IL-6-type cytokines because it does not signal through glycoprotein-130 (GP-130), the common signaling receptor subunit. IL-31 uses an earlier described orphan receptor, the glycoprotein 130-like monocyte receptor or glycoprotein 130-like receptor (GPL)[48], in combination with the oncostatin M receptor (OSMR)[45], which is expressed on epithelial cells and keratinocytes[45,49].

IL-31 binds directly to the GPL, and OSMR mainly plays a role in delivering the signaling information into the cells. GPL and OSMR are widely expressed in various tissues, including the gastrointestinal tract. This suggests a role for IL-31 in the immune and inflammatory responses of the intestine. In response to IL-31, its receptor complex recruits the Jak1, Jak2, STAT-1, STAT-3 and STAT5 signaling pathways, as well as the PI3-kinase/AKT cascade[49]. SHP-2 and Shc adaptor molecules are also recruited, and contribute to an increased activation of the MAP kinase pathway in response to IL-31[49]. Despite the extensive study of intracellular signaling pathways activated by IL-31 stimulation, the cellular responses to IL-31 were barely investigated in any cell type.

IL-31 stimulates inflammatory responses in colon myofibroblasts

To define the role of IL-31 in the intestinal mucosa, we investigated how IL-31 modulates mRNA expression in human colonic SEMFs. An analysis of the cDNA microarrays indicated that IL-31 effectively induced the secretion of chemokines [CXCL8 (IL-8), CXCL1 (growth-related oncogene; GRO-α)], CCL7 (monocyte chemoattractant protein-3; MCP-3), CXCL3, CCL13, CCL15), proinflammatory cytokines (IL-6, IL-16 and IL-32), and matrix metalloproteinases (MMP-1, MMP-3, MMP-25 and MMP-7). The stimulatory effects of IL-31 were comparable to the effects of IL-17A. Furthermore, simultaneous stimulation with IL-31 and IL-17A showed additive effects on IL-6, IL-8, GRO-α, MCP-3, MMP-1 and MMP-3 secretion. Similar effects for IL-31 have been reported in bronchial epithelial cells[50]. In bronchial epithelial cells, IL-31 could significantly elevate both gene and protein expressions of epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1/CCL2). The combination of IL-31 with either IL-4 or IL-13 further enhanced VEGF and CCL2 production. In these cells, IL-31 could activate p38 MAPK, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK).

As mentioned above, IL-31 is a Th2 type cytokine, and these findings indicate that Th2 cells may be involved in the immune and inflammatory responses of the intestinal mucosa through IL-31secretion. Since IL-31 and IL-17A stimulate the secretion of proinflammatory mediators in an additive manner, Th2-derived IL-31 and Th17-derived IL-17A cooperate in the pathophysiology of IBD.

IL-32

IL-32 cytokine family

IL-32 is a recently described cytokine produced by T lymphocytes, natural killer cells, monocytes, and epithelial cells[51,52]. IL-32 is a proinflammatory cytokine originally described as a transcript termed NK4, found in activated natural killer (NK) cells and T lymphocytes[53]. Although IL-32 was first reported as a transcript in IL-2 activated NK and T cells, it appears that the epithelial cells are the dominant and widespread source[54]. The gene encoding IL-32 is located on human chromosome 16p13.3, and is organized into eight exons[55]. There are four splice variants (IL-32α, IL-32β, IL-32δ and IL-32γ), and IL-32α is the most abundant transcript. Of particular importance, IL-32 is prominently induced by interferon (IFN)-γ in lung epithelial cells and monocytes[51]. IL-32 stimulates the secretion of proinflammatory cytokines and chemokines such as IL-1β, TNF-α, IL-6 and IL-8 by *via* the activation of NF-κB and p38 mitogen-activated protein kinases (MAPKs)[51,52]. IL-32 has been implicated in inflammatory disorders such as rheumatoid arthritis[54,56-58], mycobacterium tuberculosis infection[59,60], and IBD[61].

Intracellular accumulation of IL-32

The amino acid sequence derived from the initial NK4 cDNA contained a signal peptide without a transmembrane domain[53,54]. But, the transcript was never expressed as a recombinant protein, and was not sequenced. Activated human T cells generate IL-32 with a molecular weight of 25 kDa, which on Western blotting analysis is found in the lysates rather than the supernatants. Similar findings were reported for 293T cells transfected with either IL-32γ or IL-32β[62]. In human peripheral blood mononuclear cells stimulated with ConA, most of the IL-32 was found in the lysates[51]. On the other hand, the overexpression of IL-32α or IL-32β in COS cells resulted in secreted IL-32[51]. It remains unclear which isoforms are secreted from which particular cell type. Activated T cells and NK cells do not secrete IL-32, or alternatively, the secreted IL-32 is derived from apoptotic cells due to the presence of GAPDH in the same supernatants. These observations suggest that IL-32 is secreted only as a result of cell death[63]. Recently, we observed that in colon cancer cell lines, proinflammatory cytokines induce the intracellular accumulation of IL-32α, but does not induce secretion[61]. Similar results were also observed in myofibroblasts isolated from the normal human pancreas[64]. Goda *et al* suggest that there is a role for intracellular IL-32 with cell death, since attenuating intracellular IL-32 levels resulted in decreased cell death[62]. These results also support the concept that high levels of intracellular IL-32β may induce cell death. One hypothesis is that the proinflammatory activity of IL-32 may act upon its release through cell death (apoptosis).

Molecular mechanisms regulating IL-32α induction

Shioya *et al* demonstrated that stimulation with IL-1β, IFN-γ and TNF-α enhanced IL-32α mRNA expression in three colon cancer cell lines[61]. TNF-α was the strongest among them. These factors also induced the intracellular accumulation of IL-32α. Since transfection with the mutant form of IκBα inhibited the effects of both IL-1β and TNF-α on IL-32α mRNA expression, NF-κB must play a role in IL-1β- and TNF-α-induced IL-32α mRNA expression.

Nishida *et al* analyzed IL-32α expression in non-transformed myofibroblasts derived from the normal human pancreas[64]. IL-32α mRNA was weakly expressed without any stimulus, and its expression was markedly enhanced by IL-1β, IFN-γ and TNF-α. IL-1β, IFN-γ and TNF-α enhanced the intracellular accumulation of IL-32α protein. But, IL-32α was not detected in the supernatants. An inhibitor of phosphatidylinositol 3-kinase, (LY294002) significantly suppressed the IL-1β-, IFN-γ- and TNF-α-induced IL-32α mRNA expression, although MAPK inhibitors had no effect. Akt activation in response to these cytokines was confirmed by Western blotting analysis. Furthermore, LY294002 suppressed both IL-1β- and TNF-α-induced NF-κB activation, as well as IL-1β-, TNF-α- and IFN-γ-induced AP-1 activation. A blockade of NF-κB and AP-1 activation by an adenovirus expressing a stable mutant form of IκBα and a dominant negative mutant of c-Jun markedly suppressed the IL-1β-, IFN-γ- and/or TNF-α-induced IL-32α mRNA expression. Thus, they concluded that IL-32α mRNA expression was dependent on interactions between the PI3K/Akt-pathway and the NF-κB/AP-1 system.

IL-32α and IBD

Shioya *et al* performed an immunohistochemical analysis to evaluate the expression of IL-32α protein in the mucosa of IBD patients[61]. IL-32α was weakly immunoexpressed by epithelial cells in the normal colonic mucosa and samples of ischemic colitis. In contrast, the epithelial expression of IL-32α was markedly enhanced in the inflamed region of active UC and CD patients[61]. In particular, IL-32α expression tends to increase in samples from active CD patients. IL-32α expression was barely detectable in leukocytes. Thus, these observations indicate that epithelial cells are the major expression site for IL-32α in the intestinal mucosa, and that IL-32α expression is enhanced in the IBD mucosa.

Netea *et al* recently demonstrated that IL-32 augments the production of IL-1β and IL-6 induced by muramyl dipeptide (MDP), a peptidoglycan fraction of bacteria, by means of nucleotide-binding oligomerization domain proteins (NOD1 and NOD2) through a caspase-1-dependent mechanism[52]. NODs are a family of intracytoplasmic bacterial sensors, and the recognition of bacterial peptidoglycans subsequently induces NF-κB activation[65]. Mutations in *NOD2* have been implicated in the pathogenesis of CD[66,67], and CD patients homozygous for the frameshift 3020insC mutated allele have defective responses to MDP in terms of cytokine production[68,69]. Recently, it has been shown that *NOD2* mutations in CD patients potentiate NF-κB activity

and IL-1β processing[70]. Thus, these findings suggest a pivotal role for IL-32 in the pathophysiology of IBD, and in particular CD. Since IL-32α is a proinflammatory cytokine characterized by NF-κB and p38 MAPK activating activities[51,57] and because IL-32 acts synergistically with NOD ligands to induce proinflammatory cytokines[52], the overexpression of IL-32α in the IBD mucosa strongly suggests that it plays an important role in the inflammatory and antibacterial responses involved in the pathogenesis of IBD.

Recent studies have focused on the role of innate immunity in the pathogenesis of IBD[4]. The initial step of innate immunity is mediated by the recognition of pathogen-associated molecular patters (PAMPs) through Toll-like receptors (TLRs) and NOD proteins (NODs)[71]. TLRs are located mainly on cell-surface membranes, but NODs function as intracellular recognition systems[65,66]. In human monocytes, IL-32 acts synergistically with NOD specific peptidoglycans for the release of IL-1β and IL-6[52]. The synergistic effects of IL-32 and the NOD ligands on cytokine production is abolished in cells from CD patients bearing the NOD2 frameshift mutation 3020insC, indicating that this synergism between IL-32 plus MDP depends on NOD2[52]. Interactions between NOD-1 and IL-32 also potentiate proinflammatory cytokine production[52]. Furthermore, Berrebi *et al* previously reported the overexpression of NOD2 in infiltrated monocytes and epithelial cells in the IBD mucosa[72]. These observations suggest that overexpressed IL-32 may cause a specific and excessive stimulation of the NOD pathways, which leads to a marked amplification in IL-1β and IL-6 production in the IBD mucosa.

IL-32 was initially characterized as an inducer of TNF-α in circulating monocytes[51], and hence inflammatory responses in the affected mucosa of IBD patients may be amplified by the consecutive loop of IL-32-induced TNF-α secretion from monocytes and TNF-α-stimulated IL-32 secretion from epithelial cells. This loop may be further amplified by the Th1 cytokine IFN-γ. Previously, it has been reported that TNF-α and IFN-γ synergistically induced the release of NOD2[73], which supports the coupled regulation of IL-32α and NOD2. The coupled regulation of IL-32α with NOD2 may account for the rapid and efficient induction of innate immune responses at the intestinal mucosa. Furthermore, these data suggest that an amelioration of IBD symptoms by TNF-α-targeting therapies may be partially dependent on interference in the TNF-α-IL-32 loop.

The apoptosis of IECs is considered a normal biological function to eliminate damaged epithelial cells, and to restore epithelial cell growth, regulation, and epithelial integrity[74]. An overexpression of cytoplasmic IL-32α might account for the induction of apoptosis in damaged epithelial cells at the inflamed mucosa of IBD patients, leading to an efficient elimination and the rapid induction of mucosal repair. Apoptosis caused by accumulated IL-32 can be considered a host defense mechanism against invading microorganisms, in which damaged epithelial cells are efficiently eliminated along with the invading microorganisms, and thus any further invasion of the microorganisms can be blocked.

CONCLUSION

In this review, we have summarized the newly reported cytokines which may play significant roles in the pathophysiology of IBD. An augmentation of TNF-α effects by IL-17A/F and a possible amplifying cascade between TNF-α and epithelial-derived IL-32 are of particular interest. The clinical efficacy of TNF-α blocking may be associated with an interruption of these cascades. The discovery of new cytokines and the determination of their biological activities may support the development of a novel therapeutic strategy for the treatment of IBD patients.

REFERENCES

1 **Mizoguchi A**, Mizoguchi E. Inflammatory bowel disease, past, present and future: lessons from animal models. *J Gastroenterol* 2008; **43**: 1-17
2 **Sands BE**. Inflammatory bowel disease: past, present, and future. *J Gastroenterol* 2007; **42**: 16-25
3 **Podolsky DK**. Inflammatory bowel disease. *N Engl J Med* 2002; **347**: 417-429
4 **Sartor RB**. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. *Nat Clin Pract Gastroenterol Hepatol* 2006; **3**: 390-407
5 **Fantini MC**, Monteleone G, Macdonald TT. New players in the cytokine orchestra of inflammatory bowel disease. *Inflamm Bowel Dis* 2007; **13**: 1419-1423
6 **Panes J**, Gomollon F, Taxonera C, Hinojosa J, Clofent J, Nos P. Crohn's disease: a review of current treatment with a focus on biologics. *Drugs* 2007; **67**: 2511-2537
7 **Kaplan GG**, Hur C, Korzenik J, Sands BE. Infliximab dose escalation vs. initiation of adalimumab for loss of response in Crohn's disease: a cost-effectiveness analysis. *Aliment Pharmacol Ther* 2007; **26**: 1509-1520
8 **Baumgart DC**, Sandborn WJ. Inflammatory bowel disease: clinical aspects and established and evolving therapies. *Lancet* 2007; **369**: 1641-1657
9 **Rouvier E**, Luciani MF, Mattei MG, Denizot F, Golstein P. CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a herpesvirus saimiri gene. *J Immunol* 1993; **150**: 5445-5456
10 **Kolls JK**, Linden A. Interleukin-17 family members and inflammation. *Immunity* 2004; **21**: 467-476
11 **Yao Z**, Painter SL, Fanslow WC, Ulrich D, Macduff BM, Spriggs MK, Armitage RJ. Human IL-17: a novel cytokine derived from T cells. *J Immunol* 1995; **155**: 5483-5486
12 **Fossiez F**, Djossou O, Chomarat P, Flores-Romo L, Ait-Yahia S, Maat C, Pin JJ, Garrone P, Garcia E, Saeland S, Blanchard D, Gaillard C, Das Mahapatra B, Rouvier E, Golstein P, Banchereau J, Lebecque S. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J Exp Med* 1996; **183**: 2593-2603
13 **Yao Z**, Fanslow WC, Seldin MF, Rousseau AM, Painter SL, Comeau MR, Cohen JI, Spriggs MK. Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. *Immunity* 1995; **3**: 811-821
14 **Jovanovic DV**, Di Battista JA, Martel-Pelletier J, Jolicoeur FC, He Y, Zhang M, Mineau F, Pelletier JP. IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. *J Immunol* 1998; **160**: 3513-3521
15 **Kawaguchi M**, Adachi M, Oda N, Kokubu F, Huang SK.

IL-17 cytokine family. *J Allergy Clin Immunol* 2004; **114**: 1265-1273; quiz 1274
16 **Kotake S**, Udagawa N, Takahashi N, Matsuzaki K, Itoh K, Ishiyama S, Saito S, Inoue K, Kamatani N, Gillespie MT, Martin TJ, Suda T. IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. *J Clin Invest* 1999; **103**: 1345-1352
17 **Ziolkowska M**, Koc A, Luszczykiewicz G, Ksiezopolska-Pietrzak K, Klimczak E, Chwalinska-Sadowska H, Maslinski W. High levels of IL-17 in rheumatoid arthritis patients: IL-15 triggers in vitro IL-17 production via cyclosporin A-sensitive mechanism. *J Immunol* 2000; **164**: 2832-2838
18 **Kawaguchi M**, Takahashi D, Hizawa N, Suzuki S, Matsukura S, Kokubu F, Maeda Y, Fukui Y, Konno S, Huang SK, Nishimura M, Adachi M. IL-17F sequence variant (His161Arg) is associated with protection against asthma and antagonizes wild-type IL-17F activity. *J Allergy Clin Immunol* 2006; **117**: 795-801
19 **Wong CK**, Ho CY, Li EK, Lam CW. Elevation of proinflammatory cytokine (IL-18, IL-17, IL-12) and Th2 cytokine (IL-4) concentrations in patients with systemic lupus erythematosus. *Lupus* 2000; **9**: 589-593
20 **Kurasawa K**, Hirose K, Sano H, Endo H, Shinkai H, Nawata Y, Takabayashi K, Iwamoto I. Increased interleukin-17 production in patients with systemic sclerosis. *Arthritis Rheum* 2000; **43**: 2455-2463
21 **Fujino S**, Andoh A, Bamba S, Ogawa A, Hata K, Araki Y, Bamba T, Fujiyama Y. Increased expression of interleukin 17 in inflammatory bowel disease. *Gut* 2003; **52**: 65-70
22 **Shin HC**, Benbernou N, Esnault S, Guenounou M. Expression of IL-17 in human memory CD45RO+ T lymphocytes and its regulation by protein kinase A pathway. *Cytokine* 1999; **11**: 257-266
23 **Weaver CT**, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity* 2006; **24**: 677-688
24 **Bettelli E**, Korn T, Kuchroo VK. Th17: the third member of the effector T cell trilogy. *Curr Opin Immunol* 2007; **19**: 652-657
25 **Stockinger B**, Veldhoen M, Martin B. Th17 T cells: linking innate and adaptive immunity. *Semin Immunol* 2007; **19**: 353-361
26 **Aujla SJ**, Dubin PJ, Kolls JK. Th17 cells and mucosal host defense. *Semin Immunol* 2007; **19**: 377-382
27 **Wilson NJ**, Boniface K, Chan JR, McKenzie BS, Blumenschein WM, Mattson JD, Basham B, Smith K, Chen T, Morel F, Lecron JC, Kastelein RA, Cua DJ, McClanahan TK, Bowman EP, de Waal Malefyt R. Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol* 2007; **8**: 950-957
28 **Iwakura Y**, Ishigame H. The IL-23/IL-17 axis in inflammation. *J Clin Invest* 2006; **116**: 1218-1222
29 **Amadi-Obi A**, Yu CR, Liu X, Mahdi RM, Clarke GL, Nussenblatt RB, Gery I, Lee YS, Egwuagu CE. TH17 cells contribute to uveitis and scleritis and are expanded by IL-2 and inhibited by IL-27/STAT1. *Nat Med* 2007; **13**: 711-718
30 **Seiderer J**, Elben I, Diegelmann J, Glas J, Stallhofer J, Tillack C, Pfennig S, Jurgens M, Schmechel S, Konrad A, Goke B, Ochsenkuhn T, Muller-Myhsok B, Lohse P, Brand S. Role of the novel Th17 cytokine IL-17F in inflammatory bowel disease (IBD): upregulated colonic IL-17F expression in active Crohn's disease and analysis of the IL17F p.His161Arg polymorphism in IBD. *Inflamm Bowel Dis* 2008; **14**: 437-445
31 **Toy D**, Kugler D, Wolfson M, Vanden Bos T, Gurgel J, Derry J, Tocker J, Peschon J. Cutting edge: interleukin 17 signals through a heteromeric receptor complex. *J Immunol* 2006; **177**: 36-39
32 **Yagi Y**, Andoh A, Inatomi O, Tsujikawa T, Fujiyama Y. Inflammatory responses induced by interleukin-17 family members in human colonic subepithelial myofibroblasts. *J Gastroenterol* 2007; **42**: 746-753
33 **Andoh A**, Bamba S, Fujiyama Y, Brittan M, Wright NA. Colonic subepithelial myofibroblasts in mucosal inflammation and repair: contribution of bone marrow-derived stem cells to the gut regenerative response. *J Gastroenterol* 2005; **40**: 1089-1099
34 **Powell DW**, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB. Myofibroblasts. II. Intestinal subepithelial myofibroblasts. *Am J Physiol* 1999; **277**: C183-C201
35 **Mahida YR**, Beltinger J, Makh S, Goke M, Gray T, Podolsky DK, Hawkey CJ. Adult human colonic subepithelial myofibroblasts express extracellular matrix proteins and cyclooxygenase-1 and -2. *Am J Physiol* 1997; **273**: G1341-G1348
36 **Powell DW**, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB. Myofibroblasts. I. Paracrine cells important in health and disease. *Am J Physiol* 1999; **277**: C1-C9
37 **Hata K**, Andoh A, Shimada M, Fujino S, Bamba S, Araki Y, Okuno T, Fujiyama Y, Bamba T. IL-17 stimulates inflammatory responses via NF-kappaB and MAP kinase pathways in human colonic myofibroblasts. *Am J Physiol Gastrointest Liver Physiol* 2002; **282**: G1035-G1044
38 **Shimada M**, Andoh A, Hata K, Tasaki K, Araki Y, Fujiyama Y, Bamba T. IL-6 secretion by human pancreatic periacinar myofibroblasts in response to inflammatory mediators. *J Immunol* 2002; **168**: 861-868
39 **Andoh A**, Yasui H, Inatomi O, Zhang Z, Deguchi Y, Hata K, Araki Y, Tsujikawa T, Kitoh K, Kim-Mitsuyama S, Takayanagi A, Shimizu N, Fujiyama Y. Interleukin-17 augments tumor necrosis factor-alpha-induced granulocyte and granulocyte/macrophage colony-stimulating factor release from human colonic myofibroblasts. *J Gastroenterol* 2005; **40**: 802-810
40 **Wolk K**, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R. IL-22 increases the innate immunity of tissues. *Immunity* 2004; **21**: 241-254
41 **Fickenscher H**, Hor S, Kupers H, Knappe A, Wittmann S, Sticht H. The interleukin-10 family of cytokines. *Trends Immunol* 2002; **23**: 89-96
42 **Liang SC**, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, Fouser LA. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* 2006; **203**: 2271-2279
43 **Chen Z**, O'Shea JJ. Th17 cells: a new fate for differentiating helper T cells. *Immunol Res* 2008; 2008; **41:** 87-102
44 **Andoh A**, Zhang Z, Inatomi O, Fujino S, Deguchi Y, Araki Y, Tsujikawa T, Kitoh K, Kim-Mitsuyama S, Takayanagi A, Shimizu N, Fujiyama Y. Interleukin-22, a member of the IL-10 subfamily, induces inflammatory responses in colonic subepithelial myofibroblasts. *Gastroenterology* 2005; **129**: 969-984
45 **Dillon SR**, Sprecher C, Hammond A, Bilsborough J, Rosenfeld-Franklin M, Presnell SR, Haugen HS, Maurer M, Harder B, Johnston J, Bort S, Mudri S, Kuijper JL, Bukowski T, Shea P, Dong DL, Dasovich M, Grant FJ, Lockwood L, Levin SD, LeCiel C, Waggie K, Day H, Topouzis S, Kramer J, Kuestner R, Chen Z, Foster D, Parrish-Novak J, Gross JA. Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice. *Nat Immunol* 2004; **5**: 752-760
46 **Heinrich PC**, Behrmann I, Muller-Newen G, Schaper F, Graeve L. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem J* 1998; **334** (Pt 2): 297-314
47 **Broxmeyer HE**, Li J, Hangoc G, Cooper S, Tao W, Mantel C, Graham-Evans B, Ghilardi N, de Sauvage FJ. Regulation of myeloid progenitor cell proliferation/survival by IL-31 receptor and IL-31. *Exp Hematol* 2007; **35**: 78-86
48 **Ghilardi N**, Li J, Hongo JA, Yi S, Gurney A, de Sauvage FJ. A novel type I cytokine receptor is expressed on monocytes, signals proliferation, and activates STAT-3 and STAT-5. *J Biol Chem* 2002; **277**: 16831-16836
49 **Diveu C**, Lak-Hal AH, Froger J, Ravon E, Grimaud L, Barbier F, Hermann J, Gascan H, Chevalier S. Predominant expression of the long isoform of GP130-like (GPL) receptor

is required for interleukin-31 signaling. *Eur Cytokine Netw* 2004; **15**: 291-302
50 **Ip WK**, Wong CK, Li ML, Li PW, Cheung PF, Lam CW. Interleukin-31 induces cytokine and chemokine production from human bronchial epithelial cells through activation of mitogen-activated protein kinase signalling pathways: implications for the allergic response. *Immunology* 2007; **122**: 532-541
51 **Kim SH**, Han SY, Azam T, Yoon DY, Dinarello CA. Interleukin-32: a cytokine and inducer of TNFalpha. *Immunity* 2005; **22**: 131-142
52 **Netea MG**, Azam T, Ferwerda G, Girardin SE, Walsh M, Park JS, Abraham E, Kim JM, Yoon DY, Dinarello CA, Kim SH. IL-32 synergizes with nucleotide oligomerization domain (NOD) 1 and NOD2 ligands for IL-1beta and IL-6 production through a caspase 1-dependent mechanism. *Proc Natl Acad Sci USA* 2005; **102**: 16309-16314
53 **Dahl CA**, Schall RP, He HL, Cairns JS. Identification of a novel gene expressed in activated natural killer cells and T cells. *J Immunol* 1992; **148**: 597-603
54 **Dinarello CA**, Kim SH. IL-32, a novel cytokine with a possible role in disease. *Ann Rheum Dis* 2006; **65** Suppl 3: iii61-iii64
55 **Chen Q**, Carroll HP, Gadina M. The newest interleukins: recent additions to the ever-growing cytokine family. *Vitam Horm* 2006; **74**: 207-228
56 **Cagnard N**, Letourneur F, Essabbani A, Devauchelle V, Mistou S, Rapinat A, Decraene C, Fournier C, Chiocchia G. Interleukin-32, CCL2, PF4F1 and GFD10 are the only cytokine/chemokine genes differentially expressed by in vitro cultured rheumatoid and osteoarthritis fibroblast-like synoviocytes. *Eur Cytokine Netw* 2005; **16**: 289-292
57 **Joosten LA**, Netea MG, Kim SH, Yoon DY, Oppers-Walgreen B, Radstake TR, Barrera P, van de Loo FA, Dinarello CA, van den Berg WB. IL-32, a proinflammatory cytokine in rheumatoid arthritis. *Proc Natl Acad Sci USA* 2006; **103**: 3298-3303
58 **Shoda H**, Fujio K, Yamaguchi Y, Okamoto A, Sawada T, Kochi Y, Yamamoto K. Interactions between IL-32 and tumor necrosis factor alpha contribute to the exacerbation of immune-inflammatory diseases. *Arthritis Res Ther* 2006; **8**: R166
59 **Kundu M**, Basu J. IL-32: an emerging player in the immune response network against tuberculosis? *PLoS Med* 2006; **3**: e274
60 **Netea MG**, Azam T, Lewis EC, Joosten LA, Wang M, Langenberg D, Meng X, Chan ED, Yoon DY, Ottenhoff T, Kim SH, Dinarello CA. Mycobacterium tuberculosis induces interleukin-32 production through a caspase- 1/IL-18/interferon-gamma-dependent mechanism. *PLoS Med* 2006; **3**: e277
61 **Shioya M**, Nishida A, Yagi Y, Ogawa A, Tsujikawa T, Kim-Mitsuyama S, Takayanagi A, Shimizu N, Fujiyama Y, Andoh A. Epithelial overexpression of interleukin-32alpha in inflammatory bowel disease. *Clin Exp Immunol* 2007; **149**: 480-486
62 **Goda C**, Kanaji T, Kanaji S, Tanaka G, Arima K, Ohno S, Izuhara K. Involvement of IL-32 in activation-induced cell death in T cells. *Int Immunol* 2006; **18**: 233-240
63 **Netea MG**, Lewis EC, Azam T, Joosten LA, Jaekal J, Bae SY, Dinarello CA, Kim SH. Interleukin-32 induces the differentiation of monocytes into macrophage-like cells. *Proc Natl Acad Sci USA* 2008; **105**: 3515-3520
64 **Nishida A**, Andoh A, Shioya M, Kim-Mitsuyama S, Takayanagi A, Fujiyama Y. Phosphatidylinositol 3-kinase/Akt signaling mediates interleukin-32alpha induction in human pancreatic periacinar myofibroblasts. *Am J Physiol Gastrointest Liver Physiol* 2008; **294**: G831-G838
65 **Peyrin-Biroulet L**, Vignal C, Dessein R, Simonet M, Desreumaux P, Chamaillard M. NODs in defence: from vulnerable antimicrobial peptides to chronic inflammation. *Trends Microbiol* 2006; **14**: 432-438
66 **Hugot JP**, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, Almer S, Tysk C, O'Morain CA, Gassull M, Binder V, Finkel Y, Cortot A, Modigliani R, Laurent-Puig P, Gower-Rousseau C, Macry J, Colombel JF, Sahbatou M, Thomas G. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 2001; **411**: 599-603
67 **Hugot JP**. CARD15/NOD2 mutations in Crohn's disease. *Ann N Y Acad Sci* 2006; **1072**: 9-18
68 **Netea MG**, Ferwerda G, de Jong DJ, Jansen T, Jacobs L, Kramer M, Naber TH, Drenth JP, Girardin SE, Kullberg BJ, Adema GJ, Van der Meer JW. Nucleotide-binding oligomerization domain-2 modulates specific TLR pathways for the induction of cytokine release. *J Immunol* 2005; **174**: 6518-6523
69 **Netea MG**, Kullberg BJ, de Jong DJ, Franke B, Sprong T, Naber TH, Drenth JP, Van der Meer JW. NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease. *Eur J Immunol* 2004; **34**: 2052-2059
70 **Maeda S**, Hsu LC, Liu H, Bankston LA, Iimura M, Kagnoff MF, Eckmann L, Karin M. Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing. *Science* 2005; **307**: 734-738
71 **Michelsen KS**, Arditi M. Toll-like receptors and innate immunity in gut homeostasis and pathology. *Curr Opin Hematol* 2007; **14**: 48-54
72 **Berrebi D**, Maudinas R, Hugot JP, Chamaillard M, Chareyre F, De Lagausie P, Yang C, Desreumaux P, Giovannini M, Cezard JP, Zouali H, Emilie D, Peuchmaur M. Card15 gene overexpression in mononuclear and epithelial cells of the inflamed Crohn's disease colon. *Gut* 2003; **52**: 840-846
73 **Rosenstiel P**, Fantini M, Brautigam K, Kuhbacher T, Waetzig GH, Seegert D, Schreiber S. TNF-alpha and IFN-gamma regulate the expression of the NOD2 (CARD15) gene in human intestinal epithelial cells. *Gastroenterology* 2003; **124**: 1001-1009
74 **Kim JM**, Eckmann L, Savidge TC, Lowe DC, Witthoft T, Kagnoff MF. Apoptosis of human intestinal epithelial cells after bacterial invasion. *J Clin Invest* 1998; **102**: 1815-1823

S- Editor Li DL **L- Editor** Roberts SE **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5162

World J Gastroenterol 2008 September 7; 14(33): 5162-5175
World Journal of Gastroenterology ISSN 1007-9327

COLORECTAL CANCER

Roscovitine synergizes with conventional chemo-therapeutic drugs to induce efficient apoptosis of human colorectal cancer cells

Mohamed Salah I Abaza, Abdul-Majeed A Bahman, Rajaa J Al-Attiyah

Mohamed Salah I Abaza, Abdul-Majeed A Bahman, Molecular Biology Program, Department of Biological Sciences, Faculty of Science, Kuwait University, Safaat 13006, Kuwait
Rajaa J Al-Attiyah, Department of Microbiology, Faculty of Medicine, Kuwait University, Safaat 13006, Kuwait
Author contributions: Abaza MSI designed research; Abaza MSI and Bahman AMA contributed equally to the experimental work execution, writing and revising the paper; and Al-Attiyah RJ carried out cell cycle analysis by fluorescence activated cell sorter.
Supported by Kuwait University, Research Project No. SL01/05
Correspondence to: Dr. Mohamed-Salah I Abaza, Molecular Biology Program, Department of Biological Sciences, Faculty of Science, Kuwait University, PO Box 5696, Safaat 13006, Kuwait. abaza@kuc01.kuniv.edu.kw
Telephone: +965-9-835274 Fax: +965-4-847054
Received: April 10, 2008 Revised: July 7, 2008
Accepted: July 14, 2008
Published online: September 7, 2008

Abstract

AIM: To examine the ability of cyclin-dependent kinase inhibitor (CDKI) roscovitine (Rosco) to enhance the antitumor effects of conventional chemotherapeutic agents acting by different mechanisms against human colorectal cancer.

METHODS: Human colorectal cancer cells were treated, individually and in combination, with Rosco, taxol, 5-Fluorouracil (5-FU), doxorubicin or vinblastine. The antiproliferative effects and the type of interaction of Rosco with tested chemotherapeutic drugs were determined. Cell cycle alterations were investigated by fluorescence-activated cell sorter FACS analysis. Apoptosis was determined by DNA fragmentation assay.

RESULTS: Rosco inhibited the proliferation of tumor cells in a time- and dose-dependent manner. The efficacies of all tested chemotherapeutic drugs were markedly enhanced 3.0-8.42 × 10^3 and 130-5.28 × 10^3 fold in combination with 5 and 10 μg/mL Rosco, respectively. The combination of Rosco and chemotherapeutic drugs inhibited the growth of human colorectal cancer cells in an additive or synergistic fashion, and in a time and dose dependent manner. Rosco induced apoptosis and synergized with tested chemotherapeutic drugs to induce efficient apoptosis in human colorectal cancer cells. Sequential, inverted sequential and simultaneous treatment of cancer cells with combinations of chemotherapeutic drugs and Rosco arrested the growth of human colorectal cancer cells at various phases of the cell cycle as follows: Taxol/Rosco (G_2/M- and S-phases), 5-FU/Rosco (S-phase), Dox/Rosco (S-phase) and Vinb/Rosco (G_2/M- and S-phases).

CONCLUSION: Since the efficacy of many anticancer drugs depends on their ability to induce apoptotic cell death, modulation of this parameter by cell cycle inhibitors may provide a novel chemo-preventive and chemotherapeutic strategy for human colorectal cancer.

Key words: Human colorectal cancer cell lines; Cyclin dependent kinase inhibition; Chemosensitization; Synergy; Apoptosis; Cell cycle

Peer reviewers: Francis Seow-Choen, Professor, Seow-Choen Colorectal Centre, Mt Elizabeth Medical Centre, Singapore, 3 Mt Elizabeth Medical Centre #09-10, 228510, Singapore; Alessandro Fichera, MD, FACS, FASCRS, Assistant Professor, Department of Surgery, University of Chicago, 5841 S. Maryland Ave, MC 5031, Chicago IL 60637, United States

Abaza MSI, Bahman AMA, Al-Attiyah RJ. Roscovitine synergizes with conventional chemo-therapeutic drugs to induce efficient apoptosis of human colorectal cancer cells. *World J Gastroenterol* 2008; 14(33): 5162-5175 Available from: URL: http://www.wjgnet.com/1007-9327/14/5162.asp DOI: http://dx.doi.org/10.3748/wjg.14.5162

INTRODUCTION

Colon cancer is the second leading cause of cancer death in the United States and is one of the most common cancers in Western countries[1]. Lack of improvement in overall survival and failure of the current systemic therapies have mandated that new approaches to this disease be explored.

A hallmark of neoplastic evolution and progression is deregulation of cell cycle control mechanisms. The key regulators of transition from one cell cycle phase to the next are the cyclin-dependent kinases (CDKs).

CDKs are serine/threonine kinases that regulate cell cycle progression in a highly coordinated manner[2]. A CDK enzyme complex becomes fully active after binding of its proper cyclin. Progression through cell cycle is mediated by the orchestrated activation and breakdown of CDK complexes[2].

A basis for selectivity of CDK-directed therapies against neoplastic cells might arise from the fact that alteration of CDK structure and function plays a key role in the pathogenesis of neoplasia[3]. At least one of the following changes is almost ubiquitously evident in human neoplasms: overexpression of cyclin D; amplification or structural alteration of CDK4; deletion or mutation of $p16^{INK4A}$; mutation of the CDK4 or 6/cyclin D substrate p^{Rb}; and loss of $p21^{WAF1/CIP1A}$ function through deletion or mutation of its transactivator P53. In relation to colon cancer, $p27^{KIP1}$ loss has been found to occur not by gene deletion or mutation, but by increased proteolysis of the CDK inhibitor (CDKI)[4]. Therefore, replacement of at least some of the missing capacity to inhibit cell cycle progression may restore some measure of cell cycle control. In contrast to their normal counterparts, transformed cells proliferate very rapidly due to the enhanced activity of the CDK[2]. Thus, inhibition of CDK/cyclin complexes offers a promising therapeutic strategy in the defense against cancer[5].

Many types of potential CDK modulators are conceivable. These include molecules that directly inhibit ATP or protein substrate binding; alter regulatory phosphorylations of the catalytic subunit; inhibit CDK catalytic subunit binding with its respective cyclin or other accessory proteins; mimic the action or increase the expression of endogenous CDK inhibitors, $p16^{INK4A}$ (or its homologues, $p15^{INK4B}$, $p18^{INK4C}$, and $p19^{INK4D}$), $p21^{WAF1}$, and $p27^{KIP1}$; interfere with the proper appearance and disappearance of cyclins; and finally alter normal signals for import of CDKs into the nucleus or localization to appropriate subcellular structures[6].

Pharmacological inhibitors of CDKs display selective anti-proliferative effects on cycling cells, especially malignant ones[7]. Depending on the selectivity profile of these novel drugs, growth inhibition in different phases of the cell cycle is observed[8]. Compounds targeting the activity of CDK4/6 block cells in early G_1, whereas selective inhibitors of CDK1/2 arrest cell cycle in G_1/S and G_2/M[8]. Interestingly, some inhibitors, especially those targeting the activity of CDK2, are able to selectively induce apoptosis in cancer cells[9,10].

CDKIs, representing a well-defined group of biologically active compounds, are structurally related to adenosine-5-triphosphate, ATP[7,8,11]. They antagonize binding of kinases to ATP. Differentially substituted adenines yielded a group of inhibitors such as roscovitine (Rosco), olomoucine and purvalanol[7]. These close analogs, characterized by increasing potency, differ in selectivity. Due to their selectivity and relative low direct cytotoxicity, CDKIs clearly provide useful anticancer drugs and offer an alternative to classic chemotherapeutics. In the present study, we have investigated whether Rosco could inhibit the growth of human colorectal cancer cells and increase their sensitivity to conventional chemotherapies.

MATERIALS AND METHODS

Cell culture and reagents

Human colorectal cancer cell lines (SW48, SW1116 and SW 837) were obtained from the American Type Culture Collection (ATCC, Rockville, Md., USA). Cells were cultured in Leibovitz's L-15 medium supplemented with 10% inactivated fetal bovine serum and 2 mmol/L glutamine. The L-15 medium formulation was devised for use in a free gas exchange with atmospheric air. CO_2 is detrimental to cells when using this medium for cultivation. All the other chemicals were purchased from Sigma Chemical Co.

Time and dose dependency of the antiproliferative effects induced in human colorectal cancer cells by treatment with Rosco

Human colorectal cancer cell lines (SW48, SW1116 and SW837) were plated (27×10^3 cells/well) into 96-well plates and incubated at 37℃ in a non-CO_2 incubator. Cells were treated with various concentrations of Rosco (0-40 μg/mL) or DMSO (0.3% final concentration) for various time periods beginning at 24 h after seeding the cells in culture. Control cells were untreated. Cell proliferation was determined at various time intervals (24-168 h) by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described[12].

In vitro efficacy of single and combined treatment of Rosco and chemotherapeutic drugs on the growth of human colorectal cancer cells

Human colorectal cancer cell lines (SW48, SW1116 and SW837) were treated with taxol (10^{-11}-10^{-6} mol/L), doxorubicin (10^{-11}-10^{-6} mol/L), 5-fluorouracil (5-FU) (10^{-9}-10^{-4} mol/L), vinblastine (10^{-12}-10^{-7} mol/L) or combinations of the tested chemotherapeutic drug and Rosco (5 and 10 μg/mL) for 96 h. At the end of treatment, control and drug treated cells were scored for proliferation using the MTT assay.

Analysis of the type of interaction between Rosco and chemotherapeutic drugs in human colorectal cancer cells

Human colorectal cancer cell lines (SW48, SW1116 and SW837) were treated with Rosco (1, 10, 15, 20, 25 μg/mL) and chemotherapeutic drugs (0.5 IC_{50}-4 IC_{50}) individually and in combination. The effect of the combinations of Rosco and chemotherapeutic drugs on cell growth was determined as previously described[12,13] using the following formulae: $SF_{A+B} > (SF_A) \times (SF_B)$, antagonistic; $SF_{A+B} = (SF_A) \times (SF_B)$; additive; $SF_{A+B} < (SF_A) \times (SF_B)$, synergistic, where SF is the surviving fraction, and A and B indicate the agents used alone, while A + B refers to the agents used in combination.

Cell cycle analysis

Cell cycle phase distribution of human colorectal cancer

Table 1 IC_{50} and sensitization ratio of taxol and its combinations with Rosco towards human colorectal cancer cell lines

Treatment with taxol and combinations with Rosco	IC_{50} (mol/L)			Sensitization ratio[1]		
	SW48	SW1116	SW837	SW48	SW1116	SW837
Taxol	4.8×10^{-8}	1.2×10^{-7}	3.8×10^{-7}	-	-	-
Taxol + 5 μg/mL Rosco	7.4×10^{-10}	1.0×10^{-8}	5.4×10^{-8}	65	12	7
Taxol + 10 μg/mL Rosco	7.5×10^{-11}	8.1×10^{-10}	6.7×10^{-10}	6.4×10^{2}	1.5×10^{2}	5.7×10^{2}

[1]Sensitization ratio = IC_{50} (taxol)/IC_{50} (taxol + Rosco).

cells treated with Rosco, chemotherapeutic drugs and their combinations was determined by flow cytometry as previously described[12]. Human colorectal cancer cells (SW837, 5×10^5 cells/well in 24 well plates) were treated with taxol (12×10^{-8} mol/L, 72 h), doxorubicin (8×10^{-7} mol/L, 72 h), 5-FU (4.8×10^{-5} mol/L, 72 h), vinblastine (2.6×10^{-7} mol/L, 72 h), Rosco (15 μg/mL) and combinations of Rosco and chemotherapeutic drugs. The combinations were added in a sequential manner, drug (24 h) followed by Rosco (48 h); inverted sequential manner, Rosco (24 h) followed by drug (48 h) and simultaneous manner (72 h). The tested cells were collected by trypsinization, and then washed with cold phosphate-buffered saline, and counted using a cell counter. A sample of 3×10^6 cells/mL was processed using DNA-Prep kit (Beckman & Coulter, Fa., USA) and a DNA-Prep Epics workstation (Beckman & Coulter). During this process, the cell sample was treated with a cell membrane-permeabilizing agent and then with propidium and RNase enzyme. The sample was then incubated at room temperature for at least 15 min before analysis by aligned flow cytometry (Epics XL, Beckman & Coulter). The percentage of cells in different cell cycle phases was evaluated using the Phoenix statistical software package, advanced DNA cell cycle software (Phoenix Flow System, San Diego, Calif., USA).

DNA fragmentation analysis

DNA fragmentation assay was performed as previously described[14]. Briefly, colorectal cancer cells (5×10^5 cells/well, SW1116 and SW837) were treated with taxol (1.2×10^{-7} mol/L), doxorubicin (8×10^{-7} mol/L), 5-FU (4.8×10^{-5} mol/L), vinblastine (2.6×10^{-7} mol/L), Rosco (15 μg/mL) and the combinations of Rosco, and tested drugs for 72 h. The cell pellets were lysed with 100 μmol/L of hypotonic buffer (10 mmol/L Tris (pH 8.0), 20 mmol/L EDTA containing 0.5 % Triton X-100) for 30 min at 4℃. Following cell lysis, the intact chromatin (pellet) was separated from DNA fragments (supernatant) by centrifugation for 15 min at 12000 *g*. The supernatants containing DNA were precipitated overnight with 0.5 mol/L NaCl and 50% isopropyl alcohol at -20℃. Pellets were recovered by centrifugation at 12000 *g* for 10 min, air dried, resuspended in 30 μL of TE buffer supplemented with 1 mg/mL RNase I at 37℃ for 30 min, and then with 2 mg/mL of proteinase K for another 1 h. DNA samples were supplemented with 3 μL of sample buffer (0.25% bromophenol blue, 30% glyceric acid), and electrophorectically separated on a 1.5% agrose gel containing 0.1 μg/mL ethidium bromide at 80 V for 2 h. DNA fragments were visualized by ultraviolet transillumination.

Statistical analysis

Results are representative of two to three individual experiments. Errors are expressed as standard errors of the percentage of the means. Where appropriate, data were analyzed using ANOVA.

RESULTS

Inhibition of the proliferation of human colorectal cancer cells by Rosco

In this study, we initially investigated the effect of Rosco on the proliferation of human colorectal cancer cells using three human colorectal cancer cell lines (SW48, SW1116 and SW837). The results, shown in Figure 1, indicated that all cell lines tested were sensitive to micromolar range of Rosco, in a dose and time dependent manner. Rosco inhibited the growth of SW48 after 24-144 h of drug treatment. It affected the growth of the colorectal cancer cell line SW1116 slightly after 48 h of treatment. However, a dramatic inhibition of cell growth was observed after 72-144 h of treatment. Rosco slightly affected the growth of SW837 after 24 h of treatment. However, a marked inhibition was observed after 48-144 h of treatment with Rosco.

Modulation of taxol cytotoxicity on human colorectal cancer cells by combination with CDKI Rosco

The ability of Rosco to enhance the sensitivity of human colorectal cancer cells to taxol was assessed by treating human colorectal cancer cell lines SW48, SW1116 and SW837 with taxol (10^{-11}-10^{-6} mol/L) or taxol (10^{-11}-10^{-6} mol/L) and Rosco (5 or 10 μg/mL) for 96 h. The results summarized in Figure 2A and Table 1 clearly indicated that the combination of taxol and 5 μg/mL Rosco enhanced (65-fold) the anticancer activity of taxol on human colorectal cancer cell line SW48 (Table 1). The difference between SW48 growth inhibition produced by treatment with taxol alone [IC_{50} (taxol) = 4.8×10^{-8} mol/L] and that produced by treatment with the combination of taxol and 5 μg/mL Rosco [IC_{50} (taxol + 5 μg/mL Rosco) = 7.4×10^{-10} mol/L] (Table 1) was statistically non-significant ($P = 0.127$). The combination of taxol and 10 μg/mL Rosco greatly increased the sensitivity of human colorectal cancer cells to taxol (640-fold) compared to treatment with

Table 2 Analysis of the combined effects of taxol and Rosco on human colorectal cancer cell lines

Combined treatment of taxol and Rosco	Combination interaction at various durations of treatment in human colorectal cancer cells[1]								
	SW48			SW1116			SW837		
	2 d	4 d	6 d	2 d	4 d	6 d	2 d	4 d	6 d
Taxol 0.5 IC_{50} + Rosco 1.0 μg/mL	ant	ant	ant	ant	ant	syn	ant	ant	syn
Taxol 1.0 IC_{50} + Rosco 10 μg/mL	ant	ant	add	ant	ant	ant	ant	ant	syn
Taxol 2.0 IC_{50} + Rosco 15 μg/mL	ant	ant	ant	syn	ant	ant	ant	ant	ant
Taxol 3.0 IC_{50} + Rosco 20 μg/mL	ant	ant	ant	ant	ant	ant	ant	ant	ant
Taxol 4.0 IC_{50} + Rosco 25 μg/mL	ant	add	add	syn	ant	add	ant	ant	ant

[1]The data are based on the mean of absorbance measurements of 3 independent experiments. ant: Antagonistic; add: Additive; syn: Synergistic.

taxol alone (Table 1). The difference in SW48 growth inhibition produced by treatment with combination of taxol and 10 μg/mL Rosco (IC_{50} = 7.5 × 10^{-11} mol/L) and that produced by treatment with taxol alone (IC_{50} = 4.8 × 10^{-8} mol/L) (Table 1) was significant (P = 0.012).

The combination of taxol and 5 μg/mL Rosco had a higher growth inhibitory effect on SW1116 (12-fold) compared to treatment with taxol alone (Table 1). The difference in SW1116 growth inhibition produced by treatment with the combination of taxol and 5 μg/mL Rosco (IC_{50} = 1 × 10^{-8} mol/L), and that produced by treatment with taxol alone (1.2 × 10^{-7} mol/L) was non-significant (P = 0.256). On the other hand, the combination of taxol and 10 μg/mL Rosco markedly increased SW1116 growth inhibition (150-fold) compared to treatment with taxol alone (Table 1). The difference in SW1116 growth inhibition produced by the combination of taxol and 10 μg/mL Rosco (IC_{50} = 8.1 × 10^{-10} mol/L) and that produced by taxol alone (1.2 × 10^{-7} mol/L) was statistically significant (P = 0.03).

Treatment of SW837 cells with various concentrations of taxol inhibited their growth in a dose dependent manner with IC_{50} = 3.8 × 10^{-7} mol/L. The combination of taxol and 5 μg/mL Rosco produced higher SW837 growth inhibition (IC_{50} = 5.4 × 10^{-8} mol/L, sensitization ratio = 7-fold) than that produced by treatment with taxol alone (Table 1). This difference in SW837 growth inhibition was non-significant (P = 0.365). Treatment of SW837 with the combination of taxol and 10 μg/mL Rosco resulted in a more significant growth inhibition (IC50 = 6.7 × 10^{-10} mol/L, sensitization ratio = 570 and P = 0.045) compared to that produced by treatment with taxol alone (Figure 2, Table 1). The combinations of taxol and Rosco produced additive and/or synergistic effects depending upon the type of cell line used, relative concentrations of the mixed drugs and exposure time (Table 2).

Cell cycle analysis of human colorectal cancer cells treated with taxol, Rosco or their combinations added in sequential (taxol followed by Rosco), inverted sequential or simultaneous manner was determined by flow cytometry as described in Materials and Methods. Treatment of colorectal cancer cells with taxol or Rosco resulted in growth arrest at G_2/M phase, 24.9% and 16.6%, respectively, compared to 4.75% for untreated cells (Figure 1B). The combination of taxol and Rosco added in sequential manner growth arrested colorectal cancer cell in G_2/M (21.8%). Also, the same combination added in an inverted sequential manner growth arrested colorectal cancer cells in G_2/M (23.4%), meanwhile when the

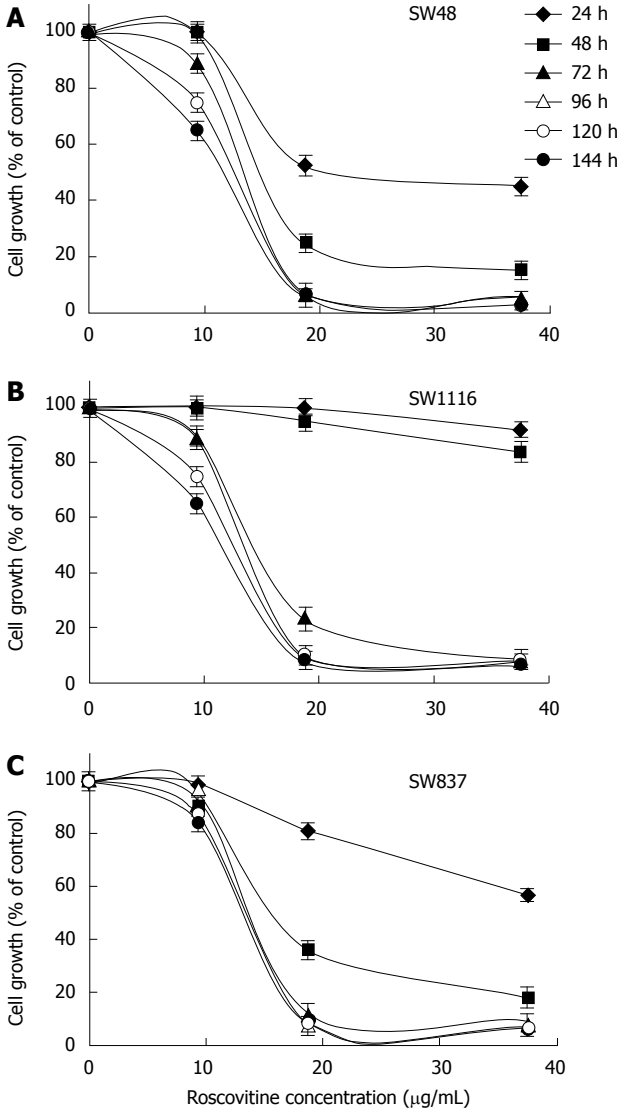


Figure 1 Time and dose dependent effect of roscovitine on the proliferation of human colorectal cancer cell lines. Human colorectal cancer cell lines SW48 (**A**), SW1116 (**B**) and SW837 (**C**) were plated (27 x 10^3 cells/well) into 96-well plates and incubated at 37℃ in a non-CO_2 incubator. Twenty four hours after starting the culture, the cells were treated with various concentrations of Rosco (0-40 μg /mL) or DMSO (0.3%, final concentration) for various time periods (24-144 h). Control (0.3% DMSO treated) and Rosco treated colorectal cancer cells were scored for proliferation using an MTT assay. Roscovitine concentrations 5 μg/mL and 10 μg/mL were used in the subsequent studies.

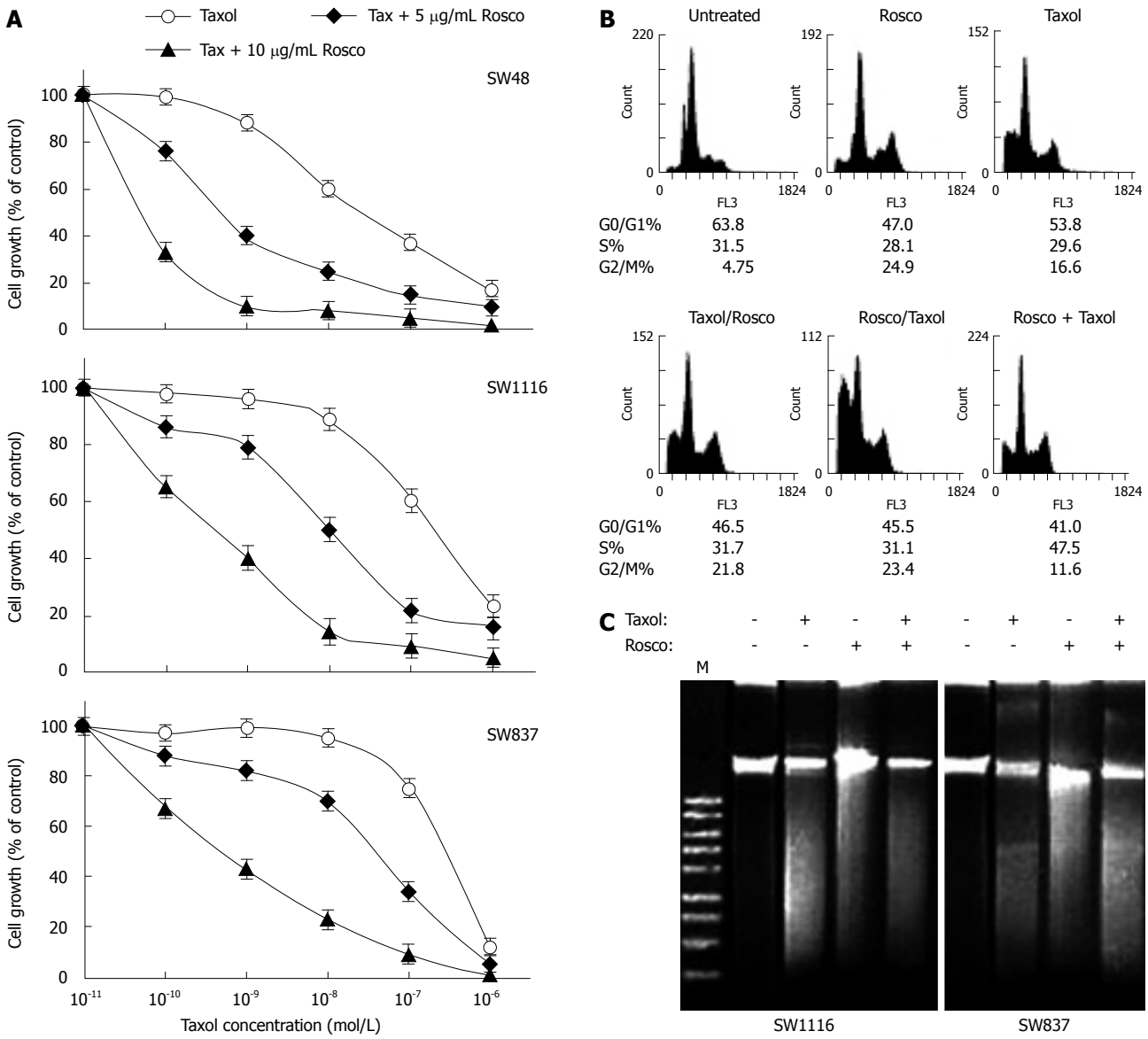


Figure 2 Potentiation of anticancer effect of taxol on human colorectal cancer cell lines by combination with cyclin dependent kinase inhibitor roscovitine. **A**: Human colorectal cancer cell lines were treated with taxol (10^{-11}-10^{-6} mol/L), or the combination of taxol (10^{-11}-10^{-6} mol/L) and Rosco (5 μg/mL or 10 μg/mL) for 96 h. At the end of treatment, control and drug treated cells were scored for proliferation using the MTT assay; **B**: Cell cycle phase distribution of human colorectal cancer cells (SW837, 5×10^5 cells/well) treated with taxol (12×10^{-8} mol/L, 72 h); Rosco (15 μg/mL, 72 h); sequential combination: taxol (12×10^{-8} mol/L, 24 h) followed by Rosco (15 μg/mL, 48 h); inverted sequential combination: Rosco (15 μg/mL, 24 h) followed by taxol (12×10^{-8} mol/L, 48 h) and simultaneous combination: taxol plus Rosco (12×10^{-8} mol/L, 15 μg/mL, 72 h) was evaluated by flow cytometry. The percentage of cells in different phases of the cycle was calculated by cell cycle analysis software, Multicycle (Phoenix Flow System, San Diego CA, USA); **C**: Human colorectal cancer cells, SW1116 and SW837 (5×10^5 cells/well), were treated with taxol (12×10^{-8} mol/L), Rosco (15 μg/mL), and the combination of taxol plus Rosco (12×10^{-8} mol/L + 15 μg/mL) for 72 h. DNA fragments were extracted and analyzed on 1% agrose gel.

same combination was used in a simultaneous manner, colorectal cancer cells were growth arrested in G_2/M (11.6%) and S (47.5%) phases, respectively, (Figure 2B). The effect of taxol, Rosco and their combination on inducing programmed cell death in human colorectal cancer cell lines SW1116 and SW837 was studied using DNA fragmentation analysis. The combination of taxol and Rosco induced apoptosis more efficiently compared to single treatment with taxol or Rosco (Figure 2C).

Modulation of 5-FU cytotoxicity on human colorectal cancer cells by combination *with the CDKI Rosco*

The anti-proliferative activities of 5-FU, Rosco and their combinations against human colorectal cancer cells are summarized in Figure 3. The combination of 5-FU (10^{-9}-10^{-4} mol/L) and Rosco (5 μg/mL) exerted a very potent anticancer effect ($P < 0.0001$) on SW48 with $IC_{50} = 5.7 \times 10^{-9}$ mol/L compared to $IC_{50} = 4.8 \times 10^{-5}$ mol/L when SW48 cells were treated with 5-FU alone (Figure 3A). These results indicate that Rosco (5 μg/mL) increased the sensitivity of SW48 to 5-FU by about 8.42×10^3-fold (Table 3). The same combination inhibited the growth of colorectal cancer cell lines SW1116 and SW837 with IC_{50} values equal to 6.7×10^{-6} mol/L and 1.8×10^{-6} mol/L compared to IC_{50} values equal to 2.5×10^{-5} mol/L and 4.8×10^{-5} mol/L obtained when these

Table 3 IC_{50} and sensitization ratio of 5-FU and its combinations with Rosco towards human colorectal cancer cell lines

Treatment with 5-FU and combinations with Rosco	IC_{50} (mol/L)			Sensitization ratio[1]		
	SW48	SW1116	SW837	SW48	SW1116	SW837
5-FU	4.8×10^{-5}	2.5×10^{-5}	4.8×10^{-5}	-	-	-
5-FU + 5 μg/mL Rosco	5.7×10^{-9}	6.7×10^{-6}	1.8×10^{-6}	8.42×10^{3}	3.73	26.7
5-FU + 10 μg/mL Rosco	5.7×10^{-9}	1.0×10^{-7}	9.1×10^{-9}	8.42×10^{3}	250	5.28×10^{3}

[1]Sensitization ratio = IC_{50} (5-FU)/IC_{50} (5-FU + Rosco).

Table 4 Analysis of the combined effects of 5-FU and Rosco on human colorectal cancer cell lines

Combined treatment of 5-FU and Rosco	Combination interaction at various durations of treatment in human colorectal cancer cells[1]								
	SW48			SW1116			SW837		
	2 d	4 d	6 d	2 d	4 d	6 d	2 d	4 d	6 d
5-FU 0.5 IC_{50} + Rosco 1.0 μg/mL	ant	ant	ant	syn	syn	syn	ant	ant	add
5-FU 1.0 IC_{50} + Rosco 15 μg/mL	ant	ant	ant	syn	syn	syn	ant	syn	add
5-FU 2.0 IC_{50} + Rosco 20 μg/mL	ant	ant	ant	syn	add	add	add	ant	ant
5-FU 3.0 IC_{50} + Rosco 25 μg/mL	ant	ant	add	ant	ant	ant	add	ant	ant

[1]The data are based on the mean of absorbance measurements of 3 independent experiments; ant: Antagonistic; add: Additive; syn: Synergistic.

cells were treated with Rosco alone, respectively, (Figure 3A, Table 3). Rosco (5 μg/mL) increased the sensitivity of SW1116 and SW837 to 5-FU by about 3.73 and 26.7 fold, respectively. The increase in SW1116 and SW837 growth inhibition after treatment with this combination was found to be statistically non-significant $P = 0.519$ and $P = 0.122$, respectively, compared to single treatment with 5-FU.

The combination of 5-FU (10^{-9}-10^{-4} mol/L) and Rosco (10 μg/mL) exerted a marked growth inhibition on all the tested colorectal cancer cells SW48 ($P < 0.0001$), SW1116 ($P = 0.05$) and SW837 ($P = 0.005$) with IC_{50} values equal to 5.7×10^{-9} mol/L, 1.0×10^{-7} mol/L and 9.1×10^{-9} mol/L, respectively, compared to IC_{50} values of 4.8×10^{-5} mol/L, 2.5×10^{-5} mol/L, and 4.8×10^{-5} mol/L exerted on SW 48, SW1116 and SW837, respectively, when treated with 5-FU alone (Figure 3A, Table 3). The combination of 5-FU (10^{-9}-10^{-4} mol/L) and Rosco (10 μg/mL) increased the sensitivity of human colorectal cancer cell lines SW48, SW1116 and SW837 by 8.42×10^{3}, 250 and 5.28×10^{3} fold, respectively (Table 3). The combination of 5-FU and Rosco had synergistic effects on SW1116, additive effects on SW837 and antagonistic/additive effects on SW48 (Table 4). The type of interaction between the mixed drugs depends upon their relative concentrations, exposure time and the tested cell line.

The effect of this combination on human colorectal caner cell cycle was also evaluated. Treatment with Rosco alone growth inhibited colorectal cancer cells in G_2/M phase (24.9% *vs* 4.75% for untreated), while treatment with 5-FU alone growth arrested cancer cells in S-phase (51.9% *vs* 31.5% for untreated). However the combination of 5-FU and Rosco added in sequential, inverted sequential and simultaneous manners growth arrested human colorectal cancer cells in S-phase: 54.8%, 56.8% and 50.7%, respectively, compared to 51.5% in the S-phase for untreated (Figure 3B). The combination of 5-FU (4.8×10^{-5} mol/L) and Rosco (15 μg/mL) had synergistic or additive apoptotic effects on SW1116 and SW837 compared to single treatment with 5-FU or Rosco (Figure 3C).

Modulation of doxorubicin cytotoxicity on human colorectal cancer cells by combination with CDKI Rosco

Human colorectal cancer cells were treated with doxorubicin (10^{-11}-10^{-6} mol/L) or combinations of doxorubicin (10^{-11}-10^{-6} mol/L) and Rosco (5 or 10 μg/mL) for 96 h. The combination of doxorubicin and Rosco had a very potent anti-proliferative effect on the colorectal cancer cell line SW48 ($IC_{50} = 5.8 \times 10^{-11}$ mol/L, $P = 0.009$) compared to the effect of doxorubicin alone ($IC_{50} = 5.4 \times 10^{-8}$ mol/L) (Figure 4A, Table 5). The combination of doxorubicin and 5 μg/mL Rosco slightly increased the growth inhibition exerted on SW1116 and SW837 with IC_{50} values equal to 2×10^{-7} mol/L and 4.1×10^{-8} mol/L, respectively, compared to IC_{50} values equal to 6×10^{-7} mol/L and 4.1×10^{-7} mol/L obtained when SW1116 and SW837 were treated with doxorubicin alone, respectively, (Figure 3A, Table 5). The combination of doxorubicin and 5 μg/mL Rosco increased the sensitivity of SW1116 (3 fold) and SW837 (10 fold) to doxorubicin (Table 5). The difference in growth inhibition produced by treatment of SW1116 ($P = 0.543$) and SW837 ($P = 0.33$) with doxorubicin plus 5 μg/mL was statistically non-significant. The combination of doxorubicin and Rosco (10 μg/mL) produced very potent anti-proliferative effects on SW48 ($IC_{50} = 5.8 \times 10^{-11}$ mol/L, $P = 0.012$), SW1116 ($IC50 = 4.5 \times 10^{-9}$ mol/L, $P = 0.068$), and SW837 ($IC_{50} = 8.2 \times 10^{-10}$ mol/L, $P = 0.049$) compared to treatment with Rosco alone (Figure 4A, Table 5). The combination of doxorubicin and Rosco exhibited an additive effect on SW48, synergistic and additive effects on SW1116 and synergistic effect on SW837 in a time and

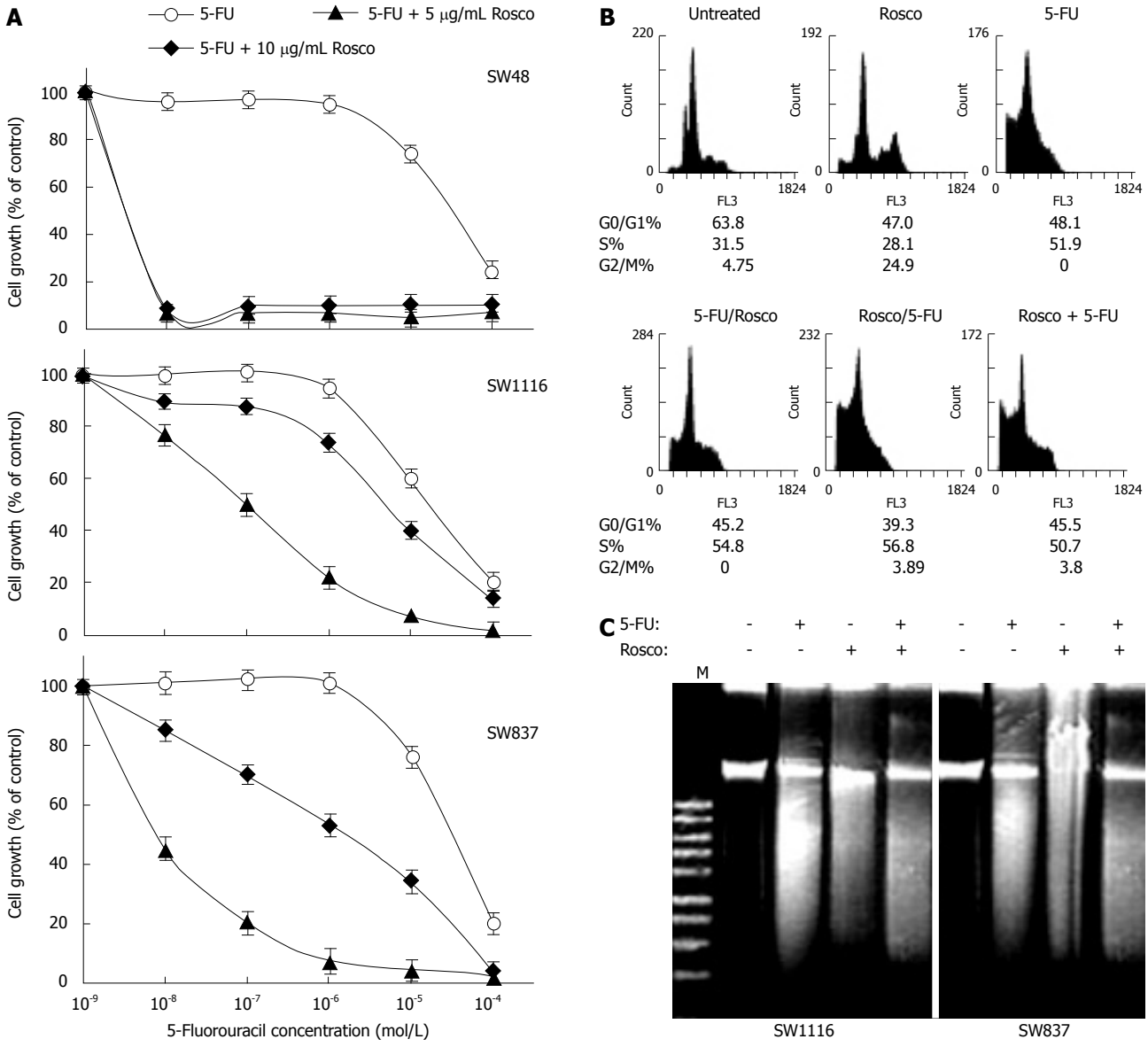


Figure 3 Potentiation of 5-FU anticancer effect on human colorectal cancer cell lines by combination with cyclin dependent kinase inhibitor roscovitine. **A**: Human colorectal cancer cell lines were treated with 5-FU (10^{-9}-10^{-4} mol/L), and the combination of 5-FU (10^{-9}-10^{-4} mol/L) and Rosco (5 μg/mL or 10 μg/mL) for 96 h. At the end of treatment, control and drug treated cells were scored for proliferation using the MTT assay; **B**: Cell cycle phase distribution of human colorectal cancer cells (SW837, 5×10^5 cells/well) treated with 5-FU (4.8×10^{-5} mol/L, 72 h); Rosco (15 μg/mL, 72 h); sequential combination: 5-FU (4.8×10^{-5} mol/L, 24 h) followed by Rosco (15 μg/mL, 48 h); inverted sequential combination: Rosco (15 μg/mL, 24 h) followed by 5-FU (4.8×10^{-5} mol/L, 48 h) and simultaneous combination: 5-FU plus Rosco (4.8×10^{-5} mol/L, 15 μg/mL, 72 h) was evaluated by flow cytometry. The percentage of cells in different phases of the cycle was calculated as described above; **C**: Human colorectal cancer cells, SW1116 and SW837 (5×10^5 cells/well), were treated with 5-FU (4.8×10^{-5} mol/L), Rosco (15 μg/L), and the combination of 5-FU plus Rosco (4.8×10^{-5} mol/L + 15 μg/mL) for 72 h. DNA fragments were extracted and analyzed on 1% agrose gel.

Table 5 IC_{50} and sensitization ratio of doxorubicin and combinations with Rosco towards human colorectal cancer cell lines

Treatment with doxorubicin and its combinations with Rosco	IC_{50} (mol/L)			Sensitization ratio[1]		
	SW48	SW1116	SW837	SW48	SW1116	SW837
Dox.	5.4×10^{-8}	6×10^{-7}	4.1×10^{-7}	-	-	-
Dox. + 5 μg/mL Rosco	5.8×10^{-11}	2×10^{-7}	4.1×10^{-8}	9.3×10^{2}	3	10
Dox. + 10 μg/mL Rosco	5.8×10^{-11}	4.5×10^{-9}	8.2×10^{-10}	9.3×10^{2}	1.3×10^{2}	5.0×10^{2}

[1]Sensitization ratio = IC_{50} (Dox.)/IC_{50} (Dox. + Rosco).

dose dependent manner (Table 6).

The effect of the combination on colorectal cancer cell cycle was also investigated. Treatment with Rosco growth arrested cancer cells in G_2/M (24.9% *vs* 4.75%

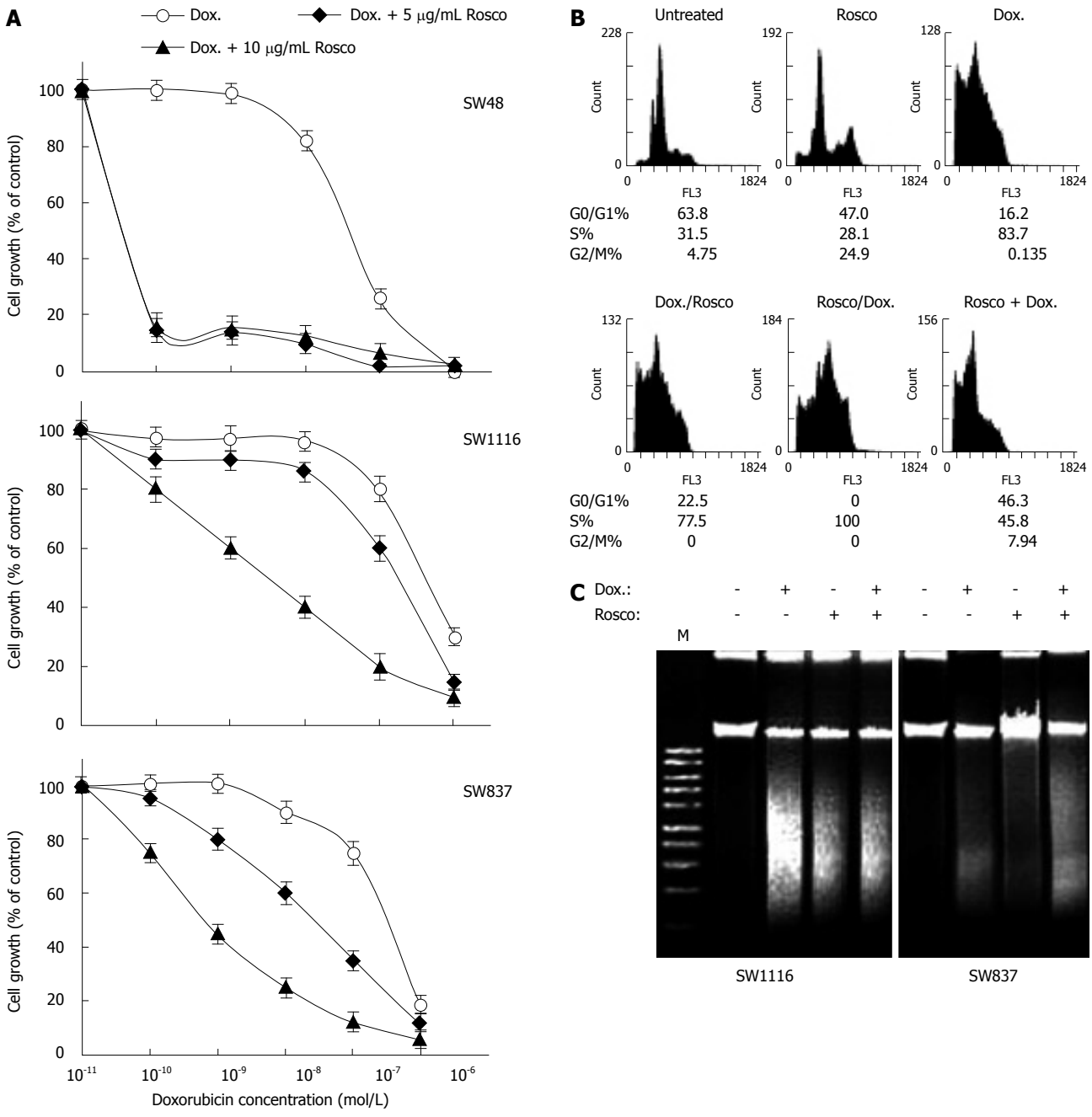


Figure 4 Potentiation of doxorubicin anticancer effect on human colorectal cancer cell lines by combination with cyclin dependent kinase inhibitor roscovitine. **A**: Human colorectal cancer cell lines were treated with doxorubicin (10^{-11}-10^{-6} mol/L), and the combination of doxorubicin (10^{-11}-10^{-6} mol/L) plus Rosco (5 μg/mL or 10 μg/mL) for 96 h. At the end of treatment, control and drug treated cells were scored for proliferation using an MTT assay; **B**: Cell cycle phase distribution of human colorectal cancer cells (SW837, 5 × 10^5 cells/well) treated with doxorubicin (8 × 10^{-7} mol/L, 72 h); Rosco (15 μg/mL, 72 h); sequential combination: doxorubicin (8 × 10^{-7} mol/L, 24 h) followed by Rosco (15 μg/mL, 48 h); inverted sequential combination: Rosco (15 μg/mL, 24 h) followed by doxorubicin (8 × 10^{-7} mol/L, 48 h) and simultaneous combination: doxorubicin plus Rosco (8 × 10^{-7} mol/L + 15 μg/mL, 72 h) was evaluated by flow cytometry. The percentage of cells in different phases of the cycle was calculated as previously described; **C**: Human colorectal cancer cells, SW1116 and SW837, (5 × 10^5 cells/well) were treated with doxorubicin (8 × 10^{-7} mol/L), Rosco (15 μg/mL), and the combination of doxorubicin plus Rosco (8 × 10^{-7} mol/L + 15 μg/mL) for 72 h. DNA fragments were extracted and analyzed on 1% agrose gel.

for untreated), while doxorubicin markedly growth arrested colorectal cancer cells in S-phase (83.7% *vs* 51.5% for untreated). The combination of doxorubicin and Rosco added to the culture in a sequential manner, i.e., doxorubicin followed by Rosco, growth arrested cancer cells in S-phase (77.5% *vs* 31.5% for untreated). The same combination added in an inverted sequential manner growth arrested cancer cells completely in S-phase (100% *vs* 31.5% for untreated). However, when the same combination was added in a simultaneous manner, the colorectal cancer cells were arrested in both S-phase (45.8% *vs* 31.5% for untreated) and G_2/M phase (7.94% *vs* 4.75% for untreated) (Figure 4B). The apoptosis inducing effect of the combination of doxorubicin (8 × 10^{-7} mol/L) and Rosco (15 μg/mL) was also tested. This combination enhanced apoptosis in SW1116 and SW837 compared to single treatment of doxorubicin and Rosco (Figure 4C).

Table 6 Analysis of the combined effects of doxorubicin and Rosco on human colorectal cancer cell lines

Combined treatment of doxorubicin and Rosco	Combination interaction at various durations of treatment in human colorectal cancer cells[1]								
	SW48			SW1116			SW837		
	2 d	4 d	6 d	2 d	4 d	6 d	2 d	4 d	6 d
Dox 0.5 IC_{50} + Rosco 1.0 μg/mL	ant	ant	ant	syn	syn	syn	syn	syn	syn
Dox 1.0 IC_{50} + Rosco 10 μg/mL	ant	ant	ant	syn	syn	syn	syn	syn	syn
Dox 2.0 IC_{50} + Rosco 15 μg/mL	ant	ant	ant	syn	syn	syn	syn	syn	syn
Dox 3.0 IC_{50} + Rosco 20 μg/mL	ant	add	add	syn	syn	add	syn	syn	add
Dox 4.0 IC_{50} + Rosco 25 μg/mL	ant	add	add	syn	ant	add	syn	ant	ant

[1]The data are based on the mean of absorbance measurements of 3 independent experiments; ant: Antagonistic; add: Additive; syn: Synergistic.

Table 7 IC_{50} and sensitization ratio of vinblastine and its combinations with Rosco towards human colorectal cancer cell lines

Treatment with vinblastine and combinations with Rosco	IC_{50} (mol/L)			Sensitization ratio[1]		
	SW48	SW1116	SW837	SW48	SW1116	SW837
Vinb.	1×10^{-8}	6.3×10^{-7}	4.2×10^{-7}	-	-	-
Vinb. + 5 μg/mL Rosco	5.4×10^{-11}	7.5×10^{-8}	5.0×10^{-8}	1.9×10^{2}	8.4	8.4
Vinb. + 10 μg/mL Rosco	5.4×10^{-11}	7.9×10^{-10}	4.6×10^{-10}	1.9×10^{2}	8.0×10^{2}	9.1×10^{2}

[1]Sensitization ratio = IC_{50} (Vinb.)/IC_{50} (Vinb. + Rosco).

Modulation of vinblastine cytotoxicity on human colorectal cancer cells by combination with CDKI Rosco

The ability of cyclin dependent kinase inhibitor Rosco to sensitize human colorectal cancer cells to vinblastine was evaluated. Treatment of cancer cells with a combination of vinblastine (10^{-12}-10^{-7} mol/L) and Rosco (5 or 10 μg/mL) dramatically growth inhibited SW48 cells ($P = 0.023$ or $P = 0.019$) with $IC_{50} = 5.4 \times 10^{-11}$ mol/L compared to $IC_{50} = 1.0 \times 10^{-8}$ mol/L for the effect of vinblastine alone (Figure 5). These results clearly indicate that Rosco (5 or 10 μg/mL) sensitized SW48 cells to vinblastine anticancer activity by 190 fold (Table 7). Treatment of SW1116 with the combination of vinblastine and Rosco (5 μg/mL) produced a higher growth inhibition of SW1116 ($IC_{50} = 7.5 \times 10^{-8}$ mol/L, sensitization ratio = 8.4 fold) (Figure 5A, Table 7) compared to treatment with vinblastine alone ($IC_{50} = 6.3 \times 10^{-7}$ mol/L). The increase in the growth inhibition induced by mixing vinblastine with 5 μg/mL of Rosco was found to be statistically non-significant ($P = 0.216$). On the other hand, treatment of SW1116 with vinblastine and 10 μg/mL Rosco markedly enhanced the cytotoxicity of vinblastine ($IC_{50} = 7.9 \times 10^{-10}$ mol/L, $P = 0.013$) towards SW1116 demonstrating a great increase in the chemo-sensitization of SW1116 (8×10^{2}-fold) to vinblastine (Table 7). Similar results were obtained with the colorectal cancer cell line SW837. The combination of vinblastine and Rosco (5 μg/mL) slightly increased the growth inhibition of SW837 ($IC_{50} = 5 \times 10^{-8}$ mol/L, sensitization ratio = 8.4) compared to single treatment with vinblastine ($IC_{50} = 4.2 \times 10^{-7}$ mol/L) (Table 7). However, the combination of vinblastine and 10 μg/mL Rosco exerted significant growth inhibition of SW837 cells ($IC_{50} = 4.6 \times 10^{-10}$ mol/L, $P = 0.023$) compared to treatment with vinblastine alone ($IC_{50} = 4.2 \times 10^{-7}$ mol/L) demonstrating a marked increase in the sensitivity of SW837 (9.1×10^{2}-fold) to vinblastine.

The combination of vinblastine ($2IC_{50}$) and Rosco (15 μg/mL) had synergistic effect on SW48 after 4 d of combination treatment (Table 8). All the tested combinations had additive or synergistic effects on SW1116 after 2 d of treatment. The combination of vinblastine (IC_{50}) and Rosco (10 μg/mL) had additive and synergistic effects on SW1116 and SW837 after 4 and 6 d of treatment, respectively (Table 8).

The effects of single and combined treatment with vinblastine (2.6×10^{-7} mol/L) and Rosco (15 μg/mL) on colorectal cancer cell cycle distribution indicated that treatment of SW837 with Rosco growth inhibited colorectal cancer cells in G_2/M (24.9% *vs* 4.75% for untreated), while, vinblastine treatment markedly growth arrest these cells in G_2/M (71.9% *vs* 4.75% for untreated). The combination of vinblastine (2.6×10^{-7} mol/L) and Rosco (15 μg/mL) added in sequential manner (vinblastine followed by Rosco) greatly growth arrested SW837 cells in G_2/M (76.6% *vs* 4.75% for untreated). The same combination added in an inverted sequential manner markedly growth arrested the cells in G_2/M (85.7% *vs* 4.75% for untreated). The same combination added simultaneously to SW837 cells growth arrested these cells both in S- (44.6% *vs* 31.6% for untreated) and G_2/M-(19.3% *vs* 4.75% for untreated) phases (Figure 5B). The combination of vinblastine (2.6×10^{-7} mol/L) and Rosco (15 μg/mL) had a marked apoptotic effect on colorectal cancer cell lines SW1116 and SW837 compared to single treatments with vinblastine (2.6×10^{-7} mol/L) or Rosco (15 μg/mL) (Figure 5C).

DISCUSSION

The central finding of the present study is that the

Table 8 Analysis of the combined effects of vinblastine and Rosco on human colorectal cancer cell lines

Combined treatment of vinblastine and Rosco	Combination interaction at various durations of treatment in human colorectal cancer cells[1]								
	SW48			SW1116			SW837		
	2 d	4 d	6 d	2 d	4 d	6 d	2 d	4 d	6 d
Vinb 1.0 IC_{50} + Rosco 10 μg/mL	ant	ant	ant	add	add	add	ant	syn	syn
Vinb 2.0 IC_{50} + Rosco 15 μg/mL	ant	syn	ant	syn	ant	ant	ant	ant	ant
Vinb 3.0 IC_{50} + Rosco 20 μg/mL	ant	ant	ant	syn	ant	ant	ant	ant	ant
Vinb 4.0 IC_{50} + Rosco 25 μg/mL	ant	ant	ant	syn	ant	ant	ant	ant	ant

[1]The data are based on the mean of absorbance measurements of 3 independent experiments; ant: Antagonistic; add: Additive; syn: Synergistic.

cyclin dependent kinase inhibitor Rosco improved the therapeutic activity of several conventional chemotherapeutic drugs namely taxol, doxorubicin, 5-FU, and vinblastine that act by different mechanisms in human colorectal cancer cells. This finding is significant because chemotherapeutic drugs cause high toxicity to normal tissues during treatment of colorectal cancer as well as other cancers.

The adverse health effects of the conventional chemotherapeutic drugs, such as immunosuppression and cardiomyopathy, which severely increase in a dose-dependent manner, as well as development of primary or secondary drug resistance in tumor cells, limit their clinical success in cancer chemotherapy[15]. The increase in systemic toxicity and drug resistance, the major drawbacks of anticancer chemotherapeutic agents, has led to a new challenge in the field of cancer research. To overcome such problems, extensive research has been directed towards reducing systemic toxicity and increasing drug activity in cancer therapy[15,16]. In this regard, combination chemotherapy has received increasing attention in the search for compounds that could increase the therapeutic index of clinical anticancer drugs[17].

Deregulation of the cell cycle and oncogenic overexpression of several cell cycle related gene products in many human cancers provide new opportunities for anticancer drug discovery. Efforts to exploit these targets are progressing quite well, with inhibition of cyclin dependent kinase activity emerging as the most productive approach at present. This has resulted in the development of several small molecules, with specific and potent CDK inhibitory effects, which are now undergoing clinical trials in phases Ⅰ and Ⅱ, and the results awaited with expectation.

CDKs are essential players in the intracellular control of the cell cycle**.** Since CDKs and their regulatory partners are frequently deregulated and exhibit enhanced activity in human cancers, their inhibition by selectively acting drugs offers a new concept in the therapeutic strategy[5,18]. Recently, a number of pharmacological inhibitors of CDKs were developed, one efficient group of such compounds is based on the substitution of purines and pyrimidines. Substituted purines represent CDK inhibitors that are structurally most similar to ATP[7]. Among a series of C2, N6, N9-substituted adenines, Rosco displays high efficiency and selectivity towards some CDKs. Out of 25 kinases investigated, only a few were significantly inhibited by Rosco with IC_{50} values lower than 1 μmol/L. CDK2/cyclin B and CDK2/cyclin A were identified as the best targets[8]. Through its high selectivity, Rosco is predestinated to be a potent anti-mitotic drug. It acts not only as a cell cycle blocker[19-22], but seems also to induce apoptosis[11,22-24].

There is still a lack of systematic knowledge about the cytotoxic effects of Rosco on normal and malignant cells. The exact discrimination between inhibition of cell proliferation and impairment of cell *via*bility is necessary. Moreover, the pro- or anti-apoptotic action of Rosco on different cells is until now contradictory and has to be conscientiously examined. The consequences of Rosco-induced cell cycle arrest and apoptosis in colorectal cancer cells upon chemo-sensitization of such cells to conventional therapeutic drugs have not yet been investigated. In this study, we addressed the ability of Rosco to synergize with conventional chemotherapeutic drugs, acting by different mechanisms, to induce efficient apoptosis in human colorectal cancer cell lines.

In the present study, human colorectal cancer cells were shown to be sensitive to the antiproliferative and cytotoxic effects of Rosco with IC_{50} values: (11.56-25 μg/mL), (11.62-14.78 μg/mL) and (13.44-16.25 μg/mL) for SW48, SW1116 and SW837, respectively, after 24-144 h, 72-144 h and 48-144 h of Rosco treatment, respectively. These results are consistent with those reported for other cell types[8,25]. To explore whether inhibition of cell growth observed in the Rosco treated human colorectal cancer cells synergize with the conventional chemotherapeutic drugs acting by different mechanisms to induce efficient apoptosis of human colorectal cancer cells, we tested the efficacy of single and combined treatments with Rosco and taxol, doxorubicin, 5-FU or vinblastine on the growth of human colorectal cancer cells. Our results indicated that Rosco (5 and 10 μg/mL) markedly sensitized the tested human colorectal cancer cells to taxol (sensitization ratio = 7.0-6.4 × 10^2), doxorubicin (sensitization ratio = 3.0-9.3 × 10^2), 5-FU (sensitization ratio = 3.73-8.42 × 10^3) and vinblastine (sensitization ratio = 9-8 × 10^3) (Tables 1, 3, 5 and 7). The combination of Rosco and chemotherapeutic drugs inhibited the growth of human colorectal cancer cells in an additive or synergistic fashion, and in a time- and dose-dependent manner (Tables 2, 4, 6 and 8). Treatment of human colorectal cancer cells with Rosco, conventional chemotherapeutic drugs or the combination of Rosco and conventional chemotherapeutic drugs add-

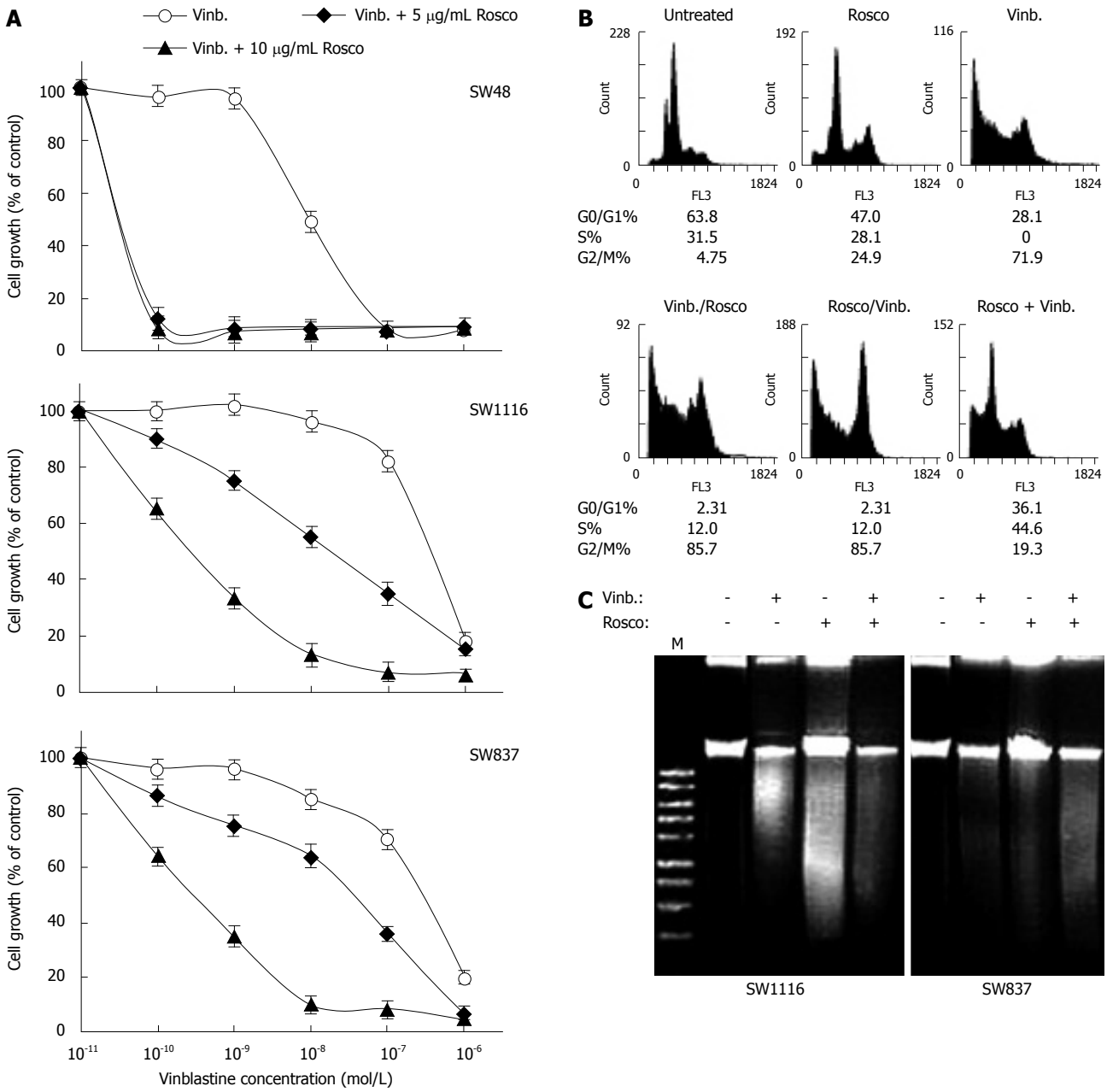


Figure 5 Potentiation of vinblastine anticancer effect on human colorectal cancer cell lines by combination with cyclin dependent kinase inhibitor roscovitine. **A**: Human colorectal cancer cell lines were treated with vinblastine (10^{-12}-10^{-7} mol/L) and the combination of vinblastine (10^{-12}-10^{-7} mol/L) plus Rosco (5 μg/mL or 10 μg/mL) for 96 h. At the end of treatment, control and drug-treated cells were scored for proliferation using an MTT assay; **B**: Cell cycle phase distribution of human colorectal cancer cells (SW837, 5 x 10^5 cells/well) treated with vinblastine (2.6 x 10^{-7} mol/L, 72 h); Rosco (15 μg/mL, 72 h); sequential combination: vinblastine (2.6 x 10^{-7} mol/L, 24 h) followed by Rosco (15 μg/mL, 48 h); inverted sequential combination: Rosco (15 μg/mL, 24 h) followed by vinblastine (2.6 x 10^{-7} mol/L, 48 h) and simultaneous combination: vinblastine plus Rosco (2.6 x 10^{-7} mol/L + 15 μg/mL, 72 h) was evaluated by flow cytometry. The percentage of cells in different phases of the cycle was calculated as described above; **C**: Human colorectal cancer cells, SW1116 and SW837, (5 x 10^5 cells/well) were treated with vinblastine (2.6 x 10^{-7} mol/L), Rosco (15 μg/mL) and the combination of vinblastine plus Rosco (2.6 x 10^{-7} mol/L + 15 μg/mL) for 72 h. DNA fragments were extracted and analyzed on 1% agrose gel.

ed in a sequential or inverted sequential manner growth arrested colorectal cancer cells in G_2/M- or S- phase of the cell cycle. While, simultaneous addition of Rosco and conventional chemotherapeutic drugs double blocked colorectal cancer cells in G_2/M- and S- phases of the cell cycle for all the tested drugs except for 5-FU, where, its combination with Rosco growth arrested colorectal cancer cells in S-phase. The growth arrest of colorectal cancer cells in G_2/M following Rosco treatment may facilitate the induction of apoptosis and sensitize the cells to conventional chemotherapeutic drugs.

Another notable observation from our morphological analysis was the extensive detachment of cells from the cell culture substratum after exposure to Rosco (data not shown). Recent evidence suggests that cellular attachment to the substratum is mediated by the interaction of integrins with ECM components such as fibronectin, collagen, and vitronectin[26]. Binding of integrins to these adhesion molecules results in the activation of focal adhesion kinase[27] accompanied by phosphorylation and recruit-

ment of a number of related cytoskeletal and signaling molecules, thereby transducing anchorage and survival messages to the nucleus[28,29]. Conversely, the uncoupling of integrins from ECM proteins leads to disruption of integrin-mediated signal transduction, inactivation of focal adhesion kinase, detachment of cells from the ECM, and apoptotic cell death[30]. Our data suggest that following Rosco treatment, human colorectal cancer cells detach from cell culture substratum, and die *via* apoptosis as indicated by the DNA fragmentation assay. This notion is consistent with previous reports demonstrating that cells deprived of matrix attachment underwent apoptosis[31]. Thus, the extensive detachment of cells from the cell culture substratum, and the apoptotic cell death observed in our experimental system may be due to the uncoupling of integrin-mediated signaling and/or disruption of cell-matrix interactions induced by Rosco. In addition to facilitating apoptosis, which will have its impact on chemosensitization of human colorectal cancer cells to conventional chemotherapeutic drugs, the loss of adhesion induced by this CDK inhibitor may deny cell anchorage and traction necessary for growth and migration and thus prevent colorectal cancer invasion and metastasis, the major cause of death in colorectal cancer patients. Because adhesion and invasion are crucial to the initiation of metastatic growth[32], additional studies on the effect of Rosco on cell adhesion to extracellular matrix components as well as the anti-invasive potential of the CDK inhibitor could be extremely rewarding. These studies are currently ongoing in our laboratory.

Rosco may prevent the assembly of actin fibers by modulating the expression and/or activity of Rho GTPases, which have been reported to be involved in the regulation of actin microfilament organization and other associated activities[33]. Disruption of actin microfilament architecture by Rosco has some biological implications. In view of the role played by actin microfilaments in various aspects of cellular physiology such as cell-cell interaction, proliferation, and secretion[33], it can be argued that all of these cellular activities could be affected in colorectal tumors following Rosco treatment.

Rosco strongly up-regulates wt p53 protein in cancer cells[19,20,22,34]. Since p53 protein plays a pivotal role in the regulation of cell cycle, the biological effect of Rosco cannot be restricted to the direct inhibition of distinct kinases. Considering the multiple p53 targets and functions, it is obvious that the Rosco-induced upregulation of p53 in cancer cells may essentially contribute to the cell cycle arrest, chromatin silencing and initiation as well as execution of apoptosis.

The role of CKDs in chemosensitization, and the potential downstream effectors of CDKs inhibition have been investigated by Crescenzi *et al*[5]. They showed that lung adenocarcinoma cell line H1299 treated with a nontoxic concentration of Rosco renders H1299 cells significantly more susceptible to doxorubicin or etoposide. In these cells, Rosco does not modulate senescence, but markedly reduces the capacity of H1299 cells to repair damage and resume proliferation after treatment. Combined treatment with Rosco and doxorubicin, or etoposide was found to enhance G_2-M accumulation, to increase the amount of γ-H2AX foci and to inhibit DNA repair. Two main repair pathways, homologous recombination and NHEJ, cooperate to repair DNA DSBs[35]. Crescenzi *et al*[5] investigated the ability of Rosco to modulate those two processes in doxorubicin-treated cells. They reported the ability of Rosco to negatively modulate DNA-PK activity in H1299 cells[5,36] and showed that Rosco significantly reduces the efficiency of recombination repair identifying a novel mechanism of action by which Rosco affects tumor cells that is inhibition of DNA DSBs repair.

The role of CDK2 and CDK1 kinases as targets for Rosco in tumor chemosensitization has also been investigated by Crescenzi *et al*[5]. In this study, experiments with inducible dn-K2 clones indicated that loss of Cdk2 and Cdk1 activity was responsible for the chemosensitizing effect of Rosco Overexpression of dn-K2 in H1299 cells potentiates doxorubicin-induced G_2-M arrest and inhibited recovery of the cells after treatment. It is worth noting that overexpression of dn-K2 results in both Cdk2 and Cdk1 inhibition[37]. Furthermore, analyses of homologous recombination in Hela cells transiently overexpressing either dn-K2 or dn-K1 or Cdk2 confirmed a role for Cdks in modulation of DNA repair processes[5]. A role for CDK in the control of DNA repair pathways has also been studied in the yeast cells[38,39]. Combined treatment of Rosco and DNA-damaging agents not only enhances drug-induced apoptosis, but also effectively hampers the recovery of mildly damaged tumor cells after treatment. Rosco, by hindering both homologous recombination and NHEJ repair processes, has the potential to inhibit recovery of mildly damaged tumor cells after chemotherapeutic drug treatment, and to increase the susceptibility of tumor cells to chemotherapy. Our results clearly indicated that Rosco synergizes with chemotherapeutic drugs to induce efficient apoptosis of human colorectal cancer cells. Important issues that need to be addressed in order to advance these agents to the clinical arena include the best drug administration schedule, testing various combinations with standard chemotherapeutic agents, the best tumor types to be targeted, and demonstration of CDK modulation of tumor samples from cancer patients.

COMMENTS

Background

Cyclin-dependent kinases (CDKs) are serine/threonine kinases that play a key role in regulating cell cycle progression. Aberrant expression or altered activity of distinct CDK complexes results in escape of cells from the cell cycle control and leads to malignant transformation. Therefore, inhibition of CDKs in malignant cells provides a new strategy in the fight against cancer. The present study examined the ability of roscovitine (Rosco), a CDK inhibitor (CDKI), to enhance the anticancer effects of chemotherapeutic drugs that act by different mechanisms on human colorectal cancer cells. The authors have also investigated whether Rosco differentially affects the cell cycle distribution of drug-treated human colorectal cancer cells.

Research frontiers

Extensive research has been directed towards reducing systemic toxicity and

increasing drug activity in cancer therapy. Combination chemotherapy has received increasing attention in the search for compounds that could increase the therapeutic index of clinical anticancer drugs. This study indicated that combinations of agents directed at different pathways or different steps of pathways involved in apoptosis can cause the cells to reach an apoptosis threshold resulting in synergistic apoptosis and increased therapeutic index of the anticancer drugs.

Innovations and breakthroughs

The central finding of this study is that the cyclin dependent kinase inhibitor Rosco improved the therapeutic activity of several conventional drugs namely taxol, 5-fluorouracil (5-FU), doxorubicin and vinblastine that act by different mechanisms. Also, Rosco differentially affected the cell cycle distribution of drug-treated colorectal cancer cells.

Applications

Chemotherapeutic drugs are highly toxic to normal tissues during treatment of colorectal cancer as well as other cancers. Rosco increases the sensitivity of several conventional chemotherapeutic drugs namely taxol, doxorubicin, 5-FU, and vinblastine. This finding is significant because increasing drug activity may reduce systemic toxicity in cancer therapy.

Terminology

CDKIs are a heterogeneous group of compounds that are able to inhibit CDKs involved in the cell cycle, transcription or neuronal functions. CDKIs are a chemically diverse, flat, hydrophobic heterocycles that compete with ATP. Rosco (CDKI) is structurally related to ATP, it blocks the cell cycle and induces apoptosis.

Peer review

A tremendous amount of *in vitro* work has been done looking at the anti colorectal cancer cell effect of Rosco, a novel CDK inhibitor, to enhance the antitumor effects of conventional chemotherapy agents. The approach is very well designed and the presentation of data is very detailed. It looks promising and may yield clinical benefits in the future.

REFERENCES

1 **Greenlee RT**, Murray T, Bolden S, Wingo PA. Cancer statistics, 2000. *CA Cancer J Clin* 2000; **50**: 7-33
2 **Nurse P**. Cyclin dependent kinases and cell cycle control (nobel lecture). *Chembiochem* 2002; **3**: 596-603
3 **Hall M**, Peters G. Genetic alterations of cyclins, cyclin-dependent kinases, and Cdk inhibitors in human cancer. *Adv Cancer Res* 1996; **68**: 67-108
4 **Loda M**, Cukor B, Tam SW, Lavin P, Fiorentino M, Draetta GF, Jessup JM, Pagano M. Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. *Nat Med* 1997; **3**: 231-234
5 **Crescenzi E**, Palumbo G, Brady HJ. Roscovitine modulates DNA repair and senescence: implications for combination chemotherapy. *Clin Cancer Res* 2005; **11**: 8158-8171
6 **Meijer L**. Chemical inhibitors of cyclin-dependent kinases. *Prog Cell Cycle Res* 1995; **1**: 351-363
7 **Gray N**, Detivaud L, Doerig C, Meijer L. ATP-site directed inhibitors of cyclin-dependent kinases. *Curr Med Chem* 1999; **6**: 859-875
8 **Meijer L**, Borgne A, Mulner O, Chong JP, Blow JJ, Inagaki N, Inagaki M, Delcros JG, Moulinoux JP. Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. *Eur J Biochem* 1997; **243**: 527-536
9 **Edamatsu H**, Gau CL, Nemoto T, Guo L, Tamanoi F. Cdk inhibitors, roscovitine and olomoucine, synergize with farnesyltransferase inhibitor (FTI) to induce efficient apoptosis of human cancer cell lines. *Oncogene* 2000; **19**: 3059-3068
10 **Mgbonyebi OP**, Russo J, Russo IH. Roscovitine induces cell death and morphological changes indicative of apoptosis in MDA-MB-231 breast cancer cells. *Cancer Res* 1999; **59**: 1903-1910
11 **De Azevedo WF**, Leclerc S, Meijer L, Havlicek L, Strnad M, Kim SH. Inhibition of cyclin-dependent kinases by purine analogues: crystal structure of human cdk2 complexed with roscovitine. *Eur J Biochem* 1997; **243**: 518-526
12 **Abaza MS**, Al-Attiyah RJ, Al-Saffar AM, Al-Sawan SM, Moussa NM. Antisense oligodeoxynucleotide directed against c-myb has anticancer activity and potentiates the antiproliferative effect of conventional anticancer drugs acting by different mechanisms in human colorectal cancer cells. *Tumour Biol* 2003; **24**: 241-257
13 **Lam PK**, To EW, Chan ES, Liew CT, Lung IW, King WK. In vitro inhibition of head and neck cancer-cell growth by human recombinant interferon-alpha and 13-cis retinoic acid. *Br J Biomed Sci* 2001; **58**: 226-229
14 **Fan XM**, Wong BC, Wang WP, Zhou XM, Cho CH, Yuen ST, Leung SY, Lin MC, Kung HF, Lam SK. Inhibition of proteasome function induced apoptosis in gastric cancer. *Int J Cancer* 2001; **93**: 481-488
15 **Raghavan D**, Koczwara B, Javle M. Evolving strategies of cytotoxic chemotherapy for advanced prostate cancer. *Eur J Cancer* 1997; **33**: 566-574
16 **Figg WD**, Arlen P, Gulley J, Fernandez P, Noone M, Fedenko K, Hamilton M, Parker C, Kruger EA, Pluda J, Dahut WL. A randomized phase II trial of docetaxel (taxotere) plus thalidomide in androgen-independent prostate cancer. *Semin Oncol* 2001; **28**: 62-66
17 **Millikan R**, Baez L, Banerjee T, Wade J, Edwards K, Winn R, Smith TL, Logothetis C. Randomized phase 2 trial of ketoconazole and ketoconazole/doxorubicin in androgen independent prostate cancer. *Urol Oncol* 2001; **6**: 111-115
18 **Blagosklonny MV**, Darzynkiewicz Z, Figg WD. Flavopiridol inversely affects p21(WAF1/CIP1) and p53 and protects p21-sensitive cells from paclitaxel. *Cancer Biol Ther* 2002; **1**: 420-425
19 **David-Pfeuty T**. Potent inhibitors of cyclin-dependent kinase 2 induce nuclear accumulation of wild-type p53 and nucleolar fragmentation in human untransformed and tumor-derived cells. *Oncogene* 1999; **18**: 7409-7422
20 **David-Pfeuty T**, Nouvian-Dooghe Y, Sirri V, Roussel P, Hernandez-Verdun D. Common and reversible regulation of wild-type p53 function and of ribosomal biogenesis by protein kinases in human cells. *Oncogene* 2001; **20**: 5951-5963
21 **Vitali L**, Yakisich JS, Vita MF, Fernandez A, Settembrini L, Siden A, Cruz M, Carminatti H, Casas O, Idoyaga Vargas V. Roscovitine inhibits ongoing DNA synthesis in human cervical cancer. *Cancer Lett* 2002; **180**: 7-12
22 **Wojciechowski J**, Horky M, Gueorguieva M, Wesierska-Gadek J. Rapid onset of nucleolar disintegration preceding cell cycle arrest in roscovitine-induced apoptosis of human MCF-7 breast cancer cells. *Int J Cancer* 2003; **106**: 486-495
23 **Mgbonyebi OP**, Russo J, Russo IH. Roscovitine induces cell death and morphological changes indicative of apoptosis in MDA-MB-231 breast cancer cells. *Cancer Res* 1999; **59**: 1903-1910
24 **Yakisich JS**, Boethius J, Lindblom IO, Wallstedt L, Vargas VI, Siden A, Cruz MH. Inhibition of DNA synthesis in human gliomas by roscovitine. *Neuroreport* 1999; **10**: 2563-2567
25 **Mihara M**, Shintani S, Kiyota A, Matsumura T, Wong DT. Cyclin-dependent kinase inhibitor (roscovitine) suppresses growth and induces apoptosis by regulating Bcl-x in head and neck squamous cell carcinoma cells. *Int J Oncol* 2002; **21**: 95-101
26 **Wen LP**, Fahrni JA, Troie S, Guan JL, Orth K, Rosen GD. Cleavage of focal adhesion kinase by caspases during apoptosis. *J Biol Chem* 1997; **272**: 26056-26061
27 **Haimovich B**, Lipfert L, Brugge JS, Shattil SJ. Tyrosine phosphorylation and cytoskeletal reorganization in platelets are triggered by interaction of integrin receptors with their immobilized ligands. *J Biol Chem* 1993; **268**: 15868-15877
28 **Leventhal PS**, Shelden EA, Kim B, Feldman EL. Tyrosine phosphorylation of paxillin and focal adhesion kinase during insulin-like growth factor-I-stimulated lamellipodial advance. *J Biol Chem* 1997; **272**: 5214-5218
29 **Marushige Y**, Marushige K. Alterations in focal adhesion and cytoskeletal proteins during apoptosis. *Anticancer Res* 1998; **18**: 301-307

30 **Fukai F**, Mashimo M, Akiyama K, Goto T, Tanuma S, Katayama T. Modulation of apoptotic cell death by extracellular matrix proteins and a fibronectin-derived antiadhesive peptide. *Exp Cell Res* 1998; **242**: 92-99
31 **Frisch SM**, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. *J Cell Biol* 1994; **124**: 619-626
32 **Wong NC**, Mueller BM, Barbas CF, Ruminski P, Quaranta V, Lin EC, Smith JW. Alphav integrins mediate adhesion and migration of breast carcinoma cell lines. *Clin Exp Metastasis* 1998; **16**: 50-61
33 **Hall A**. Rho GTPases and the actin cytoskeleton. *Science* 1998; **279**: 509-514
34 **Wesierska-Gadek J**, Schmid G. Dual action of the inhibitors of cyclin-dependent kinases: targeting of the cell-cycle progression and activation of wild-type p53 protein. *Expert Opin Investig Drugs* 2006; **15**: 23-38
35 **Jackson SP**. Sensing and repairing DNA double-strand breaks. *Carcinogenesis* 2002; **23**: 687-696
36 **Maggiorella L**, Deutsch E, Frascogna V, Chavaudra N, Jeanson L, Milliat F, Eschwege F, Bourhis J. Enhancement of radiation response by roscovitine in human breast carcinoma in vitro and in vivo. *Cancer Res* 2003; **63**: 2513-2517
37 **Hu B**, Mitra J, van den Heuvel S, Enders GH. S and G2 phase roles for Cdk2 revealed by inducible expression of a dominant-negative mutant in human cells. *Mol Cell Biol* 2001; **21**: 2755-2766
38 **Ira G**, Pellicioli A, Balijja A, Wang X, Fiorani S, Carotenuto W, Liberi G, Bressan D, Wan L, Hollingsworth NM, Haber JE, Foiani M. DNA end resection, homologous recombination and DNA damage checkpoint activation require CDK1. *Nature* 2004; **431**: 1011-1017
39 **Aylon Y**, Liefshitz B, Kupiec M. The CDK regulates repair of double-strand breaks by homologous recombination during the cell cycle. *EMBO J* 2004; **23**: 4868-4875

S- Editor Li DL **L- Editor** Alpini GD **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5176

World J Gastroenterol 2008 September 7; 14(33): 5176-5185
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Enhanced therapeutic effects for human pancreatic cancer by application K-ras and IGF-IR antisense oligodeoxynucleotides

Yong-Mei Shen, Xiao-Chun Yang, Chen Yang, Jun-Kang Shen

Yong-Mei Shen, Radioimmunoassay Center & Clinical Laboratory, the Second Affiliated Hospital of Soochow University, Suzhou 215004, Jiangsu Province, China
Xiao-Chun Yang, Jun-Kang Shen, Department of MRI, the Second Affiliated Hospital of Soochow University, Suzhou 215004, Jiangsu Province, China
Chen Yang, Radioimmunoassay Center, the Municipal Hospital of Suzhou, Suzhou 215002, Jiangsu Province, China
Author contributions: Shen YM, Yang XC and Yang C contributed equally to this work; Shen YM, Yang XC, Yang C designed research and performed research; Shen YM, Yang XC, Yang C and Shen JK analyzed data; and Shen YM and Yang XC wrote the paper.
Supported by Social development foundation of Suzhou, China, No. SZD0614; Young teacher foundation of Soochow University; Foundation of health department of Jiangsu Province, China, No. Z200622
Correspondence to: Dr. Xiao-Chun Yang, Department of MRI, the Second Affiliated Hospital of Soochow University, Suzhou 215004, Jiangsu Province, China. szyxc@yahoo.com.cn
Telephone: +86-512-67783460 Fax: +86-512-68284303
Received: April 9, 2008 Revised: July 21, 2008
Accepted: July 28, 2008
Published online: September 7, 2008

Abstract

AIM: To investigate the combined effects of K-ras antisense oligodeoxynucleotide (K-ras ASODN) specific to GTT point mutation at codon 12 and type I insulin-like growth factor receptor (IGF-IR) antisense oligodeoxynucleotide (IGF-IR ASODN) on proliferation and apoptosis of human pancreatic cancer Patu8988 cells *in vitro* and *in vivo*.

METHODS: *K-ras* gene point mutation and its style at codon 12 of human pancreatic cancer cell line Patu8988 were detected by using polymerase chain reaction with special sequence primers (PCR-SSP) and sequence analysis. According to the mutation style, K-ras mutation ASODN specific to K-ras point mutation at codon 12 was designed and composed. After K-ras ASODN and IGF-IR ASODN treated on Patu8988 cells respectively or cooperatively, the proliferation and morphological change of Patu8988 cells were analyzed by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, colony forming assay and transmission electron microscopy; the expression of K-ras and IGF-IR mRNA and protein in the treated cells was measured by reverse-transcript polymerase chain reaction (RT-PCR) and flow cytometry respectively; apoptosis was determined by flow cytometry. The combined antitumor activity of K-ras ASODN and IGF-IR ASODN was evaluated in BALB/c nude mice bearing human pancreatic cancer inoculated with Patu8988 cells.

RESULTS: The results of PCR-SSP and sequence analysis showed that the human pancreatic cancer cell line Patu8988 had point mutation at codon 12, and the mutation style was GGT→GTT. 2-32 μg/mL K-ras ASODN and 2-32 μg/mL IGF-IR ASODN could inhibit Patu8988 cells' growth, induce apoptosis and decrease the expression of K-ras and IGF-IR mRNA and protein alone. However, there was much more effective inhibition of growth and induction of apoptosis by their combination than by each one alone. In tumor bearing mice, the combination of K-ras ASODN and IGF-IR ASODN showed a significant inhibitory effect on the growth of transplanted pancreatic cancer, resulting in a statistically significant difference compared with each alone.

CONCLUSION: It has been found that K-ras ASODN combined with IGF-IR ASODN could cooperatively inhibit the growth of Patu8988 cells, and induce their apoptosis *via* reinforcing specific down regulation of K-ras and IGF-IR mRNA and protein expression.

Key words: Pancreatic cancer; Antisense oligodeoxynucleotide; K-ras; Type I insulin-like growth factor receptor; Patu8988

Peer reviewer: Minoti Vivek Apte, Associate Professor, Pancreatic Research Group, South Western Sydney Clinical School, the University of New South Wales, Level 2, Thomas and Rachel Moore Education Centre, Liverpool Hospital, New South Wales 2170, Liverpool, Australia

Shen YM, Yang XC, Yang C, Shen JK. Enhanced therapeutic effects for human pancreatic cancer by application K-ras and IGF-IR antisense oligodeoxynucleotides. *World J Gastroenterol* 2008; 14(33): 5176-5185 Available from: URL: http://www.wjgnet.com/1007-9327/14/5176.asp DOI: http://dx.doi.org/10.3748/wjg.14.5176

INTRODUCTION

Pancreatic cancer is the fatal cancer of the digestive system with the worst prognosis. The 5-year survival rate is approximately 1%-2%, and the median survival time after diagnosis ranges only 4-6 mo[1-3]. The reasons for poor prognosis include: (1) the difficulty of early diagnosis due to its anatomical location, and lack of specific early syndromes; (2) the high potential to infiltrate to the surrounding tissues and metastasize even in the early stage; and (3) the poor responsiveness to conventional treatments such as chemotherapy, radiotherapy and immunotherapy[4-6]. Surgery represents the only opportunity for possible cure, but it is restricted to early stage pancreatic cancer and most patients who undergo tumor resection show recurrence or distant metastases and die within a few years. At least, at the present time, an emphasis on early diagnosis alone may not be sufficient for significant improvement in the current poor prognosis of pancreatic cancer, which necessitates the search for novel treatment strategies to improve the prognosis.

Previous studies have demonstrated that a high percentage of pancreatic cancers harbors *K-ras* gene point mutation and overexpresses insulin-like growth factor receptor type 1 (IGF-IR)[7-11]. These alterations may together contribute to the progression and aggressiveness of pancreatic cancer from different pathways. Consequently, targeting expression of K-ras or IGF-IR has a potential value in pancreatic cancer therapy, and has led to the development of new therapeutic strategies based on the use of agents able to selectively inhibit targeted gene expression. In particular, antisense oligodeoxynucleotides (ASODNs) have proved their efficacy as targeted therapy, and are able to modulate target protein expression in pancreatic cancer studies[12-15]. In the practical application of the ASODN approach, many key problems need to be solved: selection of a single agent does not seem particularly promising because of the multigenic alterations of pancreatic cancer; finding a targeting site of *K-ras* mRNA or *IGF-IR* mRNA that is likely to be accessible to ASODNs; selection of an adaptable vector for mediating ASODNs; optimization of transfection concentration in a cell line, *etc*. Based on these considerations, we used polymerase chain reaction with special sequence primers (PCR-SSP) and sequence analysis to detect a K-ras point mutation at codon 12, and its mutation style on pancreatic cancer Patu8988 cells, designed and prepared ASODN (K-ras ASODN) specific for the K-ras point mutation at codon 12, and then combined it with strongest efficient IGF-IR ASODN designed by Resnicoff *et al*[16] to transfect pancreatic cancer Patu8988 cells with highly efficient vector Lipofectamine 2000. 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, reverse-transcript polymerase chain reaction (RT-PCR), flow cytometry and transmission electron microscope were used to evaluate the effects of cell proliferation, apoptosis and target gene expression. Therapeutic efficacy of the combination treatment was also evaluated in xenografts.

MATERIALS AND METHODS

Cell culture

Human pancreatic cancer cell lines Patu8988 and BXPC-3 used in this study were preserved in our laboratory. The cells were grown in RPMI 1640 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sijiqing, Hangzhou, China), 5 mmol/L HEPES, 100 U/mL penicillin and 100 U/mL streptomycin in 5% atmospheric CO_2 at 37℃. Cells were passaged every 3 d, checked routinely, and found to be free of contamination. When cells grew to 75% confluence, they were digested and used for *in vitro* and *in vivo* studies.

K-ras gene point mutation at condon 12 detected by PCR-SSP and sequence analysis

Genomic DNAs for Patu8988 cells and BXPC-3 cells were extracted according to the protocol. With regard to the sequences of K-ras cDNA in Genbank and the three high frequency mutation styles (CGT, GTT and GAT) at codon 12, three kinds of special sequence primers (SSP) for polymerase chain reaction were designed to detect *K-ras* gene point mutation at codon 12 for Patu8988 cells and BXPC-3 cells. Primers were as following: R1: 5'-GGTAGTTGGAGCTC-3'; R2: 5'-GTAGTTGGAGCTGT-3'; R3: 5'-GTAGTTGGAGCTGA-3'; R4: 5'-CTATTGTTGGATCATATTCG-3'. The pairing of R1-R4 amplified CGT mutation with a 89 base pair fragment; the pairing R2-R4 amplified GTT mutation with a 88 base pair fragment; the pairing R3-R4 amplified GAT mutation with a 88 base pair fragment. The amplification products were loaded on 8% acrylamide gels, and stained with ethidium bromide to detect mutation styles. In addition, *K-ras* gene was amplified from Patu8988 cells and BXPC-3 cells using RT-PCR, and the PCR products were directly sequenced.

Proliferation assay of Patu8988 cells treated with K-ras ASODN or IGF-IR ASODN alone

Based on the results of PCR-SSP and sequence analysis, the antisense phosphorothioate oligodeoxynucleotides 5'-TACGCCAACAGCTCCAAC-3' (K-ras ASODN) specific to the *K-ras* gene point mutation at codon 12 were designed and synthesized. The antisense phosphorothioate oligodeoxynucleotides 5'-TCCTCCGGAGCCAGACTT-3' (IGF-IR ASODN) specific to *IGF-IR* gene were synthesized according to the report from Resnicoff *et al*[16]. Exponentially growing Patu8988 cells at 1×10^5/well were seeded in 96-well microtiter plate, and treated with K-ras ASODN or IGF-IR ASODN mediated by Lipofectamine™ 2000 at concentration of 2-32 mg/L for 24 , 48, 72 and 96 h. The culture medium was changed every 24 h with fresh RPMI 1640 medium, which contained the same

concentration of K-ras ASODN or IGF-IR ASODN. The control cultures were left untreated at 37℃ for the same period of time, with triplicate wells for each concentration. After incubating for 24, 48, 72 and 96 h, 20 μL of 5 g/L MTT (Sigma, USA) in PBS was added to each well, followed by incubation for 4 h at 37℃. Formazan crystals were dissolved in DMSO for 15 min at 37℃. Absorbance was determined with an enzyme-linked immunosorbent assay reader at 570 nm. The cell proliferation curves were drawn according to the absorbance. The optimal concentration able to inhibit cell growth was selected for further experiments.

Proliferation assay of Patu8988 cells treated with combination of K-ras ASODN and IGF-IR ASODN

Patu8988 cells were seeded in a 96-well plate at a concentration of 1×10^5/well, and divided into three groups: (1) 16 mg/L K-ras ASODN group; (2) 16 mg/L IGF-IR ASODN group; (3)16 mg/L K-ras ASODN + 16 mg/L IGF-IR ASODN group. The cell cultures were measured for cell proliferation at different time points (0, 24, 48, 72 and 96 h after transfection) using MTT assay as described above. The cell proliferation curves were drawn according to the absorbance.

Apoptosis detection by annexin V-FITC/PI dual staining

Cells at the concentration of 1×10^5/mL were plated in 6-well plates, divided into three groups as described above. After being incubated for 48 h at 37℃, cells were harvested by trypsinization and rinsed with cold PBS twice. After centrifugation, cells were suspended by 250 μL conjugated buffer solution and then treated with 5 μL Annexin V-FITC and 10 μL propidium iodide (PI) for 15 min in the dark at room temperature. Finally, each sample was added into 300 μL of conjugated buffer solution and analyzed with flow cytometry. The experiments were performed in triplicate and the results were given as mean ± SE.

K-ras or IGF-IR protein expression detected by flow cytometry

Patu8988 cells, treated as described above, were removed from the plate by brief trypsinization with 0.25% trypsin, and then washed with PBS twice, stained with primary K-ras Ab or IGF-IR Ab, followed by FITC-conjugated goat anti-mouse IgG. After two rinses with PBS containing 2% FBS, these cells were analyzed with flow cytometry. Controls consisted of incubation with no antibodies or incubation with only the secondary antibody. The experiment was repeated three times.

K-ras or IGF-IR mRNA expression detected by semi-quantitive RT-PCR

Cells were plated in 6-well plates and performed as described above. Total cellular RNA was extracted by using Trizol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The purity and concentration were determined by measuring the absorbance (A) at 260 nm and 280 nm (A_{260}/A_{280}). To generate first-strand cDNA, an oligo (dT) 18 was used as primer, and 2 μg RNA was reverse-transcribed in the light of MMLV First Strand cDNA Synthesis Kit (Fermentas, USA) protocols. Amplification of human β-actin served as an internal control. The primers used were 5'-GGACCTGACTGACTACCTC-3' (forward) and 5'-TCATACTCCTGCTTGCTG-3' (reverse). The amplification products were 540 bp. The primers for K-ras were 5'-CGCGGATCCATGACTGAATATAAACTTGTG-3' (forward) and 5'-CGCAAGCTTTTACATAATTACACACTTTGT-3' (reverse). The amplification products were 585 bp. The primers for IGF-IR were 5'-CCAAAACTGAAGCCGAGAAG-3' (forward) and 5'-TGCAGCTGTGGATATCGATG-3' (reverse). The amplification products were 300 bp. K-ras was amplified 35 cycles under the following conditions: denaturing at 94℃ for 5 min followed by 94℃ for 1 min, annealing at 51℃ for 30 s and extension at 72℃ for 1 min; the final extension was at 72℃ for 10 min. *IGF-IR* gene and β-*actin* were amplified 30 cycles under the following conditions: denaturing at 94℃ for 5 min followed by 94℃ for 1 min, annealing at 55℃ for 1 min and extension at 72℃ for 90 s; the final extension was at 72℃ for 10 min. PCR products were separated in 1.5% agarose gels, stained with ethidium bromide, and visualized by UV absorption. Densitometric scanning of the bands was performed, and the relative amount of each gene mRNA expression was estimated by normalization to the β-*actin* mRNA detected in the same sample.

Transmission electron microscopic examination

Patu8988 cells treated with the combination of K-ras ASODN and IGF-IR ASODN for 48 h were harvested, and washed in PBS. The cell pellets were prefixed in 2.5% glutaraldehyde, postfixed in 1% osmic acid, dehydrated in gradient acetone and embedded in the resin. Ultrathin sections were cut, stained with lead citrate and assessed for the morphological changes under transmission electron microscope.

Colony assays

Twenty Patu8988 cells treated with 16 mg/L K-ras ASODN, 16 mg/L IGF-IR ASODN or 16 mg/L K-ras ASODN + 16 mg/L IGF-IR ASODN were seeded in 6-well plate and cultured in 5% atmospheric CO_2 at 37℃ for 2 wk. The control was with the same volume of culture medium.

Treatment in vivo

To investigate whether the combination of K-ras ASODN with IGF-IR ASODN would alter the tumorigenicity of Patu8988, male 4-wk -old BALB/c nude mice were purchased from the Animal Center of Shanghai. 1×10^7 cells in 0.1 mL PBS were injected subcutaneously into the right flank of nude mice. Fourteen days later, 16 mice with about the same tumor size were divided into four groups randomly. Intratumoral injections were given with K-ras ASODN, IGF-IR ASODN or K-ras ASODN + IGF-IR

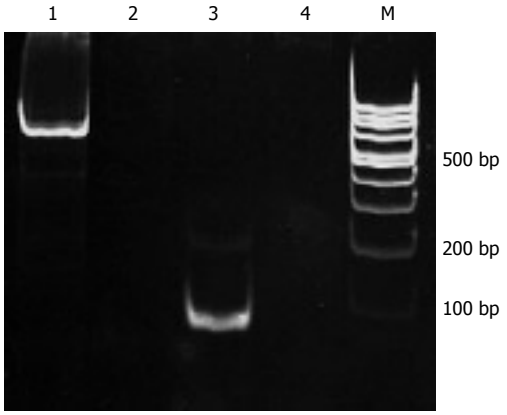


Figure 1 Detection of K-ras point mutation at 12 codon and its style in pancreatic cancer cell line Patu8988 by PCR-SSP. 1: β-actin; 2: R1-R4 pairing (CGT mutation); 3: R2-R4 pairing (GTT mutation); 4: R3-R4 pairing (GAT mutation); M: 100 bp DNA marker.

ASODN, and the control with 100 μL physiological saline. The injection was repeated every 48 h and 5 times in all. Tumor sizes were measured every 7 d and calculated by the formula: volume (mm^3) = $1/2(width)^2$ × length. After a 49-d follow-up period, mice were sacrificed. The tumors were removed, fixed by 4% polyformaldehyde, paraffin embedded and sectioned for immunohistochemical analysis.

Statistical analysis

All experiments were performed in triplicate and data were expressed as mean ± SD. Statistical analyses were conducted by one-factor analysis of variance and performed with SPSS 10.0 software. $P < 0.05$ was considered statistically significant.

RESULTS

K-ras point mutation at codon 12 of Patu8988 cell line

Detection of K-ras point mutation at codon 12 in the pancreatic cancer cell line Patu8988 is shown as Figure 1. The pairing R2-R4 had the amplification product of GTT mutation with an 88 bp fragment. But, the pairings of R1-R4 and R3-R4 had no amplification product of any mutation. Therefore, K-ras point mutation at codon 12 was found in pancreatic cancer cell line Patu8988, and the mutation style was GTT; no other mutation styles were found. For wild type pancreatic cancer cell line BXPC-3, no amplification products were found in pairings of R1-R4, R2-R4 and R3-R4. The direct sequencing results were consistent with the results from the PCR-SSP (Figure 2).

Inhibition of Patu8988 cell proliferation by K-ras ASODN and IGF-IR ASODN alone or combination

As shown in Figure 3A and B, when Patu8988 cells were exposed to K-ras ASODN and IGF-IR ASODN respectively, the growth of the cells was suppressed as compared to untreated cells ($P < 0.01$) except at the concentration of 2 mg/L. Moreover, when cells were exposed to different doses of K-ras ASODN

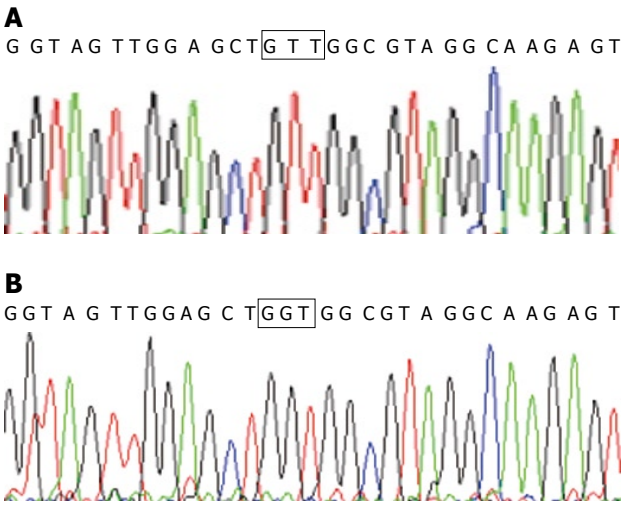


Figure 2 Sequence analysis of pancreatic cancer cell line Patu8988 (**A**) and BXCP-3 (**B**). Patu8988 had K-ras point mutation at codon 12 and its style was GGT→GTT (indicated in the square). BXCP-3 was a *K-ras* gene wild type cell line with normal GGT at codon 12; No other mutation styles were found.

and IGF-IR ASODN individually, growth inhibition was dose dependent: obvious inhibition was seen at the concentration of 16 mg/L, and the greatest effect was seen at a concentration of 32 mg/L. However, no statistical significance was found between 16 mg/L and 32 mg/L ($P > 0.05$). So, combination treatment with 16 mg/L K-ras ASODN and 16 mg/L IGF-IR ASODN was employed, and the ASODNs were transfected into Patu8988 cells for 24, 48, 72 and 96 h. Patu8988 cell growth was inhibited at a significantly higher rate in the combination treatment than that in K-ras ASODN or IGF-IR ASODN alone at different transfection times ($P < 0.01$) (Figure 3C). The inhibition peak was reached at 48 h. Subsequently, the inhibition ability wore off, and the tumor cells recovered proliferation. Further experiments were conducted to assess the combined effects on the expression of K-ras or IGF-IR mRNA and protein, apoptosis, clone formation and tumor growth inhibition *in vivo* with the combination treatment of 16 mg/L K-ras ASODN and 16 mg/L IGF-IR ASODN at 37℃ for 48 h.

Inhibition of colony formation by K-ras ASODN and IGF-IR ASODN alone or combination

Patu8988 cell proliferation treated in different groups was analyzed by soft agar colony formation assays. The average numbers of colonies in the control, K-ras ASODN, IGF-IR ASODN and combination group were 18.8, 11, 12 and 3, respectively. The Patu8988 cells in the combination groups formed significantly fewer colonies (6 fold decrease) in soft agar than those in the control groups did ($P < 0.05$ *vs* control). However, there were no statistical differences between K-ras ASODN groups and IGF-IR ASODN groups, although the number of colonies of IGF-IR ASODN groups was a little larger than those of K-ras ASODN groups ($P > 0.05$). At the same time, we noticed that the size of most of the colonies in the combination groups were much smaller than those in the control groups.

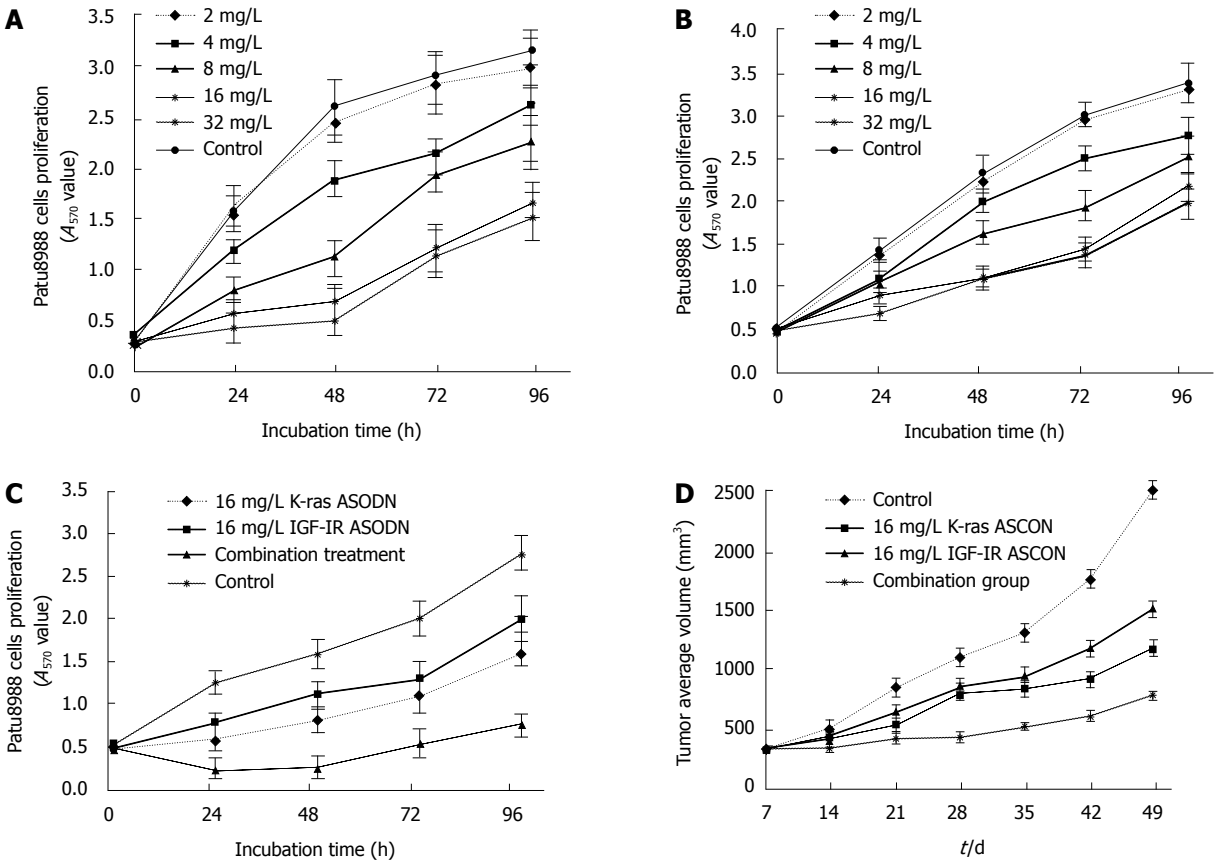


Figure 3 Each value represents the mean ± SD from triplicate determinations. **A**: Growth curves of Patu8988 cells treated with different concentrations of K-ras ASODN at different incubation times; **B**: Growth curves of Patu8988 cells treated with different concentrations of IGF-IR ASODN at different incubation times; **C**: Growth curves of Patu8988 cells treated with the combination treatment of 16 mg/L K-ras ASODN and 16 mg/L IGF-IR ASODN at different incubation times; **D**: The inhibition effect of the combination treatment with K-ras ASODN and IGF-IR ASODN on tumor growth in nude mice.

Table 1 Protein expression for K-ras and IGF-IR on Patu8988 cells detected by flow cytometry (%)

Groups	Positive rate	
	K-ras protein	IGF-IR protein
K-ras ASODN	25.95 ± 0.18[b]	85.25 ± 0.99
IGF-IR ASODN	69.18 ± 0.87	40.78 ± 1.42[b]
Combination	19.69 ± 1.15[b]	38.25 ± 1.22[b]
Control	76.15 ± 1.62	91.53 ± 1.62

All values are presented as mean ± SD of triplicate determinations. [b]$P < 0.01$ *vs* control group.

Apoptotic influence of Patu8988 cells treated with combination treatment of K-ras ASODN and IGF-IR ASODN

To further confirm the occurrence of apoptosis, we subjected the ASODNs-treated cells (48 h of ASODNs exposure) to annexin V-FITC/PI dual staining followed by flow cytometry analyses. The ratios of apoptosis cells were 21.54% ± 0.93%, 12.76% ± 0.74%, 8.43% ± 0.51% and 1.60% ± 0.19% in combination group, K-ras ASODN group, IGF-IR group and control group, respectively. Compared with the control group, statistically significant differences were observed ($P < 0.01$). The apoptotic rate of combination group was significantly higher than that of K-ras ASODN group alone or IGF-IR ASODN alone ($P < 0.05$). No difference existed between K-ras ASODN group and IGF-IR group ($P > 0.05$) (Figure 4).

K-ras and IGF-IR protein expression of Patu8988 cells detected by flow cytometry

Flow cytometry, results showed that the positive rate of K-ras protein was 76.15% ± 1.62% and 69.18% ± 0.87% in control group and IGF-ASODN group, respectively. No statistical difference was found between the two groups ($P > 0.05$). But, in K-ras ASODN group and the combination group, K-ras protein was significantly decreased by 25.95% ± 0.18% and 19.69% ± 1.15%, respectively, compared with that of control group ($P < 0.01$). Flow cytometric analysis by using IGF-IR antibody showed that there was high expression in K-ras ASODN group, and control group with a positive rate of 91.53% ± 1.62% and 85.25% ± 0.99%, respectively. But, in IGF-IR ASODN, and combination group, IGF-IR protein expression was reduced to 40.78% ± 1.42% and 38.25% ± 1.22%, respectively. Significant differences were found when compared with control group ($P < 0.01$). All above results revealed that antisense oligodeoxynucleotides can inhibit corresponding protein expression. But, K-ras ASODN can not obviously inhibit the expression of IGF-IR protein, and IGF-IR ASODN can not obviously inhibit the expression of K-ras protein (Table 1).

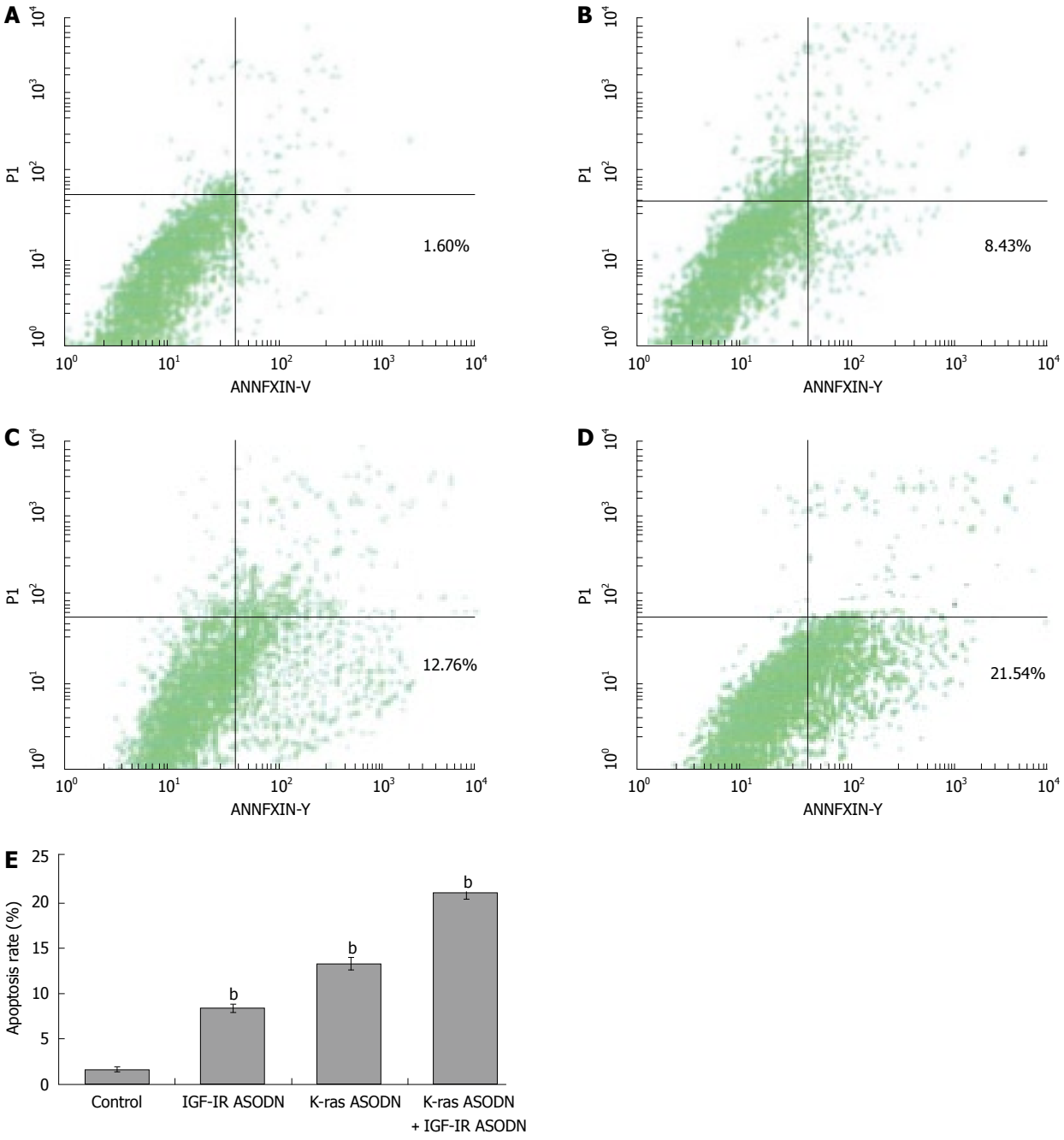


Figure 4 Apoptosis of Patu8988 cells detected by flow cytometry with Annexin V-FITC dual staining. **A**: Control group; **B**: IGF-IR ASODN group; **C**: K-ras ASODN group; **D**: K-ras ASODN + IGF-IR ASODN group; **E**: Apoptosis rate of Patu8988 cells for different groups. $^{b}P < 0.01$, *vs* control group. Each value represents the mean ± SD from triplicate determinations.

K-ras and IGF-IR mRNA expression of Patu8988 cells detected by RT-PCR

The mRNA expression intensities of *K-ras* gene and *IGF-IR* gene were analyzed by semiquantitive RT-PCR. The mRNA levels were normalized by internal control β-actin. At 48 h post-transfection, *K-ras* mRNA intensity levels were 0.389 ± 0.018 for IGF-IR ASODN group, 0.213 ± 0.027 for K-ras ASODN + IGF-IR ASODN group, 0.275 ± 0.023 for K-ras ASODN group and 0.391 ± 0.021 for control group. The statistical analysis showed that *K-ras* mRNAs of Patu8988 cells in K-ras ASODN group, and combination group were reduced significantly, compared with that of control group ($P < 0.05$). The inhibition rate reached 45.5% in the combination group. IFG-IR ASODN had no significant inhibitory effect on the expression of K-ras mRNA ($P > 0.05$, *vs* control) (Figure 5). As for *IGF-IR* gene, the relative mRNA levels were 0.642 ± 0.017 for *K-ras* ASODN, 0.355 ± 0.020 for the combination group, 0.387 ± 0.025 for IGF-IR ASODN group, and 0.630 ± 0.029 for control group. The statistical analysis showed that both IGF-IR ASODN group, and combination group could have a significant down-regulation effect on the mRNA expression of *IGF-IR* in Patu8988 cells ($P < 0.05$, *vs* control). The inhibition rate was 43.7% in the combination group. However, K-ras ASODN showed

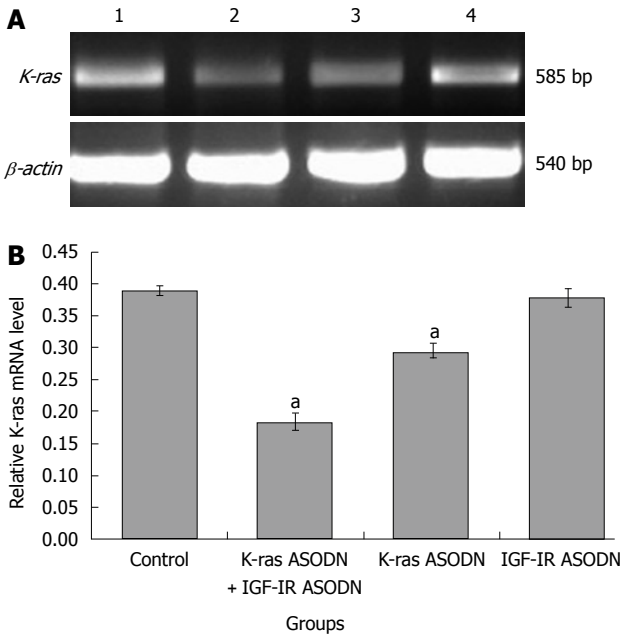


Figure 5 mRNA expression of *K-ras* gene in Patu8988 cells at 48 h post-transfection. **A**: RT-PCR analysis for *K-ras* gene in different groups. The *β-actin* gene was included as internal control. 1: IGF-IR ASODN group; 2: K-ras ASODN + IGF-IR ASODN; 3: K-ras ASODN group; 4: Control group; **B**: K-ras products quantified relative to the internal control β-actin. $^aP < 0.05$, *vs* control. Each value represents the mean ± SD from triplicate determinations.

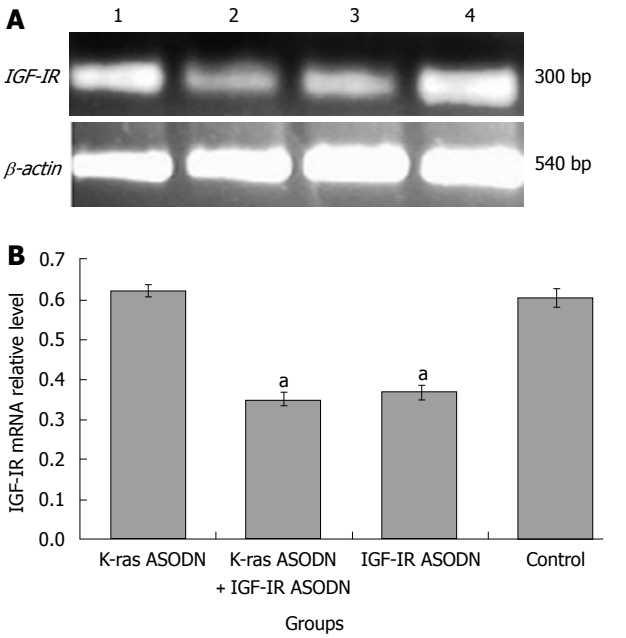


Figure 6 mRNA expression of *IGF-IR* gene in Patu8988 cells at 48 h post-transfection. **A**: RT-PCR analysis for *IGF-IR* gene in different groups. The β-*actin* gene was included as internal control. 1: K-ras ASODN group; 2: K-ras ASODN + IGF-IR ASODN; 3: IGF-IR ASODN group; 4: Control group; **B**: IGF-IR products quantified relative to the internal control β-actin. $^aP < 0.05$ *vs* control. Each value represents the mean ± SD from triplicate determinations.

no obvious inhibition for *IGF-IR* mRNA expression ($P > 0.05$ *vs* control) (Figure 6).

Inhibition of in vivo tumor growth by K-ras and IGF-IR downregulation

All nude mice were bearing pancreatic tumors from 7 to 10 d, and survived during the therapy with no red swelling, and disruption at the inoculation point. Before therapy, there were no significant difference for nude mice in weight and volume. As shown in Figure 3D, the tumor volume increased gradually in control group. The long diameter reached above 1.0 cm, and the volume reached 2230.0 ± 65.6 mm^3 on the 49th d after inoculation. The tumor growth in K-ras ASODN group, IGF-IR ASODN group and combined group was inhibited with significant difference when compared with control group ($P < 0.01$). The therapeutic effect in the combined group was greater than that of K-ras ASODN or IGF-IR ASODN alone ($P < 0.01$). These results indicated that combination group exerted a strong growth-suppressive effect on pancreatic cancer. However, between K-ras ASODN and IGF-IR ASODN group, there was no obvious difference ($P > 0.05$). The results of immunohistochemical showed that K-ras and IGF-IR protein expression decreased in tumor tissues (data not shown).

Morphologic change under transmission electron microscopy

Using transmission electron microscope, we saw that the normal Patu8988 cells had intact cell membranes and nuclear membranes, distributed nuclear chromosomes, distinct organelles, big nuclei and excessive nuclear division, which indicated that Patu8988 cells were highly malignant (Figure 7A). When Patu8988 cells were treated with K-ras ASODN combined with IGF-IR ASODN for 48 h, changes such as apoptosis, cell shrinkage, separation from neighboring cells, plasma condensation, plasma vacuolation, karyopyknosis, margination of condensed chromatin and membrane-bounded apoptotic bodies were observed (Figure 7B); some cells exhibited distinct deformation and disruption (Figure 7C).

DISCUSSION

The rapid development of molecular techniques has made it clear that tumorigenesis is actually a process of gene abnormalities. The strong invasiveness and rapid diffusive ability of pancreatic cancer are also closely associated with gene abnormalities. The study results of many years show that many genes' cooperation and many factors' participation contribute to the development of pancreatic cancer. Gene therapy brings hope for patients of pancreatic cancer. But, single gene therapy does not achieve ideal results. If two or more genes are combined to treat pancreatic cancer, in theory therapeutic effects will be better.

Since Almoguera *et al*[17] first reported that K-ras mutation occurred in patients with pancreatic cancer, 85%-95% patients with pancreatic cancer have been found to have K-ras mutation, and most of these were point mutations at codon 12. Among those point mutations, GAT, GTT and CGT mutation styles comprised more than 95% of the point mutations at

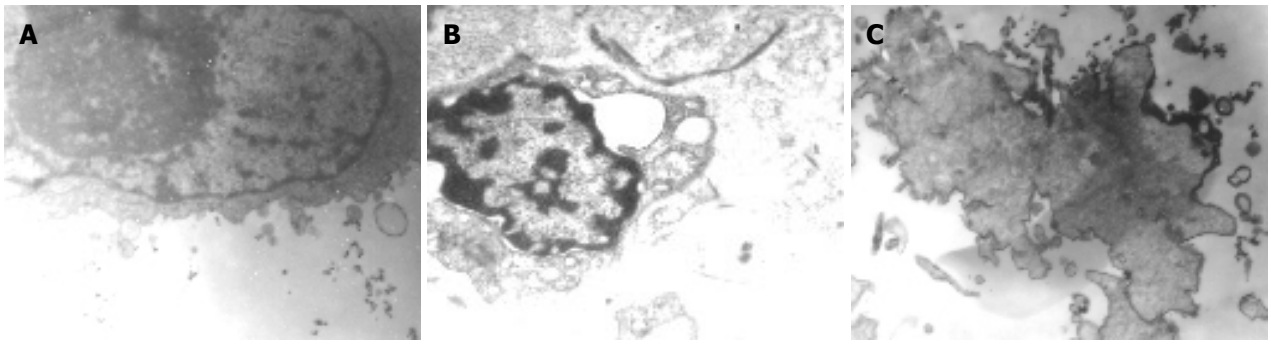


Figure 7 Transmission electron microscopic appearance of Patu8988 cell. **A**: Normal Patu8988 cell with intact cell membrane and nuclear membrane (× 12 000); **B**: Patu8988 cell treated with K-ras ASODN + IGF-IR ASODN for 48 h. The margination of condensed chromatin and membrane-bounded apoptotic bodies are observed (× 15 000); **C**: Necrotic cell (× 15 000).

codon 12[18]. Therefore, K-ras point mutation at codon 12 is an early event for pancreatic cancer, which can be used as a target for early diagnosis and gene therapy[19,20]. *K-ras* gene mutation destroys the GTP enzyme activity of ras protein and makes K-ras active constantly, which makes K-ras protein unable to block signals for growth. Recently, K-ras antisense oligodeoxynucleotids (ASODNs) have been transfected into pancreatic cells in China and abroad. Wang *et al*[21] first detected and confirmed *K-ras* gene mutation type CGT in human pancreatic carcinoma cell line PC-2. Then, K-ras mutation ASODN specific to CGT at codon 12 was used to explore its inhibitory effects on target gene in cell line PC-2. The results show that ASODN specific to K-ras point mutation CGT had significant inhibitory effects on target gene expression in human pancreatic carcinoma cells *in vitro*. Nakada *et al*[22] used ASODN specific to K-ras point mutation GAT at codon 12 to transfect into human pancreatic cancer cell line PANC-1 (with GAT mutation at codon 12), and the invasive activity was investigated using *in vitro* chemoinvasion assay. The results show that K-ras mutation ASODN specific to GAT at codon 12 strongly inhibited the invasive activity of the cell line PANC-1, but not in that with a wild type K-ras (BxPC-3). So, ASODNs specific to mutated *K-ras* genes can inhibit the proliferation and invasiveness of human pancreatic cancer cell lines. Specific antisense therapy to the point mutation of K-ras might be a new anticancer strategy for pancreatic cancer. However, these studies also indicate that adopting K-ras ASODN alone could not eradicate all the tumor cells. For exploring more effective therapy methods, some scholars abroad recently started to explore therapeutic alliance with diverse antisense oligodeoxynucleotides to treat pancreatic cancer, such as simultaneous transfection with mda-7 ASODN and K-ras ASODN into pancreatic cancer *in vitro* and *in vivo*. The results of their studies showed that the therapeutic effects of combination methods were better than that of one alone[23,24].

IGF-IR is a receptor protein tyrosine kinase (RPTK) expressed in a wide variety of cell types including mesenchymal, epithelial, and hematopoietic cells. The receptor is a transmembrane heterotetramer consisting of two α-subunits and two β-subunits linked by disulfide bonds. The binding of IGF-I to its receptor results in receptor oligomerization, activation of PTK, inter-molecular receptor autophosphorylation and phosphorylation of cellular substrates that consequently lead to gene activation, DNA synthesis and cell proliferation. Overexpression of IGF-IR stimulated cells not only to transform toward malignance and sustain malignant phenotype, but also to promote tumor cells' anti-apoptosis, mitosis, proliferation and invasiveness. Min *et al*[10] reported that IGF-IR overexpressed in pancreatic cancer and down-regulation of IGF-IR expression using monoclonal antibodies or antisense oligodeoxynucleotides could inhibit tumor cell growth both *in vitro* and *in vivo*[25-27]. However, the inhibition ratio was not high[24].

Considering the important effects of *K-ras* gene point mutation at codon 12 in pancreatic cancer and the broad tumorigenesis of *IGF-IR* gene, our study explored the effects of Patu8988 cell proliferation, apoptosis and target gene expression using combined antisenses with K-ras ASODN against K-ras point mutation at codon 12 and IGF-IR ASODN against insulin-like growth factor-1. We noticed that different doses for K-ras ASODN or IGF-IR ASODN could inhibit Patu8988 cell growth. But, combinations could produce greater effects ($P < 0.01$). The results were also confirmed in animal experiments. Compared with the single method, combination could obviously induce Patu8988 cell apoptosis, and reduce protein and mRNA expression of K-ras and IGF-IR. In our study, the inhibition effects of K-ras ASODN were better than that of IGF-IR ASODN. Ras protein is not only one of the signal pathways of IGF-IR, but also the pathway for many other growth factors, such as VEGF. Therefore, mutated ras protein not only amplifies the IGF-IR signal, but also amplifies signals for many other growth factors to inhibit cell apoptosis, and also induces vascular growth and cell proliferation. IGF-IR ASODN can inhibit only IGF-IR signal. But, K-ras ASODN can inhibit signals for many factors. Therefore, the inhibition effects of K-ras ASODN was better than that of IGF-IR ASODN. So, combined therapy can inhibit signals on two sides. After being treated with K-ras ASODN and IGF-IR ASODN together, some cells appeared in the

form of apoptosis, some others in the shape of edema or deformation, which indicated that antisense not only induced apoptosis, but also promoted cell death directly.

Our study shows that K-ras ASODN combined with IGF-ASODN obviously inhibited Patu8988 cell growth, and induced cell apoptosis and death. The mechanism may be associated with the inhibition of mRNA, and protein expression of K-ras and IGF-IR in Patu8988 cells. Cooperation with two synergistic antisense oligodeoxynucleotides could provide a new gene therapeutic strategy against pancreatic cancer. Meanwhile, the results of our study show that K-ras ASODN and IGF-IR ASODN inhibited tumor growth alone or in combination. However, a rapid cell proliferation tendency was seen in later stage of combined therapy. We speculated that this phenomenon might be associated with the degradation of ASODN in the late stage of treatment. We need to do further study to learn the relationship between dose-effect and time-effect. On the other hand, the development of pancreatic cancer involves many genes; we can not inhibit tumor growth completely by suppressing two genes of *K-ras* and *IGF-IR*, only partial.

COMMENTS

Background

Pancreatic carcinoma is the cancer that has the highest *K-ras* gene mutation rate. 95% of mutations happen at codon 12. Three major mutation types have been reported, including CGT, GAT and GTT. Antisense oligodeoxynucleotides (ASODNs) specific to CGT and GAT point mutations in human pancreatic cancer cell lines were reported; the ASODN against GTT point mutation in pancreatic cancer remains unclear. Some studies reported that type I insulin-like growth factor receptor (IGF-IR) is overexpressed in pancreatic cancer and down-regulation of IGF-IR expression using ASODNs could inhibit tumor cell growth. In this article, whether K-ras ASODN specific to GTT mutation in alliance with IGF-IR ASODN regulate Patu8988 proliferation, apoptosis, target gene expression *in vitro* and *in vivo* was investigated.

Research frontiers

In previous studies, antisense oligodeoxynucleotides (ASODNs) specific to CGT and GAT point mutation of *K-ras* gene were demonstrated to inhibit proliferation in pancreatic cancer.

Innovations and breakthroughs

It was found in the present study that K-ras ASODN combined with IGF-IR ASODN could cooperatively inhibit the growth of Patu8988 cells and induce their apoptosis via reinforcing specific down regulation of *K-ras* and *IGF-IR* mRNA and protein expression.

Applications

Cooperation with two synergistic antisense oligodeoxynucleotides could provide a new gene therapeutic strategy against pancreatic cancer.

Terminology

PCR-SSP is polymerase chain reaction with special sequence primers; K-ras ASODN is an antisense oligodeoxynucleotide against *K-ras* gene; IGF-IR ASODN is an antisense oligodeoxynucleotide against *IGF-IR* gene; GAT, CGT and GGT are three major point mutation types at codon 12 of *K-ras* gene.

Peer review

This is an interesting study that identifies molecular pathways that may be therapeutically targeted to inhibit pancreatic cancer growth.

REFERENCES

1 **Nitecki SS**, Sarr MG, Colby TV, van Heerden JA. Long-term survival after resection for ductal adenocarcinoma of the pancreas. Is it really improving? *Ann Surg* 1995; **221**: 59-66

2 **Coppola D**. Molecular prognostic markers in pancreatic cancer. *Cancer Control* 2000; **7**: 421-427

3 **Welsch T**, Kleeff J, Friess H. Molecular pathogenesis of pancreatic cancer: advances and challenges. *Curr Mol Med* 2007; **7**: 504-521

4 **Boeck S**, Heinemann V. The role of second-line chemotherapy after gemcitabine failure in patients with advanced pancreatic cancer. **Future Oncol** 2008; **4**: 41-50

5 **Brasiuniene B**, Juozaityte E. The effect of combined treatment methods on survival and toxicity in patients with pancreatic cancer. *Medicina* (Kaunas) 2007; **43**: 716-725

6 **Plate JM**. Current immunotherapeutic strategies in pancreatic cancer. *Surg Oncol Clin N Am* 2007; **16**: 919-943, xi

7 **Deramaudt T**, Rustgi AK. Mutant KRAS in the initiation of pancreatic cancer. *Biochim Biophys Acta* 2005; **1756**: 97-101

8 **Fryzek JP**, Garabrant DH, Schenk M, Kinnard M, Greenson JK, Sarkar FH. The Association Between Selected Risk Factors for Pancreatic Cancer and the Expression of p53 and K-ras Codon 12 Mutations. *Int J Gastrointest Cancer* 2006; **37**: 139-145

9 **Jiao L**, Zhu J, Hassan MM, Evans DB, Abbruzzese JL, Li D. K-ras mutation and p16 and preproenkephalin promoter hypermethylation in plasma DNA of pancreatic cancer patients: in relation to cigarette smoking. *Pancreas* 2007; **34**: 55-62

10 **Min Y**, Adachi Y, Yamamoto H, Ito H, Itoh F, Lee CT, Nadaf S, Carbone DP, Imai K. Genetic blockade of the insulin-like growth factor-I receptor: a promising strategy for human pancreatic cancer. *Cancer Res* 2003; **63**: 6432-6441

11 **Liu W**, Bloom DA, Cance WG, Kurenova EV, Golubovskaya VM, Hochwald SN. FAK and IGF-IR interact to provide survival signals in human pancreatic adenocarcinoma cells. *Carcinogenesis* 2008; **29**: 1096-1107

12 **Wang YX**, Gao L, Ji ZZ. Inhibitary effects of antisense oligonucleotide specific to K-ras point mutation on the target gene expression in human pancreatic carcinoma cells. *Chin Med J* (Engl) 2007; **120**: 1448-1450

13 **Zhou NX**, Fen YQ, Huang ZQ, Wang YZ, Xu QS. Growth inhibition of pancreatic cancer by antisense oligonucleotide against the insulin-like factor-I receptor. *Zhonghua Putong Waike Zazhi* 1999; **29**: 247-251

14 **Masui T**, Hosotani R, Ito D, Kami K, Koizumi M, Mori T, Toyoda E, Nakajima S, Miyamoto Y, Fujimoto K, Doi R. Bcl-XL antisense oligonucleotides coupled with antennapedia enhances radiation-induced apoptosis in pancreatic cancer. *Surgery* 2006; **140**: 149-160

15 **Hotz HG**, Hines OJ, Masood R, Hotz B, Foitzik T, Buhr HJ, Gill PS, Reber HA. VEGF antisense therapy inhibits tumor growth and improves survival in experimental pancreatic cancer. *Surgery* 2005; **137**: 192-199

16 **Resnicoff M**, Coppola D, Sell C, Rubin R, Ferrone S, Baserga R. Growth inhibition of human melanoma cells in nude mice by antisense strategies to the type 1 insulin-like growth factor receptor. *Cancer Res* 1994; **54**: 4848-4850

17 **Almoguera C**, Shibata D, Forrester K, Martin J, Arnheim N, Perucho M. Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. *Cell* 1988; **53**: 549-554

18 **Berndt C**, Haubold K, Wenger F, Brux B, Muller J, Bendzko P, Hillebrand T, Kottgen E, Zanow J. K-ras mutations in stools and tissue samples from patients with malignant and nonmalignant pancreatic diseases. *Clin Chem* 1998; **44**: 2103-2107

19 **Fleming JB**, Shen GL, Holloway SE, Davis M, Brekken RA. Molecular consequences of silencing mutant K-ras in pancreatic cancer cells: justification for K-ras-directed therapy. *Mol Cancer Res* 2005; **3**: 413-423

20 **Zhu H**, Liang ZY, Ren XY, Liu TH. Small interfering RNAs targeting mutant K-ras inhibit human pancreatic carcinoma cells growth in vitro and in vivo. *Cancer Biol Ther* 2006; **5**: 1693-1698

21 **Wang YX**, Gao L, Ji ZZ. [The study of the effect of antisense oligonucleotide specific to K-ras point mutation on human

pancreatic carcinoma cell PC-2] *Zhonghua Waike Zazhi* 2005; **43**: 1387-1390

22 **Nakada Y**, Saito S, Ohzawa K, Morioka CY, Kita K, Minemura M, Takahara T, Watanabe A. Antisense oligonucleotides specific to mutated K-ras genes inhibit invasiveness of human pancreatic cancer cell lines. *Pancreatology* 2001; **1**: 314-319

23 **Su Z**, Lebedeva IV, Gopalkrishnan RV, Goldstein NI, Stein CA, Reed JC, Dent P, Fisher PB. A combinatorial approach for selectively inducing programmed cell death in human pancreatic cancer cells. *Proc Natl Acad Sci USA* 2001; **98**: 10332-10337

24 **Lebedeva IV**, Su ZZ, Emdad L, Kolomeyer A, Sarkar D, Kitada S, Waxman S, Reed JC, Fisher PB. Targeting inhibition of K-ras enhances Ad.mda-7-induced growth suppression and apoptosis in mutant K-ras colorectal cancer cells. *Oncogene* 2007; **26**: 733-744

25 **Maloney EK**, McLaughlin JL, Dagdigian NE, Garrett LM, Connors KM, Zhou XM, Blattler WA, Chittenden T, Singh R. An anti-insulin-like growth factor I receptor antibody that is a potent inhibitor of cancer cell proliferation. *Cancer Res* 2003; **63**: 5073-5083

26 **Pan YZ**, Sun CY, Wang YZ. The growth inhibition of human pancreatic cancer cells by lipofectin mediated IGF-1R antisense oligonucleotides. *Zhonghua Heyixue Zazhi*; 2005; **25**: 212-215

27 **Adachi Y**, Lee CT, Carbone DP. Genetic blockade of the insulin-like growth factor 1 receptor for human malignancy. *Novartis Found Symp* 2004; **262**: 177-189; discussion 190-192, 265-268

S- Editor Li DL **L- Editor** Li M **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5186

World J Gastroenterol 2008 September 7; 14(33): 5186-5191
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Phosphatidylinositol 3-kinase/Akt pathway regulates hepatic stellate cell apoptosis

Yan Wang, Xiao-Yu Jiang, Li Liu, Hui-Qing Jiang

Yan Wang, Li Liu, Hui-Qing Jiang, Department of Gastroenterology, The Second Hospital of Hebei Medical University, The Hebei Institute of Gastroenterology, Shijiazhuang 050000, Hebei Province, China
Xiao-Yu Jiang, Hebei Medical University, Shijiazhuang 050017, Hebei Province, China
Author contributions: Jiang HQ, Liu L, and Wang Y designed the research; Wang Y and Jiang XY performed the research; Wang Y analyzed data; and Jiang HQ, Liu L, Wang Y and Jiang XY wrote the paper.
Supported by (in part) The Natural Science Foundation of Hebei Province, China, No.C2007000843
Correspondence to: Hui-Qing Jiang, MD, PhD, Department of Gastroenterology, The Second Hospital of Hebei Medical University, The Hebei Institute of Gastroenterology, No. 215, Heping West Road, Shijiazhuang 050000, Hebei Province, China. huiqingjiang@yahoo.com.cn
Telephone: +86-311-66002955 Fax: +86-311-87061012
Received: May 6, 2008 Revised: August 13, 2008
Accepted: August 20, 2008
Published online: September 7, 2008

Abstract

AIM: To investigate the role of phosphatidylinositol 3-kinase (PI 3-K)/Akt signaling pathway in the balance of HSC activation and apoptosis in rat hepatic stellate cells (HSC).

METHODS: An activated HSC cell line was used in this study. LY 294002, the PI 3-K/Akt signal pathway blocker was used to investigate the molecular events on apoptosis in HSC and to interpret the role of this pathway in HSC apoptosis. Immunocytochemistry, Western blot and reverse transcription polymerase chain reaction (RT-PCR) analysis were applied to detect the expression of PI 3-K, and simultaneously phosphorylated-Akt (p-Akt) and total-Akt were determined by Western blot. The HSC apoptosis was examined by annexin-V/propidium iodide double-labelled flow cytometry and transmission electron microscopy.

RESULTS: The apoptosis rates in LY 294002 (30.82% ± 2.90%) and LY 294002 + PDGF-BB (28.16% ± 2.58%) groups were significantly increased compared with those of control (9.02% ± 1.81%) and PDGF-BB (4.35% ± 1.18%). PDGF-BB augmented PI 3-K and p-Akt expression. LY 294002 significantly reduced the contents of PI 3-K and p-Akt. mRNA transcription evaluated by RT-PCR showed similar tendencies as protein expression.

CONCLUSION: Inhibition of PI 3-K/Akt signaling pathway induces apoptosis in HSC.

Key words: Hepatic fibrosis; Hepatic stellate cells; Phosphatidylinositol 3-kinase; Akt; Cell apoptosis

Peer reviewer: Trond Berg, Professor, Department of Molecular Biosciences, University of Oslo, PO Box 1041 Blindern, Oslo 0316, Norway

Wang Y, Jiang XY, Liu L, Jiang HQ. Phosphatidylinositol 3-kinase/Akt pathway regulates hepatic stellate cell apoptosis. *World J Gastroenterol* 2008; 14(33): 5186-5191 Available from: URL: http://www.wjgnet.com/1007-9327/14/5186.asp DOI: http://dx.doi.org/10.3748/wjg.14.5186

INTRODUCTION

The activation and proliferation of hepatic stellate cells (HSC) is a key event in fibrogenesis. On the other hand, HSC apoptosis results in fibrolysis and fibrotic regression[1-3]. Therefore, by understanding pro- and anti-apoptogenic factors, new therapeutic targets will be identified for the treatment of liver fibrosis. However, intracellular signals that regulate HSC apoptosis are still obscure. Our previous study has demonstrated that a cross talk between platelet-derived growth factor (PDGF), the most potent proliferative cytokines for HSC, and focal adhesive kinase (FAK), a nonreceptor tyrosine kinase, is involved in an integrin signaling pathway[4-6]. This interaction is essential for PDGF to induce HSC proliferation[7].

Phosphatidylinositol 3-kinase (PI 3-K)/Akt signaling molecules, which are downstream of FAK, are also activated by PDGF. The activated PI 3-K/Akt participate in regulation of HSC migration, proliferation, collagen secretion and adhesion[5]. PI 3-K is involved in regulating a number of cellular responses, such as cell growth, survival and migration. Akt, a serine/threonine kinase, is downstream of PI 3-K and an important anti-apoptotic factor. The purpose of the present study was to determine the effects and the molecular mechanisms whereby PI 3K/Akt influence apoptosis in HSC.

MATERIALS AND METHODS

Reagents and instruments

RPMI 1640 culture medium was obtained from Gibco (Invitrogen Corporation Carlsbad, California USA), fetal

bovine serum (FBS) from Sijiqing Company of Bio-products (Hangzhou, China) and LY 294002 from Sigma (Saint Louis, Missouri USA). Rat PDGF-BB was from Cytolab Biochemicals (Rehovot, Israel USA). Rabbit anti-phospho-Ser^{473} Akt polyclonal antibody and rabbit anti-PI 3-K p85α polyclonal antibody were from Santa Cruz Biotechnology (Santa Cruz, CA. USA). Rabbit anti-total Akt polyclonal antibody was purchased from Cell Signaling Biotechnology (Beverly, MA USA). RT-PCR amplification system was from Promega (Madison, WI USA). Annexin-V/Propidium iodide double-labelled flow cytometry kit was purchased from Baosai Company of Bio-products (Beijing, China). CO_2 incubator was from SANYO Company (Chatsworth, CA Japan). Flow cytometer type FACS-420 was purchased from Becton Dickinson Company (Franklin Lakes, NJ USA).

Cell line and cell culture

The activated HSC phenotype, from CCl_4 induced cirrhotic liver, was provided by Professor Greenwel (USA). This cell line is similar to that of primary cultured HSC except for absent expression of collagen type Ⅳ[8]. HSC was cultured in RPMI 1640 media supplemented with 10% FBS, penicillin (100 IU/mL)/streptomycin (100 μg/mL), and glutamine (4 mmol/L) in a 95% air and 5% CO_2 humidified atmosphere. Cell viability was determined by trypan blue exclusion staining. When exponential growing HSC populations were found to be nearly 100% confluence, HSC (2×10^5/mL) was incubated in serum free media for 24 h and then split into four groups: control, PDGF-BB (PDGF 20 ng/mL), PDGF-BB + LY 294002 group (LY 294002 50 μmol/L, PDGF-BB 20 ng/mL) and LY 294002 (LY 294002 50 μmol/L). Activated HSC was serum-starved for 24 h, and then treated as described above. One set of the cells was harvested for 4 h later for mRNA transcription and protein expression assay. The same set of cells was harvested 20 h later for electron microscopic examination, flow cytometric analysis and immunostaining.

Electron microscopic study

The cultured HSC of the four groups were collected, digested, washed with phosphate buffered solution (PBS), fixed with 4% glutaraldehyde for 2 h, and then fixed with osmium tetroxide for 1 h, stained with uranium acetate, embedded into 6.8# epoxide resin, after sectioning into ultra-thin slices, the cells were stained with lead citrate and examined under transmission electron microscopy.

Flow cytometric analysis

Four groups of HSC were collected, washed with pre-cooled PBS twice, fixed with cold 70% ethanol, digested with 50 μg/mL RNase at 37℃, and stained with 65 μg/mL propidium iodide at 4℃ for 1 h, and then the flow cytometric analysis was conducted. 10^4 cells were used in apoptotic analyses. Avian red blood cells were used as control. The variation coefficient (CV) of the assay was less than three percent. The data collected were analyzed in a computer (type HP-300 Consort 30), and analyzed with Histogram Statistics software.

RT-PCR

Total RNA from cultured HSC was extracted by the Trizol method. After RT (41℃, 45 min) and pre-denaturation (94℃, 2 min), PCR amplification was carried out for 35 cycles. The primers, as designed according to GenBank, were as follows: PI 3-K p85: sense 5'-CCCAGGAGCGGTACAGCAAAGAA-3', antisense 5'-TGGGGCAAATCCTCATCATCTTC-3'; β-actin: sense 5'-ACAGAGTACTTGCGCTCAGGAG-3', antisense 5'-GTCACCCACACTGTGCCCATCT-3'. The initial reaction mixture contained 1 μmol/L primers, AMV reverse transcriptase, RNA Tfl DNA polymerase and 25 mmol/L $MgSO_4$. Total volume was 50 μL. The amplicon lengths of the PCR products were: PI 3-K p85 355 bp and β-actin 585 bp. Denaturation (94℃ for 40 s), annealing (52℃ for 1 min), and extension (72℃ for 1.5 min). PCR products were analyzed by electrophoresis in a 1.5% agarose gel. β-actin amplification was used as internal control.

Immunostaining

Cultured cells were seeded onto glass coverslips and allowed to adhere for 20 h under routine culture conditions. After incubation, cells were washed twice with cold PBS, fixed and permeabilized by cold methanol for 8 min, and coverslips were then air-dried at room temperature. Immunostaining was performed using the SP kit according to the instructions of the manufacturer. Briefly, HSC was washed with washing buffer (0.2% Tween PBS), incubated with peroxidase-blocking reagent and normal goat serum. After rinsing, coverslips were incubated with primary antibodies (rabbit anti-phospho-Ser^{473}, Akt polyclonal antibody (1:100) and rabbit anti-PI 3-K p85α polyclonal antibody (1:50) at room temperature for 12 h. After rinsing primary antibody with washing buffer, horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was added, and incubated at 37℃ for 30 min. Following rinsing with washing buffer, 3,3-diamino benzidine/enhancer (DAB) solution was added and incubated at room temperature for 5 min. After washing, the coverslips were counterstained with hematoxylin for 1 min, rinsed with water and dried for 10 min. The slides were dehydrated with 100% ethanol for 20 s twice, and finally with xylene for 20 s. The coverslips were mounted onto glass slices and viewed under microscopy.

Western blot analysis of PI 3-K, Akt and p-Akt

Cultured HSC was washed with PBS and lysed with protein sample buffer (50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, 1 mmol/L EDTA, 1 mmol/L phenylmethyl sulfonylfluoride, and 2 mg/L leupeptin). The protein concentrations were measured using Coomassie brilliant blue chromatometry. Equal amounts (100 μg) of the denatured proteins per lane were loaded and separated on sodium dodecyl sulfate-10% polyacrylamide gels for p-Akt (Ser^{473}), Akt and β-actin, and 8% SDS-polyacrylamide gels for PI 3-K. After electrophoresis, proteins were

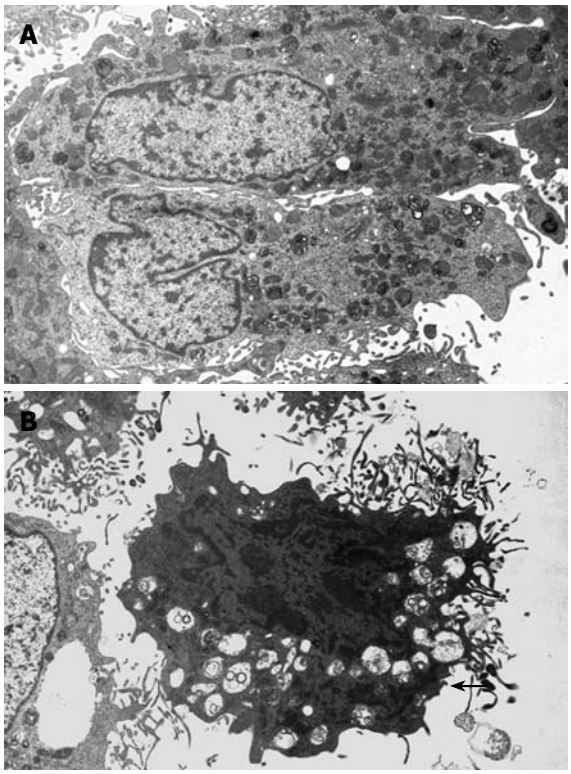


Figure 1 Transmission electron micrography of cultured HSC. **A**: Control HSC showing nuclear is intact and the mitochondria is smooth; **B**: LY 294002 treated HSC: Chromatins condensed, shrunk and aggregated along inside the nuclear membrane. The arrows points at the apoptotic bodies (× 5000).

transferred to nitrocellulose. The membranes were blocked for 12 h with 5% powdered skim milk in Tris-HCl-buffered saline containing 0.05% Tween 20 (TBS-T). After washed with TBS-T for three times, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:3000). After washing 5 times, the blots were detected with the enhanced chemiluminescence method (ECL) assay.

Statistical analysis

Results were expressed as mean ± SD. Statistical analysis was performed by one-way ANOVA, and, when the *F* value was significant, by Student-Newman-Keuls test. *P* value less than 0.05 was considered statistically significant.

RESULTS

LY 294002 induces apoptosis in rat HSC

Under the transmission electron microscopy (Figure 1), the cells in LY 294002 and LY 294002 + PDGF-BB groups showed condensed chromatin, shrunk and aggregated along inside the nuclear membrane. The morphology of the cells showed spherical, petal or crescent shape, apoptotic bodies were found in some cells, while in control and PDGF-BB groups, HSC revealed normal silhouettes.

As listed in Table 1, the apoptotic rates in LY 294002 and LY 294002 + PDGF-BB groups were significantly increased compared with that of control group (30.82% ± 2.90%, 28.16% ± 2.58% and 9.02% ± 1.81%, respectively; $P < 0.01$). There was no significant difference between LY 294002 and LY 294002 + PDGF-BB groups ($P = 0.12$).

Table 1 Apoptosis induction of PDGF-activated HSC by LY 294002 (mean ± SD)

Groups	Apoptotic rate of HSC
Control	9.02 ± 1.81
PDGF-BB	4.35 ± 1.18[a]
PDGF-BB + LY 294002	28.16 ± 2.58[b]
LY 294002	30.82 ± 2.90[b]

[a]$P = 0.005$ *vs* control group; [b]$P < 0.0001$ *vs* control group.

Table 2 Protein expression of PI3K and p-Akt by immunocytochemistry (mean ± SD)

Groups	p-Akt (%)	PI3K (%)
Control	33.65 ± 1.90	35.82 ± 1.87
PDGF-BB	58.86 ± 1.89[a]	60.85 ± 1.75[a]
LY294002 + PDGF-BB	24.24 ± 0.84[a]	25.18 ± 1.05[a]
LY294002	23.65 ± 1.90[a]	23.63 ± 1.64[a]

[a]$P < 0.0001$ *vs* control group.

PDGF-induced HSC activation abolished by LY 294002

PI 3-K assay: PDGF-BB could significantly increase PI 3-K expression in rat HSC. LY 294002 not only decreased the PI 3-K positive cells in control group, but also reduced PI 3-K content in PDGF-BB activated cells (Figure 2 and Table 2). These immunocytochemical results were supported by Western blots: the band density in PDGF-BB group was the strongest. LY 294002 not only decreased the PI 3-K expression in control cells, but also decreased the protein content in PDGF-BB activated cells (Figure 3). The effects of LY 294002 were reflected not only by the protein expression levels, but also by mRNA transcription (Figure 4).

Akt assay: Immunocytochemistry showed that p-Akt expression was parallel with PI 3-K: PDGF-BB stimulated p-Akt protein expression, while LY 294002 not only inhibited p-Akt concentration in control group, but also abolished PDGF stimulated p-Akt expression (Figure 2 and Table 2). Western blots showed the same pattern as immunocytochemistry for p-Akt. Noticeably, the total Akt levels assessed by Western blotting revealed no change in all four groups (Figure 5).

All the results above showed that PDGF activated the whole PI 3-K/Akt/p-Akt, pathway, while LY 294002 decreased the entire pathway activity not only in control cells, but also in PDGF stimulated circumstances.

DISCUSSION

It is activation of HSC that initiates liver fibrosis, and regardless of the etiology, is the final pathway is to activate HSC. The activated HSC is proliferative, fibrogenic,

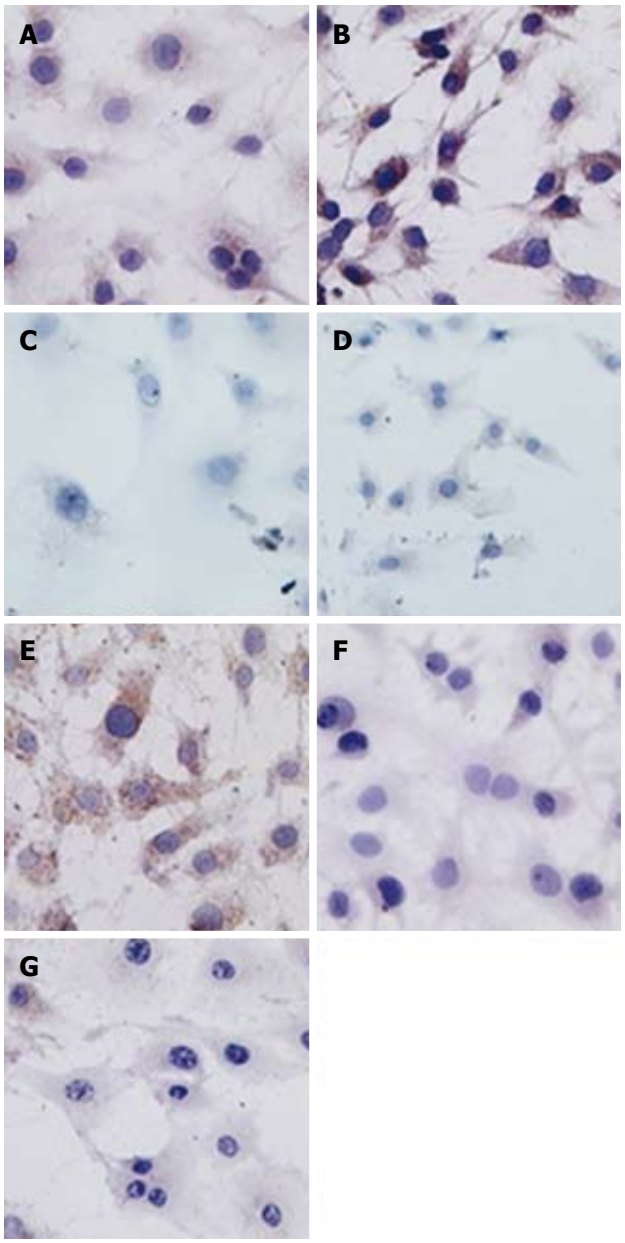


Figure 2 Immunocytochemistry (× 400). **A**: In negative control, the primary antibody was omitted; **B** and **E**: PI 3-K p85 and p-Akt^{473} staining in the PDGF-BB group; **C** and **F**: PI 3-K p85 and p-Akt473 expression in the PDGF-BB and LY 294002 groups; **D** and **G**: PI 3-K p85 and p-Akt^{473} staining in the LY 294002 group. Rabbit anti-PI 3-K p85α polyclonal antibody (1:50) and rabbit anti-phospho-Ser^{473} Akt polyclonal antibody (1:100) used as primary antibodies.

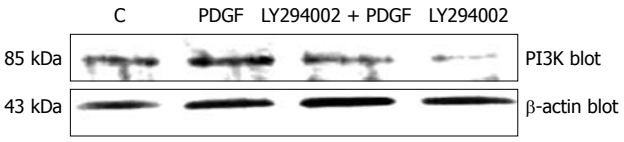


Figure 3 Representative Western blot analysis of PI 3-K protein expression in HSC with β-actin as internal control. From left, 1st lane, control HSC; 2nd lane, PDGF stimulated HSC; 3rd lane, PDGF + LY 294002 group and 4th lane, LY 294002 treated HSC.

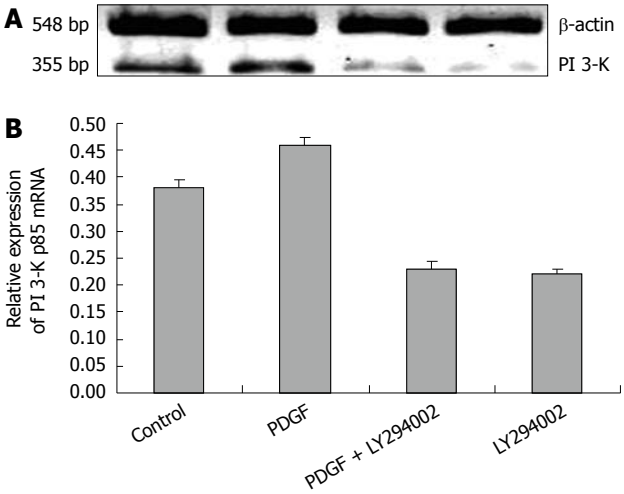


Figure 4 Representative RT-PCR photography of PI 3-K mRNA transcription from rat HSC, β-actin as internal control. **A**: From left, 1st lane, control HSC; 2nd lane, PDGF stimulated HSC; 3rd lane, PDGF + LY 294002 group and 4th lane, LY 294002 treated HSC. **B**: A graphic analysis of the RNase protection assay.

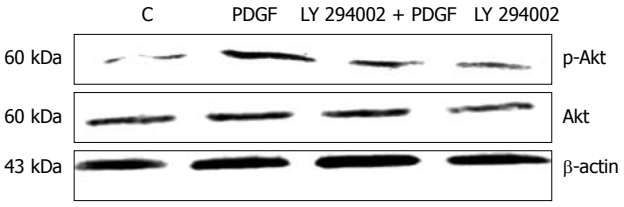


Figure 5 Representative Western blot analysis of p-Akt and total Akt protein expressions in HSC, β-actin as internal control. From left, 1st lane, control HSC; 2nd lane, PDGF stimulated HSC; 3rd lane, PDGF + LY 294002 group and 4th lane, LY 294002 treated HSC.

and contractile myofibroblasts[5,9]. The proliferation and apoptosis of HSC keep balance in physiological situation. Once this equilibrium is broken under chronic injury, continuing HSC proliferation and collagen secretion will cause fibrosis[10]. Therefore, inhibiting the proliferation of HSC and inducing its apoptosis are two ways to delay or stop fibrogenesis. PI 3-K and Akt are two molecules that stimulate HSC proliferation[1,2].

PI 3-K is comprised of an 85-kDa regulatory subunit, and a catalytic 110-kDa subunit. Activated by growth factors such as PDGF and insulin-like growth factor (IGF), PI 3-K catalyzes the phosphorylation of phosphatidylinositol (PI) at the 3-OH position to generate phosphatidylinositol triphosphate (PIP3). Activated growth factor receptor migrates to the cellular membrane, and this results in PI 3-K activation, that later combines with its substrates. Akt, a serine/threonine kinase, is also known as protein kinase B (PKB). Three isoforms have been identified thus far in the mammalian cells: Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ. The three isoforms have similar phosphorylation sites. One is in the N-terminal Thr^{308}. And the other in the COOH-terminal hydrophobic motif Ser^{473}. The phosphorylation of both sites is necessary for full activity. Activated PI 3-K/Akt signaling inhibits apoptosis, and stimulates cell proliferation, migration, survival as well as other biological behaviours[11].

Among the three PDGF isoforms, PDGF-AA, PDGF-BB and PDGF-AB, PDGF-BB has been identified as the most potent mitogen for HSC[7,12]. Liver

fibrosis is associated with an increase in PDGF protein expression and increased PDGF receptor expression. PDGF receptors have intrinsic tyrosine kinase activity and upon binding to its ligand, PDGF receptors can phosphorylate itself at tyrosine residues. In cultured human HSC, PDGF can activate PI 3-K which is necessary for both mitogenesis and chemotaxis[13-15]. This pathway is up-regulated during liver injury *in vivo*. Wortmannin and LY 294002, two specific PI 3-K inhibitors, can both dose-dependently block PI 3-K activity induced by PDGF and inhibit DNA synthesis[16].

Our immunocytochemistry in the present study found that PI 3-K and p-Akt were mainly in HSC cytoplasm. Immunocytochemistry, Western blot and RT-PCR demonstrated that PDGF-BB can significantly stimulate PI 3-K and p-Akt productions in both mRNA transcription (PI 3-K) and protein expression levels, LY 294002 not only reduced PI 3-K and p-Akt contents in control group, but also reduced PDGF stimulated PI 3-K expression and Akt phosphorylation. There was no significant difference between the groups of LY 294002 only and LY 294002 + PDGF. This means that LY 294002 can abolish the simulatory effect of PDGF. Our results also showed that there was no significant difference of total-Akt levels among four groups, indicating that instead of total Akt syntheses, LY 294002 mainly inhibits Akt phosphorylation. This *in vitro* study is consistent with the well-documented data which confirmed Akt phosphorylation being inhibited by LY 294002 in bile duct-ligated (BDL) animals. In addition, PDGF can induce Akt phosphorylation and LY 294002 blocks this phosphorylation.

The Akt family represents pivotal factors to promote cell survival, proliferation and inhibit apoptosis. Akt plays an important role in inhibiting apoptosis in variety of cells such as uterine leiomyoma cell lines, hematopoietic progenitor/stem cell lines, pancreatic beta cells and islet beta cells *etc*[17-20]. Our results showed that Akt also inhibited apoptosis in HSC. Following PI 3-K/Akt pathway inhibition, HSC revealed typical apoptotic morphous under transmission electron microscopy. Microvilli on the cell surface decreased，became short, and even disappeared. Cells shrank, cytoplasm condensed, ribosome and mitochondria aggregated. The chromatin condensed and shrank and aggregated along inside of the nuclear membrane to form of balls, petals and crescents. Sometimes，apoptotic bodies formed. The apoptosis rates of both LY 294002 treated groups were significantly increased compared with that of the control group and the PDGF group and thus, we conclude that PI 3-K/Akt inhibition enhances HSC apoptosis.

In summary, the data obtained have shown that inhibition of PI 3-K/Akt signaling pathway can induce apoptosis in rat HSC, even under strong mitogen stimulator (PDGF). The activation of HSC is essential for fibrogenesis, and PI 3-K/Akt signaling is indispensable to support HSC activation. Therefore, blocking the PI 3-K/Akt signaling pathway may provide a potential therapeutic benefit for liver fibrosis.

COMMENTS

Background

Hepatic fibrosis represents a reversible and dynamic process in response to a variety of chronic stimuli. Activation of the hepatic stellate cell (HSC), a perisinusoidal cell that resides in the liver in a quiescent state, is responsible for the increased synthesis and deposition of extracellular matrix (ECM) in the liver, and plays a critical role in fibrogenesis. The paradigms of HSC activation and apoptosis remain valuable frameworks for understanding pathways of hepatic fibrogenesis and fibrosis regression. HSC apoptosis results in fibrolysis and fibrotic regression. Therefore, by understanding the pro- and anti-apoptogenic factors, new therapeutic targets will be identified for the treatment of liver fibrosis. Phosphatidylinositol 3-kinase (PI 3-K)/Akt are components of the major intracellular signaling pathways elicited by platelet-derived growth factor (PDGF) in HSC. Intracellular signals that regulate HSC apoptosis are still obscure. Our previous study has demonstrated that a cross talk between PDGF, the most potent proliferative cytokines for the HSC, and focal adhesion kinase (FAK), a nonreceptor tyrosine kinase, is involved in integrin signaling pathway. This interaction is essential for PDGF to induce HSC proliferation.

Research frontiers

The activated HSC is primarily responsible for excessive deposition of ECM proteins and collagen deposition during liver fibrosis. Substantial insight is being gained into the molecular mechanisms responsible for apoptosis in the HSC. The activated HSC becomes responsive to both proliferative and fibrogenic cytokines. These cytokines activate both mitogen-activated protein kinase (MAPK) signaling, involving p38, and FAK-PI 3-K-Akt-p70 (S6K) signaling cascades. PI 3-K/Akt constitutes an important pathway regulating the signaling of multiple biological processes such as apoptosis, metabolism, cell proliferation and cell growth. Activating mutations, which have been reported for PI 3-K and Akt in tumors, are able to confer tumorigenic properties in several cellular systems. These regulate the proliferative response, activating cell cycle progression as well as collagen gene expression. It is anticipated that by understanding the molecular mechanisms responsible for HSC proliferation, apoptosis, and excess ECM production new therapeutic targets will be identified for the treatment of liver fibrosis.

Innovations and breakthroughs

HSC apoptosis results in fibrolysis and further on, fibrotic regression. Intracellular signals that regulate HSC apoptosis are still obscure. Our previous study has demonstrated that a cross talk between PDGF and FAK is involved in integrin signaling pathway. PI 3-K/Akt signaling molecules, which are downstream of FAK, are also activated by PDGF. Up to now, few studies have addressed the function of PI 3-K/Akt signal transduction pathways on the apoptosis of HSC. The present study determined the effects and the molecular mechanisms whereby PI 3K/Akt influences apoptosis in HSC, and showed that PDGF activated the whole PI 3-K/Akt/p-Akt, pathway, while LY 294002 decreased the entire pathway activity not only in control cells, but also in PDGF stimulated circumstances. PI 3K/Akt signal molecules will be new therapeutic targets for the treatment of liver fibrosis.

Applications

The present study has shown that inhibition of PI 3-K/Akt signaling pathway can induce apoptosis in rat HSC, even under strong mitogen stimulators (PDGF). The activation of HSC is essential for fibrogenesis, and PI 3-K/Akt signaling is indispensable to support HSC activation. Therefore, blocking the PI 3-K/Akt signaling pathway may provide a potential therapeutic benefit for liver fibrosis.

Peer review

The paper by Wang *et al* presents an interesting study of the role of PI 3-K/Akt in controlling apoptosis of hepatic stellate cells. The purpose of the present work was to determine whether inhibition of PI 3-K affected apoptosis in HSC. The methods used to measure expression of PI 3-K and phosphorylation of Akt as well as total Akt were acceptable, and the assessment of apoptosis in HSC also reliable. The results very clearly show that the apoptotic rate was markedly increased in presence of LY294002.

REFERENCES

1 **Murphy FR**, Issa R, Zhou X, Ratnarajah S, Nagase H, Arthur MJ, Benyon C, Iredale JP. Inhibition of apoptosis of activated hepatic stellate cells by tissue inhibitor of metalloproteinase-1 is mediated via effects on matrix

metalloproteinase inhibition: implications for reversibility of liver fibrosis. *J Biol Chem* 2002; **277**: 11069-11076
2 **Saile B**, Knittel T, Matthes N, Schott P, Ramadori G. CD95/CD95L-mediated apoptosis of the hepatic stellate cell. A mechanism terminating uncontrolled hepatic stellate cell proliferation during hepatic tissue repair. *Am J Pathol* 1997; **151**: 1265-1272
3 **Iredale JP**. Hepatic stellate cell behavior during resolution of liver injury. *Semin Liver Dis* 2001; **21**: 427-436
4 **Parsons JT**. Focal adhesion kinase: the first ten years. *J Cell Sci* 2003; **116**: 1409-1416
5 **Friedman SL**. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *J Biol Chem* 2000; **275**: 2247-2250
6 **Jiang HQ**, Zhang XL, Liu L, Yang CC. Relationship between focal adhesion kinase and hepatic stellate cell proliferation during rat hepatic fibrogenesis. *World J Gastroenterol* 2004; **10**: 3001-3005
7 **Carloni V**, Defranco RM, Caligiuri A, Gentilini A, Sciammetta SC, Baldi E, Lottini B, Gentilini P, Pinzani M. Cell adhesion regulates platelet-derived growth factor-induced MAP kinase and PI-3 kinase activation in stellate cells. *Hepatology* 2002; **36**: 582-591
8 **Greenwel P**, Schwartz M, Rosas M, Peyrol S, Grimaud JA, Rojkind M. Characterization of fat-storing cell lines derived from normal and CCl4-cirrhotic livers. Differences in the production of interleukin-6. *Lab Invest* 1991; **65**: 644-653
9 **Friedman SL**. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol Rev* 2008; **88**: 125-172
10 **Guo J**, Friedman SL. Hepatic fibrogenesis. *Semin Liver Dis* 2007; **27**: 413-426
11 **Parsons CJ**, Takashima M, Rippe RA. Molecular mechanisms of hepatic fibrogenesis. *J Gastroenterol Hepatol* 2007; **22** Suppl 1: S79-S84
12 **Rovida E**, Navari N, Caligiuri A, Dello Sbarba P, Marra F. ERK5 differentially regulates PDGF-induced proliferation and migration of hepatic stellate cells. *J Hepatol* 2008; **48**: 107-115
13 **Robino G**, Parola M, Marra F, Caligiuri A, De Franco RM, Zamara E, Bellomo G, Gentilini P, Pinzani M, Dianzani MU. Interaction between 4-hydroxy-2,3-alkenals and the platelet-derived growth factor-beta receptor. Reduced tyrosine phosphorylation and downstream signaling in hepatic stellate cells. *J Biol Chem* 2000; **275**: 40561-40567
14 **Bell CA**, Tynan JA, Hart KC, Meyer AN, Robertson SC, Donoghue DJ. Rotational coupling of the transmembrane and kinase domains of the Neu receptor tyrosine kinase. *Mol Biol Cell* 2000; **11**: 3589-3599
15 **Gutierrez-Ruiz MC**, Gomez-Quiroz LE. Liver fibrosis: searching for cell model answers. *Liver Int* 2007; **27**: 434-439
16 **Marra F**, Gentilini A, Pinzani M, Choudhury GG, Parola M, Herbst H, Dianzani MU, Laffi G, Abboud HE, Gentilini P. Phosphatidylinositol 3-kinase is required for platelet-derived growth factor's actions on hepatic stellate cells. *Gastroenterology* 1997; **112**: 1297-1306
17 **Yin XJ**, Wang G, Khan-Dawood FS. Requirements of phosphatidylinositol-3 kinase and mammalian target of rapamycin for estrogen-induced proliferation in uterine leiomyoma- and myometrium-derived cell lines. *Am J Obstet Gynecol* 2007; **196**: 176.e1-e5
18 **Wandzioch E**, Edling CE, Palmer RH, Carlsson L, Hallberg B. Activation of the MAP kinase pathway by c-Kit is PI-3 kinase dependent in hematopoietic progenitor/stem cell lines. *Blood* 2004; **104**: 51-57
19 **Maeda H**, Rajesh KG, Maeda H, Suzuki R, Sasaguri S. Epidermal growth factor and insulin inhibit cell death in pancreatic beta cells by activation of PI3-kinase/AKT signaling pathway under oxidative stress. *Transplant Proc* 2004; **36**: 1163-1165
20 **Scassa ME**, Guberman AS, Varone CL, Canepa ET. Phosphatidylinositol 3-kinase and Ras/mitogen-activated protein kinase signaling pathways are required for the regulation of 5-aminolevulinate synthase gene expression by insulin. *Exp Cell Res* 2001; **271**: 201-213

S- Editor Zhong XY **L- Editor** Ma JY **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5192

World J Gastroenterol 2008 September 7; 14(33): 5192-5196
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Ketamine anesthesia reduces intestinal ischemia/reperfusion injury in rats

Carlos Rodrigo Cámara, Francisco Javier Guzmán, Ernesto Alexis Barrera, Andrés Jesús Cabello, Armando Garcia, Nancy Esthela Fernández, Eloy Caballero, Jesus Ancer

Carlos Rodrigo Cámara, Francisco Javier Guzmán, Ernesto Alexis Barrera, Andrés Jesús Cabello, Armando Garcia, Nancy Esthela Fernández, Department of Physiology, Universidad Autonoma de Nuevo Leon, School of Medicine, Monterrey, Nuevo Leon 64460, Mexico
Eloy Caballero, Jesus Ancer, Department of Pathology, Universidad Autonoma de Nuevo Leon, School of Medicine, Monterrey, Nuevo Leon 64460, Mexico
Author contributions: Camara CR, and Guzman FJ contributed equally to this work; Camara CR, Barrera EA, Cabello AJ, and Garcia A performed research; Camara CR, Fernandez NE, and Guzman FJ designed research; Caballero E and Ancer J contributed morphological analytic tools for tissue samples, Camara CR, Guzman FJ and Caballero E analyzed data, and Camara CR wrote the paper.
Correspondence to: Carlos Rodrigo Cámara, Department de Fisiología, Facultad de Medicina, U.A.N.L. Monterrey N.L, Av. Francisco I Madero y Dr. Eduardo Aguirre Pequeño Col. Mitras Centro, Monterrey, Nuevo Leon 64460, Mexico. crcamara83@hotmail.com
Telephone: +51-81-83294203 Fax: +51-81-83294203
Received: March 23, 2008 Revised: July 10, 2008
Accepted: July 17, 2008
Published online: September 7, 2008

Abstract

AIM: To investigate the effects of ketamine anesthesia on the motility alterations and tissue injury caused by ischemia/reperfusion in rats.

METHODS: Thirty male Wistar rats weighing 200-250 g were used. Ischemia was induced by obstructing blood flow in 25% of the total small intestinal length (ileum) with a vascular clamp for 45 min, after which either 60 min or 24 h of reperfusion was allowed. Rats were either anesthetized with pentobarbital sodium (50 mg/kg) or ketamine (100 mg/kg). Control groups received sham surgery. After 60 min of reperfusion, the intestine was examined for morphological alterations, and after 24 h intestinal basic electrical rhythm (BER) frequency was calculated, and intestinal transit determined in all groups.

RESULTS: The intestinal mucosa in rats that were anesthetized with ketamine showed moderate alterations such as epithelial lifting, while ulceration and hemorrhage was observed in rats that received pentobarbital sodium after 60 min of reperfusion. Quantitative analysis of structural damage using the Chiu scale showed significantly less injury in rats that received ketamine than in rats that did not (2.35 ± 1.14 *vs* 4.58 ± 0.50, $P < 0.0001$). The distance traveled by a marker, expressed as percentage of total intestinal length, in rats that received pentobarbital sodium was 20% ± 2% in comparison with 25.9% ± 1.64% in rats that received ketamine ($P = 0.017$). BER was not statistically different between groups.

CONCLUSION: Our results show that ketamine anesthesia is associated with diminished intestinal injury and abolishes the intestinal transit delay induced by ischemia/reperfusion.

Key words: Ischemia/reperfusion; Ketamine; N-methyl-D-aspartate; Intestinal motility; Tissue damage

Peer reviewer: Anthony J Bauer, PhD, Department of Medicine/Gastroenterology, University of Pittsburgh, S-849 Scaife Hall, 3550 Terrace Street, Pittsburgh 15261, United States

Cámara CR, Guzmán FJ, Barrera EA, Cabello AJ, Garcia A, Fernández NE, Caballero E, Ancer J. Ketamine anesthesia reduces intestinal ischemia/reperfusion injury in rats. *World J Gastroenterol* 2008; 14(33): 5192-5196 Available from: URL: http://www.wjgnet.com/1007-9327/14/5192.asp DOI: http://dx.doi.org/10.3748/wjg.14.5192

INTRODUCTION

Mesenteric ischemia is a clinical entity with a mortality rate between 60% and 100% that usually requires surgical resection of the necrotic intestinal segment[1]. Although there have been advancements in the treatment of ischemic injury, an ideal treatment has not been defined, and new options should be considered. A promising strategy is the use of anesthetic and sedative agents that might exert protective effects on the injured tissue. Ketamine is an agent that has been recommended for this purpose in clinical situations of sepsis, renal ischemia, cerebral ischemia and serious burn injuries[2-4].

The small intestine is very sensitive to ischemic insult[5]. Reperfusion causes additional damage through the release of free radicals, pro-inflammatory cytokines, leukotrienes and other related products[6]. Intestinal

ischemia/reperfusion (I/R) damages the intestinal mucosa and alters gastrointestinal motility[7]. The histological damage induced by I/R includes alterations in capillary permeability, mucosal barrier dysfunction, necrosis, epithelial shredding. The damage is reversed in a period of approximately 24 h[8].

The NMDA (N-methyl-D-Aspartate) receptor family is a heterogeneous group of excitatory ionotropic voltage and ligand gate dependent receptors. Ketamine is a parenteral anesthetic that non-competitively blocks the NMDA receptor[9]. It has been shown that ketamine protects various tissues from I/R injury, such as brain[10], myocardium[11] and skeletal muscle[12]. To the best of our knowledge, no previous studies have evaluated the effects of ketamine on intestinal motility impairment and tissue damage induced by I/R.

MATERIALS AND METHODS

Procedures

Animal procedures were performed in accordance with the proper use and care of laboratory animals. Experiments were performed on 30 male Wistar rats weighing 200-250 g. Animals were maintained under standard conditions, such as stable room temperature (24 ± 3℃), a 12 h light/12 h dark cycle, and access to commercial rat pellets and water *ad libitum*.

Animal models

Briefly, after anesthesia with pentobarbital sodium (Anestesal, Pfizer Inc, Mexico) or ketamine (Anesket, Pisa Agropecuaria, Hidalgo, Mexico), midline laparotomy was performed, and the small intestine was externalized and kept wrapped in humid sterile gauze to prevent dehydration. Rats were placed over a heating mat to maintain constant core temperature (37℃). Intestinal I/R was induced by selective clamping of the vascular supply of an ileum segment consisting of 25% of the total intestinal length with microvascular clamps for 45 min (ischemia) followed by either 60 min or 24 h reperfusion. Ischemia was confirmed by absence of pulsations as well as characteristic changes in intestinal coloration.

Thirty male Wistar rats were randomized into 3 groups (n = 10) depending on the type of anesthetic used. Each group was then divided into 2 subgroups. In subgroup A (n = 5), reperfusion lasted 60 min, and tissue was obtained for histological examination only, while in subgroup B (n = 5) it was extended to 24 h and the animals used to evaluate intestinal transit and intestinal electrical activity.

The control group (n = 10) animals were anesthetized with pentobarbital sodium (50 mg/kg) and sham-operated (only midline laparotomy and gut manipulation). In the SK group (n = 10) animals were anesthetized with pentobarbital sodium (50 mg/kg) and intestinal I/R was induced as described. In the KET group (n = 10) animals were anesthetized with ketamine (100 mg/kg) and intestinal I/R was induced as described.

Intestinal transit and basic electrical rhythm

In subgroup B, after 24 h of reperfusion, animals were anesthetized with pentobarbital sodium (50 mg/kg), mid line laparotomy was performed, and 3 subserosal bipolar electrodes were used to record the electrical activity of the ileum segment that had been subjected to I/R injury using a Power Lab 4/20T (ADInstruments, USA) equipped with 3 DC pre-amplifiers (ADInstruments, USA) to analyze and calculate intestinal Basic Electrical Rhythm (BER). Immediately after 30 min, 2 mL/kg of a non-absorbable charcoal solution marker was instilled through a canula into the proximal end of the ileum segment studied, and after another 30 min the intestine was removed, and the distance traveled by the marker was registered. The distance traveled by a marker was then expressed as percentage of total intestinal length from pylorus to cecum. This was used as an indicator of intestinal transit. Finally, tissue samples were obtained for morphologic examination.

Morphological examination

After the I/R procedure, rats were sacrificed by exsanguination from the aorta. Immediately, tissue samples were obtained and fixed in 10% neutral buffered formalin and embedded in paraffin, then 5 micrometer-thick sections were stained with hematoxylin and eosin and examined under light microscope by a blinded pathologist. The Chiu scale[13] of mucosal injury was used to evaluate the degree of histological alteration on 10 sections of 1 mm each to complete 1 cm per animal and then averaged. The scale consists of values from 0 to 5, where 0 normal mucosa; 1, development of sub epithelial (Gruenhagen's) spaces; 2, extension of the sub epithelial space with moderate epithelial lifting from the lamina propria; 3, extensive epithelial lifting with occasional denuded villi tips; 4, denuded villi with exposed lamina propria and dilated capillaries; and 5, disintegration of the lamina propria, hemorrhage, and ulceration.

Statistical analysis

Data were analyzed with SPSS 11.0 (SPSS Inc. Software, Chicago, Illinois, USA) statistical software using one-way analysis of variance (ANOVA) and with Tukey-Kramer test so as to determine comparison between groups, and differences between groups, respectively. All values were expressed as mean ± SD and $P < 0.05$ was considered statistically significant.

RESULTS

Morphological examination

Structural changes in intestinal mucosa in rats of group KET A (ketamine anesthesia) showed moderate alteration such as epithelial lifting, while ulceration, hemorrhage and villi disintegration was observed in rats of group SK A after 45 min of ischemia and 60 min of reperfusion (Figure 1). The results of the quantitative evaluation of structural damage using the Chiu scale were as follows: control A 0.7 ± 0.45, SK A 4.58 ± 0.50

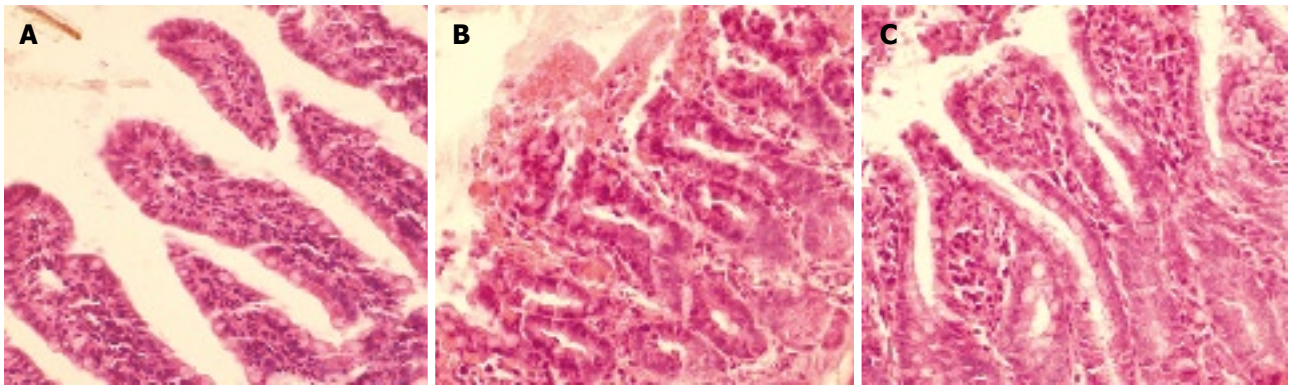


Figure 1 **A**: Control A group showing normal mucosa; **B**: SK A. Changes in intestinal mucosa structure following 45 min of ischemia and 60 min reperfusion; **C**: KET A. Less damage was observed (all x 40).

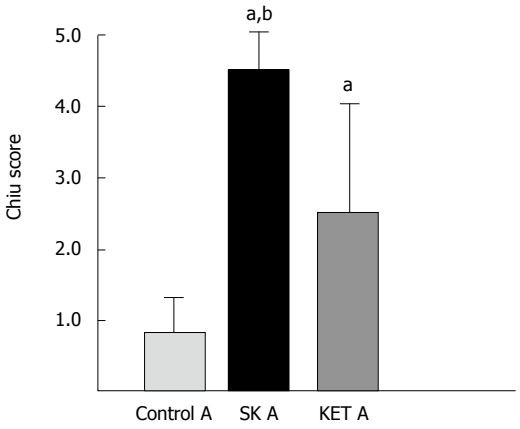


Figure 2 Bar diagram illustrating the Chiu score of mucosal injury after 60 min of reperfusion. Lower damage score was observed in a KET A group than in the SK A group. [a]$P < 0.0001$ *vs* control A; [b]$P < 0.0001$ *vs* KET A.

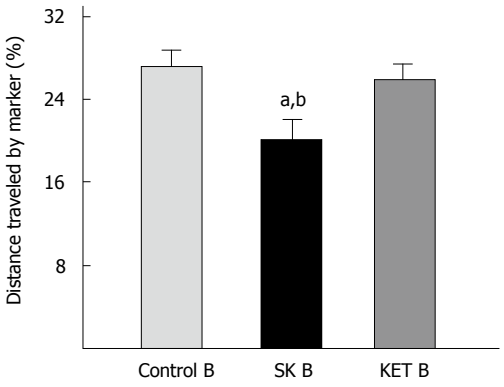


Figure 3 Bar diagram illustrating the distance travelled by the marker, expressed as a percentage of total intestinal length from pylorus to cecum. Intestinal I/R caused transit delay that was corrected by the administration of ketamine as anesthetic. [a]$P = 0.00019$ *vs* control B; [b]$P = 0.0004$ *vs* KET B.

and KET A 2.35 ± 1.14 (Figure 2). Damage in group KET A was significantly reduced in comparison with group SK A ($P < 0.0001$). However, the damage score in the KET A group did not reach control A group levels, indicating only moderate protection. The degree of mucosal damage after 24 h of reperfusion (subgroups KET B and SK B) was the same as in control rats and showed no statistical significance.

Intestinal transit and BER

The distance traveled by the marker, expressed as percentage of total intestinal length, in subgroup SK B was 20% ± 2% in comparison with subgroup KET B 25.9% ± 1.64%. In the control B group, the distance traveled was 27% ± 1.4% (Figure 3). Intestinal transit showed statistically significant delay in the SK subgroup compared with the other groups in this study ($P = 0.0004$). Group KET B showed no significant alteration in transit speed compared to the control B group. The frequency of the BER was not statistically different between any of the groups after 24 h reperfusion: Control B 11.5 ± 1.1/min; SK B 11.2 ± 0.5/min; KET B 11.75 ± 0.95/min.

DISCUSSION

The alterations on intestinal motility caused by I/R are directly related to the length of both ischemia and reperfusion[14]. The functional alterations caused by I/R have been previously identified[15]. These changes consist of reversible alterations in smooth muscle contractility and intestinal transit, as well as characteristic changes in electrical activity such as a decrease in BER frequency during ischemia[7,16]. Nitric oxide is one of the mediators thought to participate in the changes caused by I/R on intestinal motility in the rat[17].

The structural damage caused by ischemia is aggravated by the restitution of blood flow. The physiopathology of intestinal mucosal damage by I/R is not completely understood. But, it is believed that cytotoxic substances such as free radicals, nitric oxide, serotonin, complement, as well as neutrophil infiltration and nuclear transcription factors play important roles[18]. Macrophages have been also implicated in the initial damage caused by intestinal I/R[19]. I/R has also been shown to induce apoptosis[20]. Bacterial translocation and mucosal barrier dysfunction have been implicated in the damage caused by I/R in the gut[21]. This damage is also related to an increase in circulating levels of pro-inflammatory cytokines such as interleukin-6 and tumor necrosis factor alpha (TNF-alpha), liberated by the intestine itself[22].

Recently, anti-inflammatory properties of ketamine

have been described in various experimental models. Interleukin-1, 6, 8 and TNF-alpha levels are reduced after the administration of ketamine[23-25]. Neutrophil adhesion, migration, and free radical release is inhibited by ketamine[26,27], as is nitric oxide production by macrophages[28]. Further studies are needed to determine whether one of these effects is responsible for the mucosal protection against I/R that we observed in this study.

Glutamate is an excitatory neurotransmitter in the central nervous system, and it participates in the modulation of intestinal function through the enteric nervous system, where NMDA receptors have been found[29]. It has been shown that the glutamate system is involved in the pathogenesis of I/R in brain and intestine[30]. Glutamate release and NMDA receptor activation induces nitric oxide, and other free radicals that cause tissue injury[31]. Glutamate release during I/R could also cause toxicity in the enteric nervous system, which could cause motility alterations[32]. One recent study found that intestinal I/R causes myenteric plexus ganglion cell reduction and death[33]. This suggests that the NMDA receptor could be related to the functional alterations induced in the intestine by I/R. In one study, the NMDA antagonist MK-801 diminished nitric oxide production, and prevented the changes in the intestinal transit caused by intestinal ischemia/reperfusion[34]. We showed that ketamine, an NMDA receptor antagonist, also corrects the intestinal transit delay caused by intestinal I/R, but we did not find any changes in the frequency of the BER after 24 h of reperfusion. Further studies are needed in order to determine whether our results are explained by ketamine acting as an NMDA receptor antagonist in the gut.

Ketamine protects the intestine against other types of non-ischemic insults. Ketamine has been shown to protect the gut against endotoxemia induced injury in rats through diminished pro-inflammatory cytokine release[35]. Ketamine also decreased enterocyte apoptosis in a model of thermal burn induced intestinal injury[36]. However, this is the first study to demonstrate that ketamine anesthesia reduces I/R induced injury in intestinal mucosa.

COMMENTS

Background

In intestinal diseases associated with ischemia and reperfusion, intestinal tissue is damaged and intestinal function is altered. In this study, the aim was to investigate the effects of ketamine anesthesia in rats in which intestinal ischemia/reperfusion was induced.

Research frontiers

The authors suggest that ketamine anesthesia may have beneficial effects that prevent the tissue damage and functional alterations that follow from intestinal ischemia/reperfusion.

Innovations and breakthroughs

This study is the first to show that ketamine anesthesia can reduce the intestinal tissue damage induced by ischemia reperfusion.

Applications

Ketamine should be further studied and the possibility of using ketamine as anesthetic in cases of intestinal ischemia should be considered.

Peer review

Methods and results are clear, but in order for the authors to come to the conclusion that ketamine is functioning as an NMDA receptor antagonist the following experiment needs to be done: Compare the effect of pentobarbital plus a selective NMDA antagonist *vs.* ketamine plus a selective NMDA antagonist. If the authors' conclusion is correct the selective NMDA receptor antagonist should "nullify" the beneficial effect of ketamine.

REFERENCES

1 **Chang RW**, Chang JB, Longo WE. Update in management of mesenteric ischemia. *World J Gastroenterol* 2006; **12**: 3243-3247

2 **Statler KD**, Alexander H, Vagni V, Dixon CE, Clark RS, Jenkins L, Kochanek PM. Comparison of seven anesthetic agents on outcome after experimental traumatic brain injury in adult, male rats. *J Neurotrauma* 2006; **23**: 97-108

3 **Lee HT**, Ota-Setlik A, Fu Y, Nasr SH, Emala CW. Differential protective effects of volatile anesthetics against renal ischemia-reperfusion injury in vivo. *Anesthesiology* 2004; **101**: 1313-1324

4 **Tsao CM**, Wu CC, Wang JJ, Wong CS, Tsai SK, Ho ST. Intravenous anesthetics in sepsis. *Acta Anaesthesiol Taiwan* 2005; **43**: 153-163

5 **Tendler DA**. Acute intestinal ischemia and infarction. *Semin Gastrointest Dis* 2003; **14**: 66-76

6 **Arumugam TV**, Arnold N, Proctor LM, Newman M, Reid RC, Hansford KA, Fairlie DP, Shiels IA, Taylor SM. Comparative protection against rat intestinal reperfusion injury by a new inhibitor of sPLA2, COX-1 and COX-2 selective inhibitors, and an LTC4 receptor antagonist. *Br J Pharmacol* 2003; **140**: 71-80

7 **Ballabeni V**, Barocelli E, Bertoni S, Impicciatore M. Alterations of intestinal motor responsiveness in a model of mild mesenteric ischemia/reperfusion in rats. *Life Sci* 2002; **71**: 2025-2035

8 **Haglund U**, Bulkley GB, Granger DN. On the pathophysiology of intestinal ischemic injury. Clinical review. *Acta Chir Scand* 1987; **153**: 321-324

9 **Annetta MG**, Iemma D, Garisto C, Tafani C, Proietti R. Ketamine: new indications for an old drug. *Curr Drug Targets* 2005; **6**: 789-794

10 **Himmelseher S**, Durieux ME. Revising a dogma: ketamine for patients with neurological injury? *Anesth Analg* 2005; **101**: 524-534, table of contents

11 **Kato R**, Foex P. Myocardial protection by anesthetic agents against ischemia-reperfusion injury: an update for anesthesiologists. *Can J Anaesth* 2002; **49**: 777-791

12 **Salman AE**, Dal D, Salman MA, Iskit AB, Aypar U. The effect of ketamine on acute muscular ischaemia reperfusion in rats. *Eur J Anaesthesiol* 2005; **22**: 712-716

13 **Chiu CJ**, McArdle AH, Brown R, Scott HJ, Gurd FN. Intestinal mucosal lesion in low-flow states. I. A morphological, hemodynamic, and metabolic reappraisal. *Arch Surg* 1970; **101**: 478-483

14 **Pawlik WW**, Thor P, Sendur R, Biernat J, Koziol R, Wasowicz P. Myoelectric bowel activity in ischemia/reperfusion damage. Role of sensory neurons. *J Physiol Pharmacol* 1998; **49**: 543-551

15 **Moore-Olufemi SD**, Kozar RA, Moore FA, Sato N, Hassoun HT, Cox CS Jr, Kone BC. Ischemic preconditioning protects against gut dysfunction and mucosal injury after ischemia/reperfusion injury. *Shock* 2005; **23**: 258-263

16 **Ladipo JK**, Seidel SA, Bradshaw LA, Halter S, Wikswo JP Jr, Richards WO. Histopathologic changes during mesenteric ischaemia and reperfusion. *West Afr J Med* 2003; **22**: 59-62

17 **Takahashi A**, Tomomasa T, Kaneko H, Watanabe T, Tabata M, Morikawa H, Tsuchida Y, Kuwano H. Intestinal motility in an in vivo rat model of intestinal ischemia-reperfusion with special reference to the effects of nitric oxide on the motility changes. *J Pediatr Gastroenterol Nutr* 2001; **33**: 283-288

18 **Cerqueira NF**, Hussni CA, Yoshida WB. Pathophysiology of mesenteric ischemia/reperfusion: a review. *Acta Cir Bras*

2005; **20**: 336-343
19 **Chen Y**, Lui VC, Rooijen NV, Tam PK. Depletion of intestinal resident macrophages prevents ischaemia reperfusion injury in gut. *Gut* 2004; **53**: 1772-1780
20 **Noda T**, Iwakiri R, Fujimoto K, Matsuo S, Aw TY. Programmed cell death induced by ischemia-reperfusion in rat intestinal mucosa. *Am J Physiol* 1998; **274**: G270-G276
21 **Chang JX**, Chen S, Ma LP, Jiang LY, Chen JW, Chang RM, Wen LQ, Wu W, Jiang ZP, Huang ZT. Functional and morphological changes of the gut barrier during the restitution process after hemorrhagic shock. *World J Gastroenterol* 2005; **11**: 5485-5491
22 **Grotz MR**, Deitch EA, Ding J, Xu D, Huang Q, Regel G. Intestinal cytokine response after gut ischemia: role of gut barrier failure. *Ann Surg* 1999; **229**: 478-486
23 **Kawasaki T**, Ogata M, Kawasaki C, Ogata J, Inoue Y, Shigematsu A. Ketamine suppresses proinflammatory cytokine production in human whole blood in vitro. *Anesth Analg* 1999; **89**: 665-669
24 **Taniguchi T**, Kanakura H, Takemoto Y, Kidani Y, Yamamoto K. Effects of ketamine and propofol on the ratio of interleukin-6 to interleukin-10 during endotoxemia in rats. *Tohoku J Exp Med* 2003; **200**: 85-92
25 **Taniguchi T**, Yamamoto K. Anti-inflammatory effects of intravenous anesthetics on endotoxemia. *Mini Rev Med Chem* 2005; **5**: 241-245
26 **Weigand MA**, Schmidt H, Zhao Q, Plaschke K, Martin E, Bardenheuer HJ. Ketamine modulates the stimulated adhesion molecule expression on human neutrophils in vitro. *Anesth Analg* 2000; **90**: 206-212
27 **Nishina K**, Akamatsu H, Mikawa K, Shiga M, Maekawa N, Obara H, Niwa Y. The inhibitory effects of thiopental, midazolam, and ketamine on human neutrophil functions. *Anesth Analg* 1998; **86**: 159-165
28 **Shimaoka M**, Iida T, Ohara A, Taenaka N, Mashimo T, Honda T, Yoshiya I. Ketamine inhibits nitric oxide production in mouse-activated macrophage-like cells. *Br J Anaesth* 1996; **77**: 238-242
29 **Liu MT**, Rothstein JD, Gershon MD, Kirchgessner AL. Glutamatergic enteric neurons. *J Neurosci* 1997; **17**: 4764-4784
30 **Xu L**, Sun J, Lu R, Ji Q, Xu JG. Effect of glutamate on inflammatory responses of intestine and brain after focal cerebral ischemia. *World J Gastroenterol* 2005; **11**: 733-736
31 **Said SI**, Berisha HI, Pakbaz H. N-methyl-D-aspartate receptors outside the central nervous system: activation causes acute lung injury that is mediated by nitric oxide synthesis and prevented by vasoactive intestinal peptide. *Neuroscience* 1995; **65**: 943-946
32 **Kirchgessner AL**, Liu MT, Alcantara F. Excitotoxicity in the enteric nervous system. *J Neurosci* 1997; **17**: 8804-8816
33 **Linhares GK**, Martins JL, Fontanezzi F, Patricio Fdos R, Montero EF. Do lesions of the enteric nervous system occur following intestinal ischemia/reperfusion? *Acta Cir Bras* 2007; **22**: 120-124
34 **Calcina F**, Barocelli E, Bertoni S, Furukawa O, Kaunitz J, Impicciatore M, Sternini C. Effect of N-methyl-d-aspartate receptor blockade on neuronal plasticity and gastrointestinal transit delay induced by ischemia/reperfusion in rats. *Neuroscience* 2005; **134**: 39-49
35 **Sun J**, Wang XD, Liu H, Xu JG. Ketamine suppresses endotoxin-induced NF-kappaB activation and cytokines production in the intestine. *Acta Anaesthesiol Scand* 2004; **48**: 317-321
36 **Yagmurdur H**, Aksoy M, Arslan M, Baltaci B. The effects of propofol and ketamine on gut mucosal epithelial apoptosis in rats after burn injury. *Eur J Anaesthesiol* 2007; **24**: 46-52

S- Editor Zhong XY **L- Editor** Alpini GD **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5197

World J Gastroenterol 2008 September 7; 14(33): 5197-5203
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Maintenance time of sedative effects after an intravenous infusion of diazepam: A guide for endoscopy using diazepam

Mitsushige Sugimoto, Takahisa Furuta, Akiko Nakamura, Naohito Shirai, Mutsuhiro Ikuma, Shingen Misaka, Shinya Uchida, Hiroshi Watanabe, Kyoichi Ohashi, Takashi Ishizaki, Akira Hishida

Mitsushige Sugimoto, Akiko Nakamura, Mutsuhiro Ikuma, Akira Hishida, First Department of Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan
Takahisa Furuta, Center for Clinical Research, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan
Naohito Shirai, Department of Gastroenterology, Enshu General Hospital, 1-1-1 Tyuou, Naka-ku, Hamamatsu 430-0929, Japan
Shingen Misaka, Shinya Uchida, Department of Pharmacokinetics and Pharmacodynamics, School of Pharmaceutics Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan
Hiroshi Watanabe, Takashi Ishizaki, Department of Clinical Pharmacology and Therapeutics, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan
Kyoichi Ohashi, Department of Clinical Pharmacology and Therapeutics, Oita University, 1-1 Idaigaoka, Hazama, Yufu 879-5593, Japan
Author contributions: Sugimoto M, Furuta T, Nakamura A and Shirai N designed research; Sugimoto M, Furuta T, Nakamura A, Shirai N, Misaka S and Uchida S performed research; Sugimoto M, Furuta T, Nakamura A, Shirai N, Ikuma M, Misaka S, Uchida S, Watanabe H, Ohashi K, Hishida A and Ishizaki T analyzed data and wrote the paper.
Supported by Grants-in-Aid for Scientific Research from YOKOYAMA Foundation for Clinical Pharmacology and a Grant-in-Aid from the Center of Excellence (COE) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan No. 17590470
Correspondence to: Dr. Mitsushige Sugimoto, First Department of Medicine, Hamamatsu University School of Medicine, Michael E. DeBakey Veterans Affairs Medical Center, 2002 Holcombe Blvd., Rm 3A-320B, Houston, Texas 77030, United States. sugimoto@bcm.edu
Telephone: +1-713-7947280 Fax: 1+713-7954471
Received: May 23, 2008 Revised: June 30, 2008
Accepted: July 7, 2008
Published online: September 7, 2008

Abstract

AIM: To examine whether the sedative effects assessed by psychomotor tests would depend on the cytochrome P450 (*CYP*) *2C19* genotypes after an infusion regimen of diazepam commonly used for gastrointestinal endoscopy in Japan.

METHODS: Fifteen healthy Japanese volunteers consisting of three different *CYP2C19* genotype groups underwent a critical flicker fusion test, an eye movement analysis and a postural sway test as a test for physical sedative effects, and a visual analog scale (VAS) symptom assessment method as a test for mental sedative effects during the 336 h period after the intravenous infusion of diazepam (5 mg).

RESULTS: The physical sedative effects assessed by the critical flicker test continued for 1 h (t values of 5 min, 30 min and 60 min later: 4.35, 5.00 and 3.19, respectively) and those by the moving radial area of a postural sway test continued for 3 h (t values of 5 h, 30 h, 60 min and 3 h later: -4.05, -3.42, -2.17 and -2.58, respectively), which changed significantly compared with the baseline level before infusion ($P < 0.05$). On the other hand, the mental sedative effects by the VAS method improved within 1 h. The *CYP2C19* genotype-dependent differences in the postinfusion sedative effects were not observed in any of the four psychomotor function tests.

CONCLUSION: With the psychomotor tests, the objective sedative effects of diazepam continued for 1 h to 3 h irrespective of *CYP2C19* genotype status and the subjective sedative symptoms improved within 1 h. Up to 3 h of clinical care appears to be required after the infusion of diazepam, although patients feel subjectively improved.

Key words: Diazepam; Sedation; cytochrome P450 (CYP) 2C19; Endoscopy; Complication; Critical flicker fusion test; Eye movement analysis; Postural sway test; Visual analog scale

Peer reviewer: William Dickey, Altnagelvin Hospital, Londonderry, Northern Ireland BT47 6SB, United Kingdom

Sugimoto M, Furuta T, Nakamura A, Shirai N, Ikuma M, Misaka S, Uchida S, Watanabe H, Ohashi K, Ishizaki T, Hishida A. Maintenance time of sedative effects after an intravenous infusion of diazepam: A guide for endoscopy using diazepam. *World J Gastroenterol* 2008; 14(33): 5197-5203 Available from: URL: http://www.wjgnet.com/1007-9327/14/5197.asp DOI: http://dx.doi.org/10.3748/wjg.14.5197

INTRODUCTION

Gastrointestinal endoscopy is commonly performed for the screening and/or diagnosis of upper gastrointestinal disorders. Recent advances in gastrointestinal endoscopy are remarkable, and gastrointestinal endoscopy now plays an important role in the treatment of several upper gastrointestinal disorders (e.g. endoscopic mucosal resection of gastric cancer in the early stage and treatment of haemorrhage from peptic ulcer). However, gastrointestinal endoscopy is invasive and accompanied with distress of patients. Therefore, benzodiazepines, such as diazepam and midazolam, are commonly used as a premedication for gastrointestinal endoscopy[1,2]. The administration of benzodiazepines as premedications for endoscopy reduces distress of patients and results in a better patient acceptance and compliance for endoscopy[1,2]. However, use of benzodiazepines sometimes causes undesirable events; e.g. residual benzodiazepine after endoscopy may cause sustained distraction, which leads to some accidents. For the prevention of such undesirable accidents, the dose of benzodiazepines should, therefore, be minimized or optimized. However, a reduced dose of benzodiazepines sometimes results in an insufficient sedation for endoscopy, which is a dilemma for performing the safe and comfortable endoscopy.

Diazepam is metabolized by cytochrome P450 (CYP) 3A4 to temazepam, and by CYP3A4 and CYP2C19 to N-desmethyldiazepam[3,4]. There are genetic differences in the activity of CYP2C19[3,4]. The pharmacokinetics of diazepam significantly depend on *CYP2C19* genotype status[5-9], as observed in proton pump inhibitors, such as omeprazole and rabeprazole[10,11]. The *CYP2C19* genotypes are classified into the three groups: homozygous extensive metabolizers (EMs), heterozygous EMs, and poor metabolizers (PMs)[12-14]. In PMs, the plasma diazepam concentrations are markedly increased due to an impaired metabolism of diazepam in comparison with those in EMs[3-7]. However, whether the pharmacodynamic effects of an intravenous infusion of diazepam would differ between the CYP2C19 EMs and PMs remain, to our knowledge, unknown.

Psychomotor tests, such as a critical flicker fusion test, an eye movement analysis, a postural sway test and a visual analog scale (VAS) symptom assessment method are well known as psychometric markers and are commonly used to quantify the pharmacodynamic responses associated with an administration of benzodiazepine sedatives[15-17]. Indeed, plasma or serum diazepam concentrations were significantly correlated with psychomotor test scores[18,19].

In this study, we aimed to examine the objective and subjective sedative effects of an intravenous 5-mg dose of diazepam commonly used for gastrointestinal endoscopy in relation to *CYP2C19* genotypes.

MATERIALS AND METHODS

Subjects and CYP2C19 genotyping

Fifteen healthy Japanese volunteers whose *CYP2C19* genotypes had been determined by a PCR-RFLP method[20,21] were enrolled in this study (5 homozygous EMs, 5 heterozygous EMs and 5 PMs of *CYP2C19*) (Table 1). A written informed consent was obtained from each subject.

Study protocol

All subjects were given a single intravenous infusion of Diazepam (Cercine®, Takeda Pharmaceutical Co. Ltd., Osaka, Japan) 5 mg at 8:00 am, which was infused over 1 min. Four psychomotor tests, such as a critical flicker fusion test, an eye movement analysis, a postural sway test and a VAS symptom assessment method[15-17], were performed at the pre- and postinfusion time points of diazepam as follows: before the infusion and 5 min and 30 min, and 1 h, 3 h, 6 h, 10 h, 24 h, 72 h and 336 h postinfusion.

The critical flicker fusion test was measured by the discrimination of fused flickering red light (DF-1, Shibata Chemical, Tokyo, Japan). The value used for the fusion time was flickers per second. The results of an eye movement test used the saccadic latency (per second), which is time from the displacement of red light signal to the response of eye movement. The stimulated horizontal displacement of red lights at random intervals was recorded (DP1200A, Nihon Denki Sanei, Tokyo, Japan). The subjects responded to and followed a signal for 30 s as quickly as possible. The moving radial area (cm^2) for 60 s by a postural sway test was measured by a sway meter (G5500, Anima, Tokyo, Japan) for the 60 s period with eyes closed. The radial area was determined by calculating the radial distance to the center of pressure at each sampling interval from the geometric center of the stance. A VAS symptom assessment method consisted of questions of both the two parameters of mental sedation and physical sedation. Subjects had to mark on the 100 mm line to show the degree of their feeling.

All subjects were provided with three meals a day (breakfast at 7:30 am, lunch at 12:30 pm, and supper at 6:00 pm). Mineral water was allowed *ad libitum*. But, no other beverages including caffeine-containing and grapefruit juice-related products were permitted. None of them drank alcohol or had a smoking habit. None had taken any drugs for 1 mo before the study, nor did they take any during it. The subjects stayed in the translational research (TR) unit of Hamamatsu University School of Medicine during the first 24 h postinfusion period. Four psychomotor tests at 72 h and 336 h postinfusion were performed in the TR unit.

The protocol was approved in advance by the Human Institutional Review Board of the Hamamatsu University School of Medicine. Written informed consent was obtained from each subject before participation in the study.

Statistical analysis

The median values of four psychomotor tests and median percent changes from the baseline value (before Diazepam infusion) were determined. Statistically

Table 1 Demographic characteristics of healthy volunteers with different *CYP2C19* genotype status (n = 5)

	Homozygous EM	Heterozygous EM	PM	*P*
Genotype status	Wild-type/Wild-type (n = 5)	Wild-type/*CYP2C19* mutation in exon 5 (n = 3) Wild-type/*CYP2C19* mutation in exon 4 (n = 2)	*CYP2C19* mutation in exon 5/CYP2C19 mutation in exon 5 (n = 2) *CYP2C19* mutation in exon 4/CYP2C19 mutation in exon 4 (n = 1) *CYP2C19* mutation in exon 5/CYP2C19 mutation in exon 4 (n = 2)	
Male/Female	3/2	3/2	5/0	0.472
Age (yr)	24 (22-31)	29 (25-44)	23 (22-35)	0.093
Height (cm)	170 (154-174)	177 (155-181)	171 (169-180)	0.400
Body weight (kg)	62 (51-67)	67 (51-78)	65 (57-78)	0.424

Age and body weight are given as median (range). EM: Extensive metabolizer; PM: Poor metabolizer.

significant differences in the median pharmacodynamic parameters among the 3 different *CYP2C19* genotype groups at the pre- and postinfusion time points were determined by the Mann-Whitney *U*-test, when a significant difference was obtained by the Kruskal-Wallis test. Statistical differences with the median parameters between the different pre- and postinfusion time points in all of the subjects enrolled in this study were determined by using the Wilcoxon's signed rank test, when significant differences were obtained by the Friedmann's test. All *P* values were two-sided, and $P < 0.05$ was taken to indicate statistical significance.

RESULTS

There were no statistically significant differences in the demographic characteristics, such as age, body weight, height and sex ratio among the 3 different *CYP2C19* genotype groups (Table 1).

Objective sedative effects assessed by critical flicker fusion, postural sway and eye movement tests

Percent changes in the critical flicker fusion test, postural sway test, and eye movement test after an intravenous administration of 5 mg of diazepam as a function of *CYP2C19* genotype status are shown in Figure 1. Sedative effects with diazepam (5 mg) assessed by those methods did not significantly differ among the different *CYP2C19* genotype groups throughout the 336 h postinfusion period ($P > 0.05$) (Figure 1). For this reasoning, the data derived from the different *CYP2C19* genotypes were combined together in the following analyses.

In a critical flicker fusion test, the median percent changes from the baseline values (before diazepam infusion) and the postinfusion values at 5 and 30 min, and at 1 h significantly differed ($P = 0.0038$, 0.0018 and 0.0090, respectively) (Figure 2A). The median percent changes from the baseline values still appeared increased for longer than 3 h postinfusion, but not significantly (Figure 1A).

In the moving radial area of a postural sway test, the median values at 5 and 30 min, and 1 h and 3 h postinfusion were 167.9% (range; 100.3%-456.7%), and 153.2% (52.3%-391.0%), and 111.2% (75.6%-281.8%) and 130.8% (51.0%-337.1%), which were significantly greater than the baseline value ($P = 0.0007$, and 0.0018, and 0.0409 and 0.0125, respectively) (Figure 2B). These objective sedative assessment values required 3 h to 6 h postinfusion to return to the respective baseline levels (Figure 2).

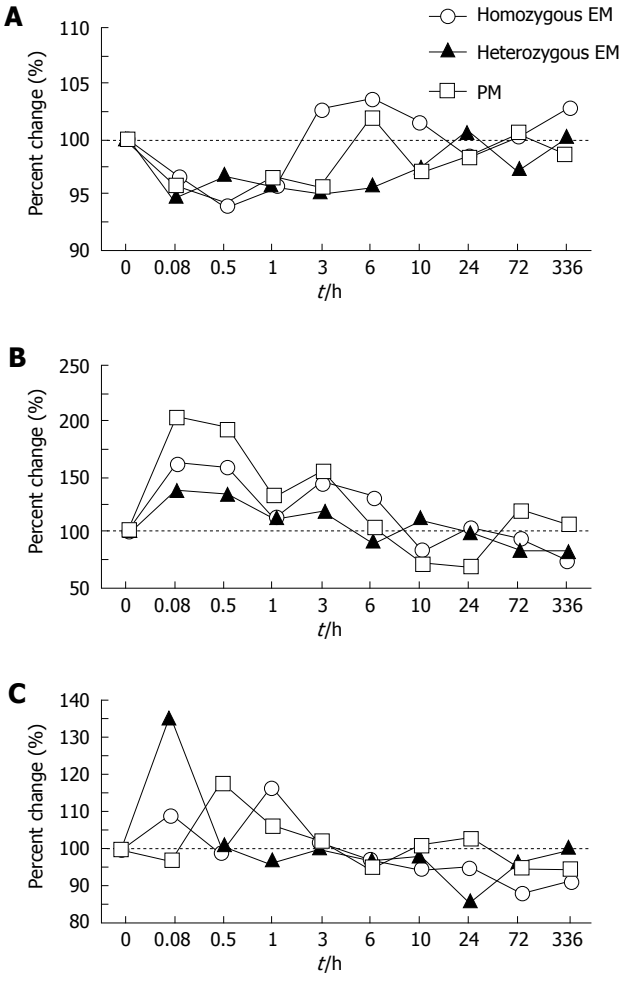


Figure 1 Median percent changes from the baseline value (before a diazepam 5 mg infusion) by a critical flicker fusion test (**A**), a postural sway test (**B**), and an eye movement test (**C**) in the 3 different *CYP2C19* genotype groups.

In the saccadic latency of an eye moving test, the median percent change from the baseline at 5 min postinfusion was 115.9% (81.9%-141.1%) ($P = 0.0171$) (Figure 2C).

The overall results indicated that the objective

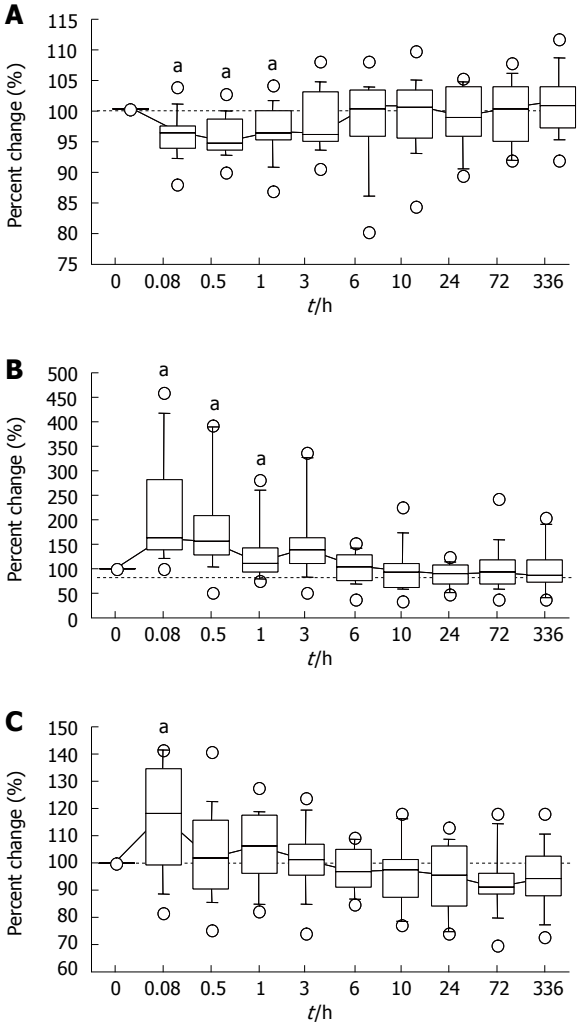


Figure 2 Median percent changes from the baseline value [before a diazepam (5-mg) infusion] by a critical flicker fusion test (**A**), a postural sway test (**B**), and an eye movement test (**C**) in all of the enrolled subjects. [a]$P < 0.05$ by using the Wilcoxon's signed rank test, when significant differences were obtained by the Friedmann's test.

sedative effects of diazepam assessed by the three methods continued for up to 3 h postinfusion.

Subjective sedative effects assessed by the VAS method

The sedative effects assessed by VAS method were fairly similar among the different *CYP2C19* genotype groups throughout the 336-h postinfusion period ($P > 0.05$) (Figure 3). The data of VAS method obtained from all of the subjects with different *CYP2C19* genotypes were, therefore, combined as mentioned above.

The median values by "alert to drowsy" of mental sedation parameter at 5 and 30 min postinfusion were significantly decreased compared with the baseline value ($P = 0.0098$ and 0.0047, respectively) (Figure 4A). This value returned to the level not different from the baseline of 1 h later (Figure 4A). In the "fuzzy to clear headed" of mental parameter, the median value at 5 min later was significantly lower ($P = 0.076$), but returned to the baseline at 30 min postinfusion (Figure 4B).

The median values of the "well-coordinated to clumsy" physical sedation parameter at 5 min and 30 min and 1 h after diazepam infusion were significantly increased compared with the baseline value ($P = 0.0031$, and 0.0038 and 0.0468, respectively) (Figure 4C). In the "lethargic to energetic" physical parameter, the median values at 5 min and 30 min postinfusion were significantly lower ($P = 0.0119$ and 0.0468, respectively), but returned to baseline level by 1 h postinfusion (Figure 4D).

These results assessed by the VAS symptom assessment method indicated that the subjective sedative effects of diazepam (5 mg) continued for less than 1 h postinfusion, although the objective sedative effects continued longer (for 1 h to 3 h postinfusion).

DISCUSSION

The development of an optimal infusion regimen of a premedication (e.g. diazepam) and an appropriate manual check at the endoscopy unit is necessary for a comfortable and safe gastrointestinal endoscopy. Although the proper premedication for gastrointestinal endoscopy should have a shorter onset of action, short elimination half-life, and faster time to recovery, diazepam is one of the first-line sedative drugs used as a premedication for gastrointestinal endoscopy in Japan. In this study, we demonstrated that the objective sedative effects by an intravenous infusion of diazepam (5 mg) continued for no less than 3 h with psychological tests, whereas the subjective sedative effects continued for no more than 1 h. Although the plasma diazepam concentrations of individuals with CYP2C19 PMs have been reported to be greater than those with homozygous EMs[5-9], we did not find any significantly different sedative effects of diazepam among the different *CYP2C19* genotype groups. Based upon these observations, we thought that patients who undergo an intravenous infusion of diazepam (5 mg) should be cared for at least 3 h postinfusion in the hospital irrespective of *CYP2C19* genotype status for the prevention of adverse events.

In the present study, the values of VAS method used for assessing the subjective symptom parameters demonstrated continuous sedative effects only during 30 min to 60 min after the infusion of diazepam (5 mg). After 1 h had passed from administration, the major parameters of the VAS assessment method returned to the respective baseline levels. However, the objective assessment by a critical flicker fusion test and a postural sway test revealed that the sedative effects of diazepam remained for approximately 3 h to 6 h postinfusion, indicating that a discrepancy exists between the subjective and objective sedative symptoms from 1 h to 6 h after the infusion of diazepam. Therefore, even if the patients feel improved or recovered from the sedation by diazepam at 1 h postinfusion, they may be at risk for some adverse effects, such as falling down and/or having driving errors. Then, we anticipate that the patients who undergo an intravenous infusion of diazepam (5 mg) should be checked for the prevention of possible sedative adverse effects during a 6 h postinfusion period at the endoscopy unit.

The sedative effects of diazepam are mediated *via* α1-$GABA_A$ receptors in the brain[22,23], and the minimum plasma concentration of diazepam to yield sedative

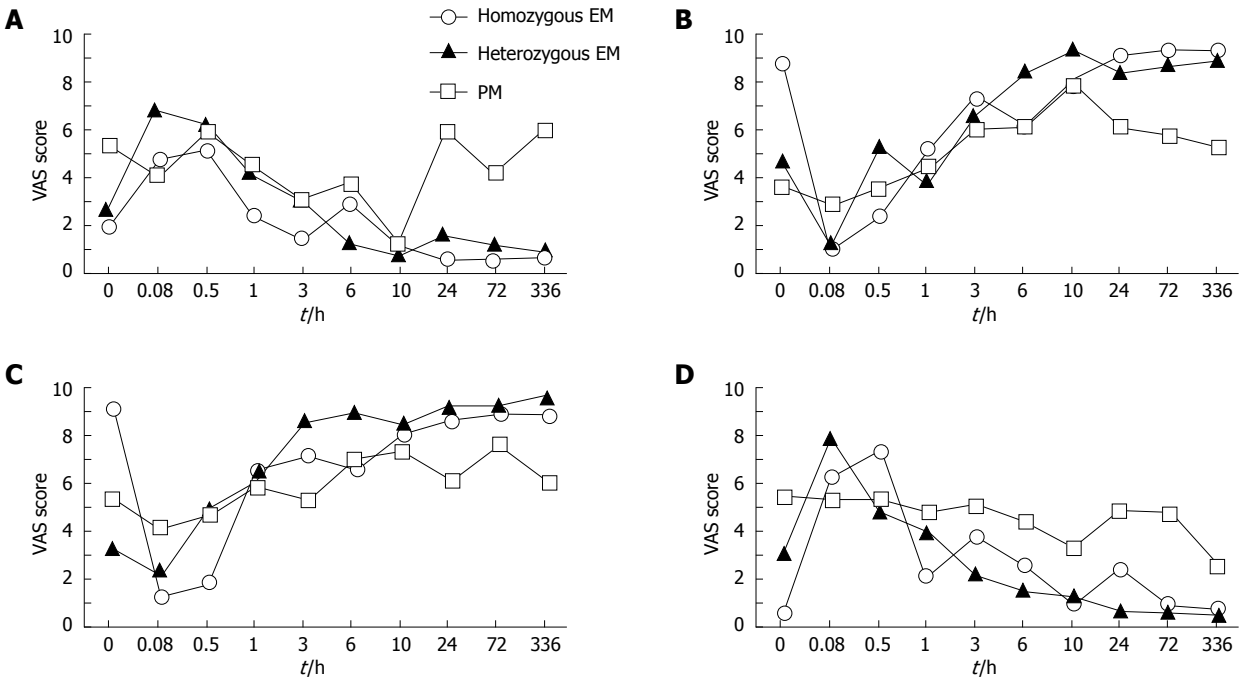


Figure 3 Median percent values for mental sedation, "alert to drowsy" (**A**), "fuzzy to clear-headed" (**B**), physical sedation, "lethargic to energetic" (**C**) and "well-coordinated to clumsy" (**D**) parameters by a VAS symptom assessment test in the 3 different *CYP2C19* genotype groups.

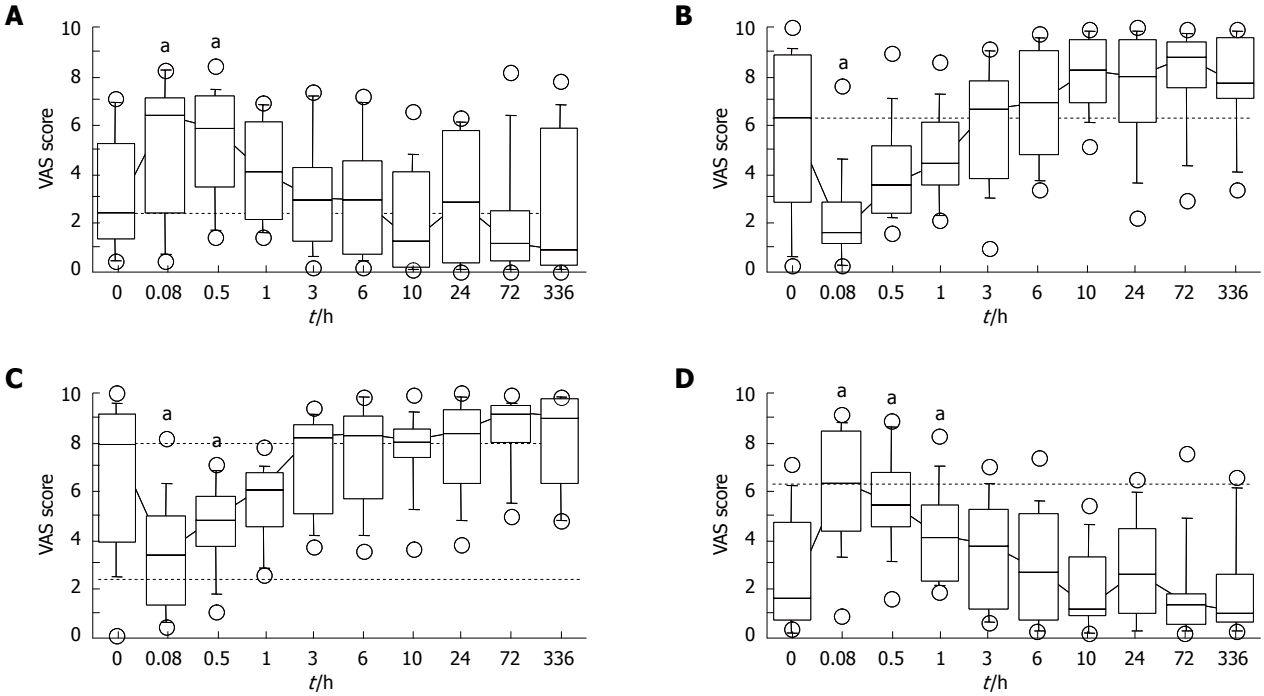


Figure 4 Median percent values for mental sedation "alert to drowsy" (**A**), "muzzy to clear-headed" (**B**), physical sedation "lethargic to energetic" (**C**) and "well-coordinated to clumsy" (**D**) parameters by a VAS symptom assessment test in all of the enrolled subjects. $^{a}P < 0.05$ by using the Wilcoxon's signed rank test, when significant differences were obtained by the Friedmann's test.

effects is more than 300 ng/mL to 400 ng/mL[18,19]. The pharmacokinetics of diazepam are affected by age[24,25], gender[26], obesity[26], liver disease[27] and *CYP2C19* genotype status[5-9]. The mean plasma elimination half-life of diazepam in PMs of CYP2C19 has been reported to be much longer than that in homozygous EMs: those values after a single oral dose of diazepam were 84.0 ± 13.7 h for PMs, 62.9 ± 9.8 h for heterozygous EMs and 20.0 ± 10.8 h for homozygous EMs[28]. However, plasma diazepam levels do not always correlate with the sedative effects[29,30]. Moreover, Kosuge *et al*[16] reported that influences of diazepam on psychomotor functions did not differ among the 3 different *CYP2C19* genotype groups, as observed in this study. Although we cannot offer appropriate explanations for the lack of difference in the pharmacodynamics of diazepam among the different

CYP2C19 genotype groups, several possible mechanisms can be raised as follows: first, the acute tolerance to diazepam may be developed, which is mediated by the dysfunction of the cortical GABA transmitter system, such as the decrease of glutaminic acid decarboxylase[31,32], reelin[33] and GABA membrane transporter[34], GABAA receptor up-regulation[35] and the decrease of dendritic spines[36]. Second, diazepam as well as its metabolites such as temazepam and N-desmethyldiazepam, have sedative effects[3,4]. Therefore, although the metabolic disposition of diazepam differs among the different *CYP2C19* genotype groups, the total amounts of diazepam plus its active metabolites would not differ among the different *CYP2C19* genotype groups, resulting in no statistical difference in the pharmacodynamics of diazepam. Third, our pharmacodynamic assessment methods may not have a sufficient power for the limited sample size (n = 15) in the psychomotor function status. Nevertheless, in a cimetidine-diazepam interaction study, only minimal changes were observed in the pharmacodynamic effects despite an increase in plasma diazepam concentration by about 60% during treatment with cimetidine[37]. Similarly, in a fluoxetine-diazepam interaction study[38], despite that the significant increment of plasma diazepam concentration occurred by about 50% with the co-administration of fluoxetine, no psychopharmacological changes were detected by the pharmacodynamic assessment methods similar to those we used. With the limitation of absent data on plasma concentrations of diazepam and its metabolites (e.g. desmethyldiazepam and temazepam) in this study, we are tempted to assume that the sedative effects assessed by the pharmacodynamic assessment methods we used would not differ among the different *CYP2C19* genotype groups when given an intravenous 5-mg infusion of diazepam as noted above.

Recently, when gastrointestinal endoscopy is performed for patients, midazolam and propofol are often used as a sedative drug. However, midazolam and propofol act quickly and potently, and therefore, advanced effects such as respiratory trouble occurs sometimes. However, the proper premedication for gastrointestinal endoscopy should have a shorter onset of action, short elimination half-life, and faster time to recovery. Therefore, we hope to evaluate the sedative effects of those drugs by using the psychopharmacological tests.

In conclusion, this study suggests that the psychopharmacological tests appear to be a useful tool for determining the optimal treatment with a benzodiazepine, such as diazepam, for gastrointestinal endoscopy. If patients undergo gastrointestinal endoscopy for sedation by an intravenous infusion of diazepam (5 mg), the sedative effects of patients should be monitored for preventing the possible adverse effects up to at least a 3-h postinfusion period in the endoscopy unit of the hospital, although patients appear to subjectively feel unimpaired or recovered.

COMMENTS

Background

Benzodiazepine is commonly used as a premedication for gastrointestinal endoscopy. However, use of benzodiazepine sometimes causes undesirable events, which leads to some accidents. For the prevention of such undesirable accidents, therefore, the dose of premedication should be minimized or optimized. For benzodiazepine, the pharmacokinetics of diazepam depends significantly on cytochrome P450 (*CYP*) *2C19* genotype status. However, whether the pharmacodynamic effects of an intravenous infusion would differ between the CYP2C19 EMs and PMs remain unknown. Moreover, there was no optimal protocol of diazepam use in the endoscopy unit.

Research frontiers

When patients receive gastrointestinal endoscopy, the development of optimal infusion regimens of sedative drug and care protocol in the endoscopy unit are required to prevent advanced effects in relation to *CYP2C19* genotypes.

Innovations and breakthroughs

There are many reports, which investigate the pharmacokinetics and pharmacodynamics of diazepam in pharmacological studies. The innovation of this study is to demonstrate the recommendation and attention of gastrointestinal endoscopy with diazepam (5 mg/body) as follows: An up to 3 h clinical care appears to be required after the infusion of diazepam irrespective of *CYP2C19* genotype status, although patients feel subjectively improved.

Applications

The physical sedative effects assessed by the critical flicker test continued for 1 h and those by the moving radial area of a postural sway test continued for 3 h, which significantly changed compared with the baseline level before infusion ($P < 0.05$). On the other hand, the mental sedative effects by the VAS method improved within 1 h. The *CYP2C19* genotype-dependent differences in the postinfusion sedative effects were not observed in any of the four psychomotor function tests. Therefore, up to 3-h of clinical care is required after the infusion of diazepam (5 mg) irrespective of *CYP2C19* genotype status, although patients feel markedly improved. Recently, many drugs, such as midazolam and propofol, were used at endoscopy as sedation drugs. Additional research, which compares with diazepam and other sedation drugs in efficacy and care time using the psychopharmacological tests, is required.

Terminology

The critical flicker fusion test: the measurement of the discrimination of fused flickering red light. The value used for the fusion time was flickers per second. The results of an eye movement test: the saccadic latency (per second), which is time from the displacement of red light signal to the response of eye movement. The subjects responded to and followed a signal for 30 s as quickly as possible. The postural sway test: the measurement of moving radial area (cm^2) for the 60 s period with eyes closed. The radial area was determined by calculating the radial distance to the center of pressure at each sampling interval from the geometric center of the stance.

Peer review

Authors examined whether the sedative effects assessed by psychomotor tests would depend on the *CYP2C19* genotypes after an infusion regimen of diazepam commonly used for gastrointestinal endoscopy in Japan. It's an interesting study, and the methodology is sound, and is a good objective assessment.

REFERENCES

1 **Bell GD**. Review article: premedication and intravenous sedation for upper gastrointestinal endoscopy. *Aliment Pharmacol Ther* 1990; **4**: 103-122
2 **Nagengast FM**. Sedation and monitoring in gastrointestinal endoscopy. *Scand J Gastroenterol Suppl* 1993; **200**: 28-32
3 **Yasumori T**, Nagata K, Yang SK, Chen LS, Murayama N, Yamazoe Y, Kato R. Cytochrome P450 mediated metabolism of diazepam in human and rat: involvement of human CYP2C in N-demethylation in the substrate concentration-dependent manner. *Pharmacogenetics* 1993; **3**: 291-301
4 **Andersson T**, Miners JO, Veronese ME, Birkett DJ. Diazepam metabolism by human liver microsomes is mediated by both S-mephenytoin hydroxylase and CYP3A isoforms. *Br J Clin Pharmacol* 1994; **38**: 131-137
5 **Andersson T**, Cederberg C, Edvardsson G, Heggelund A, Lundborg P. Effect of omeprazole treatment on diazepam plasma levels in slow versus normal rapid metabolizers of omeprazole. *Clin Pharmacol Ther* 1990; **47**: 79-85

6 **Ishizaki T**, Chiba K, Manabe K, Koyama E, Hayashi M, Yasuda S, Horai Y, Tomono Y, Yamato C, Toyoki T. Comparison of the interaction potential of a new proton pump inhibitor, E3810, versus omeprazole with diazepam in extensive and poor metabolizers of S-mephenytoin 4'-hydroxylation. *Clin Pharmacol Ther* 1995; **58**: 155-164

7 **Sohn DR**, Kusaka M, Ishizaki T, Shin SG, Jang IJ, Shin JG, Chiba K. Incidence of S-mephenytoin hydroxylation deficiency in a Korean population and the interphenotypic differences in diazepam pharmacokinetics. *Clin Pharmacol Ther* 1992; **52**: 160-169

8 **Wan J**, Xia H, He N, Lu YQ, Zhou HH. The elimination of diazepam in Chinese subjects is dependent on the mephenytoin oxidation phenotype. *Br J Clin Pharmacol* 1996; **42**: 471-474

9 **Schmider J**, Greenblatt DJ, von Moltke LL, Shader RI. Relationship of in vitro data on drug metabolism to in vivo pharmacokinetics and drug interactions: implications for diazepam disposition in humans. *J Clin Psychopharmacol* 1996; **16**: 267-272

10 **Sugimoto M**, Furuta T, Shirai N, Kajimura M, Hishida A, Sakurai M, Ohashi K, Ishizaki T. Different dosage regimens of rabeprazole for nocturnal gastric acid inhibition in relation to cytochrome P450 2C19 genotype status. *Clin Pharmacol Ther* 2004; **76**: 290-301

11 **Shirai N**, Furuta T, Moriyama Y, Okochi H, Kobayashi K, Takashima M, Xiao F, Kosuge K, Nakagawa K, Hanai H, Chiba K, Ohashi K, Ishizaki T. Effects of CYP2C19 genotypic differences in the metabolism of omeprazole and rabeprazole on intragastric pH. *Aliment Pharmacol Ther* 2001; **15**: 1929-1937

12 **Chang M**, Dahl ML, Tybring G, Gotharson E, Bertilsson L. Use of omeprazole as a probe drug for CYP2C19 phenotype in Swedish Caucasians: comparison with S-mephenytoin hydroxylation phenotype and CYP2C19 genotype. *Pharmacogenetics* 1995; **5**: 358-363

13 **Chang M**, Tybring G, Dahl ML, Gotharson E, Sagar M, Seensalu R, Bertilsson L. Interphenotype differences in disposition and effect on gastrin levels of omeprazole--suitability of omeprazole as a probe for CYP2C19. *Br J Clin Pharmacol* 1995; **39**: 511-518

14 **Ishizaki T**, Horai Y. Review article: cytochrome P450 and the metabolism of proton pump inhibitors--emphasis on rabeprazole. *Aliment Pharmacol Ther* 1999; **13** Suppl 3: 27-36

15 **Norris H**. The action of sedatives on brain stem oculomotor systems in man. *Neuropharmacology* 1971; **10**: 181-191

16 **Kosuge K**, Jun Y, Watanabe H, Kimura M, Nishimoto M, Ishizaki T, Ohashi K. Effects of CYP3A4 inhibition by diltiazem on pharmacokinetics and dynamics of diazepam in relation to CYP2C19 genotype status. *Drug Metab Dispos* 2001; **29**: 1284-1289

17 **Sundstrom I**, Ashbrook D, Backstrom T. Reduced benzodiazepine sensitivity in patients with premenstrual syndrome: a pilot study. *Psychoneuroendocrinology* 1997; **22**: 25-38

18 **Bowden CL**, Fisher JG. Relationship of diazepam serum level to antianxiety effects. *J Clin Psychopharmacol* 1982; **2**: 110-114

19 **Dasberg HH**, van der Kleijn E, Guelen JP, van Praag HM. Plasma concentrations of diazepam and of its metabolite N-desmethyldiazepam in relation to anxiolytic effect. *Clin Pharmacol Ther* 1974; **15**: 473-483

20 **de Morais SM**, Wilkinson GR, Blaisdell J, Meyer UA, Nakamura K, Goldstein JA. Identification of a new genetic defect responsible for the polymorphism of (S)-mephenytoin metabolism in Japanese. *Mol Pharmacol* 1994; **46**: 594-598

21 **de Morais SM**, Wilkinson GR, Blaisdell J, Nakamura K, Meyer UA, Goldstein JA. The major genetic defect responsible for the polymorphism of S-mephenytoin metabolism in humans. *J Biol Chem* 1994; **269**: 15419-15422

22 **Tauber M**, Calame-Droz E, Prut L, Rudolph U, Crestani F. alpha2-gamma-Aminobutyric acid (GABA)A receptors are the molecular substrates mediating precipitation of narcosis but not of sedation by the combined use of diazepam and alcohol in vivo. *Eur J Neurosci* 2003; **18**: 2599-2604

23 **van Rijnsoever C**, Tauber M, Choulli MK, Keist R, Rudolph U, Mohler H, Fritschy JM, Crestani F. Requirement of alpha5-GABAA receptors for the development of tolerance to the sedative action of diazepam in mice. *J Neurosci* 2004; **24**: 6785-6790

24 **Herman RJ**, Wilkinson GR. Disposition of diazepam in young and elderly subjects after acute and chronic dosing. *Br J Clin Pharmacol* 1996; **42**: 147-155

25 **Divoll M**, Greenblatt DJ, Ochs HR, Shader RI. Absolute bioavailability of oral and intramuscular diazepam: effects of age and sex. *Anesth Analg* 1983; **62**: 1-8

26 **Greenblatt DJ**, Abernethy DR, Locniskar A, Harmatz JS, Limjuco RA, Shader RI. Effect of age, gender, and obesity on midazolam kinetics. *Anesthesiology* 1984; **61**: 27-35

27 **Branch RA**, Morgan MH, James J, Read AE. Intravenous administration of diazepam in patients with chronic liver disease. *Gut* 1976; **17**: 975-983

28 **Qin XP**, Xie HG, Wang W, He N, Huang SL, Xu ZH, Ou-Yang DS, Wang YJ, Zhou HH. Effect of the gene dosage of CgammaP2C19 on diazepam metabolism in Chinese subjects. *Clin Pharmacol Ther* 1999; **66**: 642-646

29 **Marks IM**, Viswanathan R, Lipsedge MS, Gardner R. Enhanced relief of phobias by flooding during waning diazepam effect. *Br J Psychiatry* 1972; **121**: 493-505

30 **Tansella M**, Siciliani O, Burti L, Schiavon M, Zimmermann C, Gerna M, Tognoni G, Morselli PL. N-desmethyldiazepam and amylobarbitone sodium as hypnotics in anxious patients. Plasma levels, clinical efficacy and residual effects. *Psychopharmacologia* 1975; **41**: 81-85

31 **Benes FM**, Vincent SL, Alsterberg G, Bird ED, SanGiovanni JP. Increased GABAA receptor binding in superficial layers of cingulate cortex in schizophrenics. *J Neurosci* 1992; **12**: 924-929

32 **Veldic M**, Guidotti A, Maloku E, Davis JM, Costa E. In psychosis, cortical interneurons overexpress DNA-methyltransferase 1. *Proc Natl Acad Sci USA* 2005; **102**: 2152-2157

33 **Guidotti A**, Auta J, Davis JM, Di-Giorgi-Gerevini V, Dwivedi Y, Grayson DR, Impagnatiello F, Pandey G, Pesold C, Sharma R, Uzunov D, Costa E. Decrease in reelin and glutamic acid decarboxylase67 (GAD67) expression in schizophrenia and bipolar disorder: a postmortem brain study. *Arch Gen Psychiatry* 2000; **57**: 1061-1069

34 **Woo TU**, Whitehead RE, Melchitzky DS, Lewis DA. A subclass of prefrontal gamma-aminobutyric acid axon terminals are selectively altered in schizophrenia. *Proc Natl Acad Sci USA* 1998; **95**: 5341-5346

35 **Lewis DA**, Volk DW, Hashimoto T. Selective alterations in prefrontal cortical GABA neurotransmission in schizophrenia: a novel target for the treatment of working memory dysfunction. *Psychopharmacology* (Berl) 2004; **174**: 143-150

36 **Rosoklija G**, Toomayan G, Ellis SP, Keilp J, Mann JJ, Latov N, Hays AP, Dwork AJ. Structural abnormalities of subicular dendrites in subjects with schizophrenia and mood disorders: preliminary findings. *Arch Gen Psychiatry* 2000; **57**: 349-356

37 **Greenblatt DJ**, Abernethy DR, Morse DS, Harmatz JS, Shader RI. Clinical importance of the interaction of diazepam and cimetidine. *N Engl J Med* 1984; **310**: 1639-1643

38 **Lemberger L**, Rowe H, Bosomworth JC, Tenbarge JB, Bergstrom RF. The effect of fluoxetine on the pharmacokinetics and psychomotor responses of diazepam. *Clin Pharmacol Ther* 1988; **43**: 412-419

S- Editor Li DL **L- Editor** Alpini GD **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5204

World J Gastroenterol 2008 September 7; 14(33): 5204-5209
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Nigella sativa relieves the deleterious effects of ischemia reperfusion injury on liver

Fahrettin Yildiz, Sacit Coban, Alpaslan Terzi, Mustafa Ates, Nurten Aksoy, Hale Cakir, Ali Riza Ocak, Muharrem Bitiren

Fahrettin Yildiz, Alpaslan Terzi, Harran University Medical Faculty, Department of General Surgery, Sanli Urfa 63300, Turkey
Sacit Coban, Gaziantep University Medical Faculty, Department of General Surgery, Gaziantep, 27310, Turkey
Mustafa Ates, Malatya State Hospital Department of General Surgery, Malatya 44100, Turkey
Nurten Aksoy, Hale Cakir, Ali Riza Ocak, Harran University Medical Faculty, Department of Biochemistry, Sanli Urfa 63300, Turkey
Muharrem Bitiren, Harran University Medical Faculty Department of Pathology, Sanli Urfa 63300, Turkey
Author contributions: Yildiz F, Coban S and Terzi A contributed equally to this work; Yildiz F, Coban S, Terzi A and Ates M designed research; Yildiz F, Coban S and Terzi A performed research; Aksoy N, Cakir H and Ocak AR analyzed biochemical data; Bitiren M evaluated the histopathology, and Yildiz F wrote the paper.
Correspondence to: Dr. Fahrettin Yildiz, Assistant Professor, Harran University Medical Faculty, Department of General Surgery, Sanliurfa 63300, Turkey. fahrettinyildiz@hotmail.com
Telephone: +90-414-3148410 Fax: +90-414-3148410
Received: July 14, 2008 Revised: August 18, 2008
Accepted: August 25, 2008
Published online: September 7, 2008

Abstract

AIM: To determine whether *Nigella sativa* prevents hepatic ischemia-reperfusion injury to the liver.
METHODS: Thirty rats were divided into three groups as sham (Group 1), control (Group 2), and *Nigella sativa* (NS) treatment group (Group 3). All rats underwent hepatic ischemia for 45 min followed by 60 min period of reperfusion. Rats were intraperitoneally infused with only 0.9% saline solution in group 2. Rats in group 3 received NS (0.2 mL/kg) intraperitoneally, before ischemia and before reperfusion. Blood samples and liver tissues were harvested from the rats, and then the rats were sacrificed. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) levels were determined. Total antioxidant capacity (TAC), catalase (CAT), total oxidative status (TOS), oxidative stress index (OSI) and myeloperoxidase (MPO) in hepatic tissue were measured. Also liver tissue histopathology was evaluated by light microscopy.
RESULTS: The levels of liver enzymes in group 3 were significantly lower than those in the group 2. TAC in liver tissue was significantly higher in group 3 than in group 2. TOS, OSI and MPO in hepatic tissue were significantly lower in group 3 than the group 2. Histological tissue damage was milder in the NS treatment group than that in the control group.
CONCLUSION: Our results suggest that *Nigella sativa* treatment protects the rat liver against to hepatic ischemia-reperfusion injury.

Key words: *Nigella sativa*; Ischemia reperfusion injury; Liver

Peer reviewers: Valentin Fuhrmann, MD, Department of Internal Medicine 4, Intensive Care Unit, Medical University Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria; Andrej Khandoga, MD, Institute for Surgical Research Ludwig-Maximilians-University of Munich, Marchioninistr. 27, Munich 81377, Germany

Yildiz F, Coban S, Terzi A, Ates M, Aksoy N, Cakir H, Ocak AR, Bitiren M. *Nigella sativa* relieves the deleterious effects of ischemia reperfusion injury on liver. *World J Gastroenterol* 2008; 14(33): 5204-5209 Available from: URL: http://www.wjgnet.com/1007-9327/14/5204.asp DOI: http://dx.doi.org/10.3748/wjg.14.5204

INTRODUCTION

Ischemia followed by reperfusion (I/R) may cause metabolic and structural hepatic damage, and may be due to trauma, sepsis, liver transplantation[1] or hepatic pedicle clamping during liver surgery[2]. This remains a significant problem for surgical procedures, and also remains limitation of liver transplantation[3].

Oxygen free radicals, produced on reperfusion, play a critical role in the injury caused by ischemia-reperfusion[4]. Reactive oxygen radicals lead to an inflammatory response and tissue damage by activating some mediators. It can also directly damage cell components[5]. Several attempts to reduce these mechanisms have been reported in the literature. Protection against reperfusion injury can be induced by assorted treatments including administration of antioxidants and anti-inflammatory drugs[4,6-8].

Various therapeutic effects, such as antioxidant, anti-inflammatory, anticancer[9], antihistaminic[10], antibacterial effects[11] have been described for *Nigella sativa*. Additionally, it has been shown that *Nigella sativa* has protective effect against ischemia reperfusion injury to various organs[12-14]. Thymoquinone, the active constituent of *Nigella sativa* seeds, is a pharmacologically active quinone, which possesses several properties including analgesic and anti-inflammatory actions[15]. It has been reported that thymoquinone prevents oxidative injury in various *in vitro* and *in vivo* studies in rats[16,17]. It has been suggested that thymoquinone may act as an antioxidant agent and prevents membrane lipid peroxidation in tissues[18]. The mechanism of action is still largely unknown. But, it seems these effects may be related to inhibition of eicosanoid generation, namely thromboxane B2 and leucotrienes B4 (by inhibiting cyclooxygenase and 5-lipooxygenase, respectively), and membrane lipid peroxidation[13].

Moreover, it has been demonstrated that *Nigella sativa* can significantly prevent hepatotoxicity[19] and might have protective effects against nephrotoxicity induced by either disease or chemicals[13]. But, the exact mechanism is not clear. There are also several clinical studies. In one study, the prophylactic effect of boiled extract of *N. sativa* on asthmatic disease was examined[20]. Similarly, black seed oil was shown to be an effective adjuvant for the treatment of patients with allergic diseases[21]. In another clinical study, significant benefits of *Nigella sativa* extract in the treatment of acute tonsillopharyngitis was shown[22]. Also, it was shown that *Nigella sativa* has anti-epileptic effects in children with refractory seizures[23].

Therefore, it seems possible that the administration of *Nigella sativa* might protect the liver against the ischemia reperfusion injury; therefore, our aim was to confirm this hypothesis. We investigated alterations in the oxidant- antioxidant balance by measuring oxidant parameters such as total oxidative status (TOS), oxidative stress index (OSI) and myeloperoxidase (MPO), and antioxidant parameters, such as total antioxidant capacity (TAC) and catalase (CAT) in the liver tissue. Also we examined histopathological changes in the liver parenchyma.

MATERIALS AND METHODS

Thirty male Wistar-albino rats weighting 190 g to 250 g were used in this experimental study. All animals were maintained under standard conditions, and were fed water and rodent chow *ad libitum* and treated in compliance with the National Institutes of Health guidelines. Rats were deprived of food, but not water, for 24 h before surgery.

Animals were divided into three groups, sham group (Group 1), control group (Group 2), and *Nigella sativa* treatment group (Group 3). All rats were anesthetized with 0.2 mL/100 g of ketamine hydrochloride intraperitoneally. After the abdomen was shaved and disinfected, a midline incision was made and rats underwent either sham surgery or ischemia-reperfusion. Ischemia was carried out by exposing the afferent and efferent blood vessels and then clamping for 45 min with a microvascular "bulldog" clamp. Forty five minutes later, the ischemic liver was reperfused by opening the clamp, and reperfusion was achieved for 60 min. *Nigella sativa* was given to the rats in treatment group, before ischemia and before reperfusion at a dose of 0.2 mL/kg by intraperitoneal route. We chose the dose of this agent according to reported studies about I/R and *Nigella sativa*, as this dose has been shown to be effective in previous studies[24,25]. Rats in the control group were infused only with saline. At the end of the procedures, the rats were killed and blood and liver tissue samples were obtained. A portion of liver was stored at -80℃ for future analyses. Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) activities were measured for evaluating the liver functions. To assess oxidative injury, TAC, TOS and OSI levels were determined.

OSI and MPO levels

The enzyme analyses of liver tissue were performed on the supernatant fractions of the tissue homogenates. The tissues were homogenized in 150 mmol/L ice-cold KCl to make a 10% homogenate, using a glass Teflon homogenizer. Then, the samples were sonicated on ice ten times for 5 s. The homogenates were centrifuged at 12 500 *g* for 30 min at 3℃, and the supernatant fractions were obtained.

Tissue samples for histological staining were obtained and fixed in 10% formalin-phosphate-buffered saline at 4℃ overnight. The samples were dehydrated and embedded in paraffin. Sections (7 μm) were cut and stained with hematoxylin and eosin. A pathologist evaluated the slides in a blinded manner.

Biochemical analyses

Plasma was used to measure AST, ALT and LDH as indicative parameters of hepatic function. The plasma activities of AST, ALT and LDH were estimated by commercially available kits using an autoanalyser (aeroset® Abbott Laboratories, Chicago, IL).

Measurement of the total antioxidant capacity

TAC of supernatant fractions was determined using a novel automated measurement method developed by Erel[26]. In this method, hydroxyl radical, which is the most potent biological radical, is produced. In the assay, ferrous ion solution, which is present in Reagent 1, is mixed with hydrogen peroxide, which is present in Reagent 2. The sequential produced radicals such as brown colored dianisidinyl radical cation, produced by the hydroxyl radical, are also potent radicals. Using this method, antioxidative effect of the sample against the potent-free radical reactions, which is initiated by the produced hydroxyl radical, is measured. The assay has excellent precision values, lower than 3%. The results are expressed as nmol Trolox Equiv./mg protein.

Measurement of total oxidant status

TOS of supernatant fractions was determined using a

novel automated measurement method, developed by Erel[27]. Oxidants present in the sample oxidize the ferrous ion-o-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of nmol H_2O_2 Equiv./mg protein.

Oxidative stress index

Percent ratio of TOS level to TAC level was accepted as OSI. OSI value was calculated according to the following formula[28]: OSI (Arbitrary Unit) = TOS (nmol H_2O_2 Equiv./mg protein)/TAC (nmol Trolox Equiv./mg protein).

Determination of myeloperoxidase activity

The MPO (EC 1.11.1.7) activity was determined, using a 4-aminoantipyrine/phenol solution as the substrate for MPO-mediated oxidation by H_2O_2 and changes in absorbance at 510 nm were recorded[29]. One unit of MPO activity was defined as that which degraded 1 mol H_2O_2/min at 25℃. The results were expressed as mU/g protein.

Determination of catalase activity

Liver catalase activity was determined by Goth's colorimetric method, in which supernatant was incubated in H_2O_2 substrate, and the enzymatic reaction stopped by the addition of ammonium molybdate. The intensity of the yellow complex formed by molybdate and H_2O_2 was measured at 405 nm[30].

Histopathologic evaluation

Liver tissues were embedded in paraffin, cut into 3 to 5-μm sections, and mounted. After deparaffinization, the tissues were stained with hematoxylin and eosin (HE) for histological examination. Histological examination was performed by a pathologist.

Statistical analysis

For statistical analyses, nonparametric independent group comparisons were made. For multiple comparisons, the Kruskal-Wallis was used for comparisons between groups and the Mann-Whitney test used if any statistical significance was found. A level of 5% ($P < 0.05$) was considered statistically significant. Data were expressed as median and range.

RESULTS

Plasma ALT, AST, and LDH levels in the *Nigella sativa* treatment group were significantly lower than those in the control and sham groups ($P < 0.01$, $P < 0.01$ and $P < 0.05$, respectively, and $P < 0.01$ for all). They were significantly higher in the control group than those in the sham group ($P < 0.01$ for all). The results are summarized in Table 1.

Table 1 Clinical parameters, oxidative and antioxidative parameters in sham, I/R and I/R + NS rats (n = 10, mean ± SD)

	Sham	I/R	I/R + NS	*P*
Clinical parameters				
AST (U/L)	132 ± 22	952 ± 251[b]	571 ± 137[d,f]	0.001
ALT (U/L)	86 ± 17	695 ± 206[b]	321 ± 128[d,f]	0.001
LDH (U/L)	534 ± 181	4334 ± 760[b]	3113 ± 729[d,e]	0.001
TAC (nmol Trolox Eqv./mg protein)	2.96 ± 0.4	2.17 ± 0.6[a]	3.07 ± 0.2[e,1]	0.029
TOS (nmol H_2O_2 Eqv./mg protein)	10.4 ± 2.2	15.9 ± 2.0[b]	12.8 ± 3.1[1,2]	0.003
OSI (Arbitrary Unite)	3.54 ± 0.7	7.76 ± 1.9[b]	3.76 ± 0.6[1,2]	0.002
MPO (U/g protein)	9.4 ± 1.8	13.2 ± 1.7[b]	11.2 ± 2.2[f,1]	0.004
CAT (U/mg protein)	18.4 ± 3.9	10.1 ± 1.9[b]	18.3 ± 2.2[1,2]	0.004

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase; TAC: Total antioxidant capacity; TOS: Total oxidative status; OSI: Oxidative stress index; MPO: Myeloperoxidase; CAT: Catalase. [a]$P < 0.05$, [b]$P < 0.01$, Sham groups *vs* I/R groups; [d]$P < 0.01$, Sham groups *vs* I/R + NS groups; [e]$P < 0.05$, [f]$P < 0.01$, I/R groups *vs* I/R + NS groups. [1]$P > 0.05$, Sham groups *vs* I/R + NS groups; [2]$P < 0.001$, I/R groups *vs* I/R + NS groups.

TAC and CAT activities in liver tissue were significantly higher in Group 3 than those in Group 2 ($P < 0.05$ and $P < 0.001$, respectively). However, TAC and CAT activities in liver tissue were significantly lower in Group 2 than those in Group 1 ($P < 0.05$ and $P < 0.01$, respectively). TOS and OSI in hepatic tissue were significantly lower in Group 3 than those in Group 2 ($P < 0.001$ for both). Also MPO levels in hepatic tissue were significantly lower in Group 3 than those in Group 2 ($P < 0.01$). However, TAS, OSI and MPO levels in hepatic tissue were significantly higher in Group 2 than those in Group 1 ($P < 0.01$ for all). There were not statistically significant differences between the *Nigella sativa* treatment group and the sham group regarding to the oxidant and antioxidant parameters ($P > 0.05$). The results are summarized in Table 1.

In histopathological evaluation, there were no pathological changes in liver tissue of the sham group (Figure 1A). Liver specimens from rats after ischemia-reperfusion exhibited focal necrosis and infiltration of leukocytes (Figure 1B). *Nigella sativa* treatment significantly decreased these pathological changes (Figure 1C). Histological tissue damage was milder in the *Nigella sativa* treatment group than that in the control group.

Nigella sativa did not produce any adverse side effects in the doses tested in our study.

DISCUSSION

An excessive production of oxygen free radicals has been reported in ischemic reperfused liver, leading to tissue damage, and this is an unavoidable process in liver transplantation and in the surgical procedures in which the Pringle maneuver is used[4]. It has been shown in many studies that supplementation of free

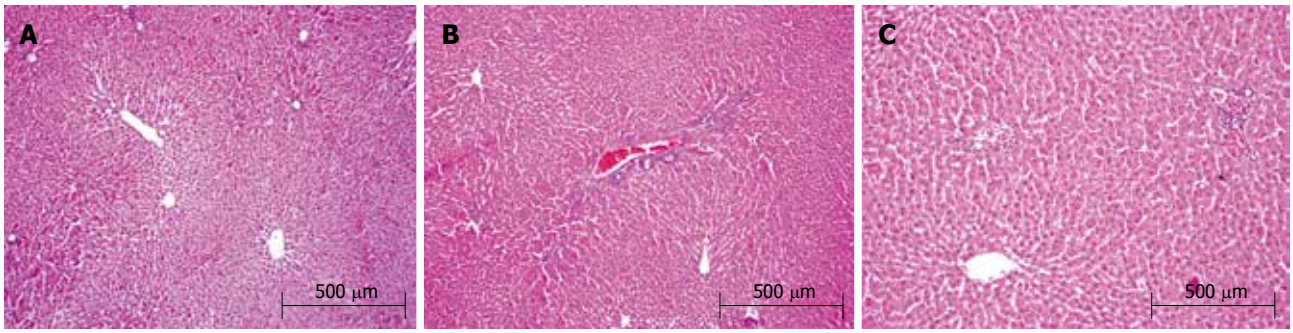


Figure 1 **A**: Normal liver tissue; **B**: Histopathological findings 60 min after reperfusion in the control group; **C**: Histopathological findings 60 min after reperfusion in the *Nigella sativa* treatment group.

radical scavengers is helpful in reducing hepatic ischemia reperfusion induced tissue damage[4,6,8]. *Nigella sativa* has been identified as a potent antioxidant acting as a free radical scavenger[9]. Therefore, it should not be surprising that *Nigella sativa* pretreatment has a protective effect on hepatic ischemia reperfusion injury in rats.

In some studies, to reduce the intestinal ischemia reperfusion injury, agents are administrated only before ischemia or reperfusion, and in some other studies both before ischemia and reperfusion. We preferred to administer both before ischemia and reperfusion as reported previously[31].

In the present study *Nigella sativa* treatment markedly attenuated ALT, AST and LDH activities which are associated with hepatic parenchymal injury. The increase of AST, ALT and LDH activities observed in control groups can be elucidated by lipid peroxidation leading to cytolysis, which is caused by the oxygen free radical formed during the reperfusion phase[32]. The decrease of AST, ALT, and LDH activities observed in the rats treated with *Nigella sativa*, when compared to the rats in control group, suggests a possible protective effect of *Nigella sativa* treatment in the hepatic ischemia/reperfusion condition.

Despite determination of either oxidants or antioxidant components alone may give information about oxidative stress, determination of oxidants along with antioxidants is more useful in this context[33]. So, we preferred to measure oxidants and antioxidant capacity simultaneously to assess oxidative stress more exactly. In the present study, we measured oxidative stress with OSI which was detected using both oxidative and antioxidative parameters. We evaluated TAC which reflects the antioxidative status and TOS to investigate oxidative status using a more recently developed measurement methods by Erel[27,28]. *Nigella sativa* treatment significantly reduced OSI and TOS levels, which show oxidative stress, and increased TAC levels, which show antioxidant capacity, in liver tissue. Oxidative stress activates mechanisms that lead to the synthesis of proinflammatory cytokines and cell adhesion molecules. Therefore, oxidative stress may contribute to an inflammatory response induced by endotoxemia after hepatic ischemia reperfusion. Our data confirm that liver ischemia reperfusion increases oxidative stress, an effect that not only produces direct tissue damage, but also modulates production of toxic cytokines leading to inflammation and leukocyte infiltration, consistent with previous studies. In addition to this, *Nigella sativa* treatment alleviated pathological structural changes.

Infiltration of neutrophils into tissues is commonly assessed by changes in activity of MPO, which is an enzyme found primarily in neutrophils. Increased MPO activity in the liver of rats after I/R suggests activation of an inflammatory response. In our study, we observed increased MPO activity in the liver tissue, and this may indicate that neutrophil accumulation and lipid peroxidation contributes to ischemia reperfusion-induced liver injury. Previously, it has been reported that the activated neutrophils located in the inflammatory foci and secreting MPO into the extracellular space can convert hydroperoxides into free radicals, triggering lipid peroxidation[34]. This is consistent with the results of our present study.

Catalase is an oxidoreductase enzyme, which transforms H_2O_2 into H_2O and O_2. It can protect cells from damage induced by ischemia reperfusion through scavenging reactive oxygen species[8]. The results of the present study showed that treatment with *Nigella sativa* can increase catalase activity, and this is consistent with its protective effect.

Although preliminary, our data indicate that *Nigella sativa* exhibits protective effects on liver tissue against ischemia reperfusion injury. The results of this present study may have clinical applications to the liver surgery associated with the Pringle maneuver and hepatic transplantation. However, more investigations are required to evaluate the protective effects of *Nigella sativa* on liver tissue damage in clinical and experimental models to verify this conclusion.

COMMENTS

Background

Hepatic ischemia-reperfusion (I/R) injury may occur in a variety of clinical settings such as trauma, sepsis, liver transplantation or hepatic pedicle clamping during a liver surgery and this remains a significant problem. Oxygen free radicals, produced on reperfusion have been shown to play a major role in hepatic I/R injury. Various therapeutic effects have been described for *Nigella sativa*. Additionally, it has been presented that *Nigella sativa* has protective effect against ischemia reperfusion injury to various organs. Therefore, it seems pos-

sible that the administration of *Nigella sativa* might protect the liver against the ischemia reperfusion injury and thus, we aimed to confirm this hypothesis.

Research frontiers

Tissue ischemia initiates a series of events that can ultimately lead to cellular dysfunction and necrosis. But, resumption of blood flow can paradoxically create more tissue injury, possibly because of the production of oxygen-derived cytotoxic products. It is important to reduce the oxidative stress mechanism and protect the liver tissue against the ischemia and reperfusion injury. This study demonstrated that *Nigella sativa* exhibits protective effects on liver tissue against ischemia reperfusion injury.

Innovations and breakthroughs

In previous studies, it has been demonstrated that *Nigella sativa* has protective effects against ischemia reperfusion injury on various organs. However, its protective effects on liver tissue against ischemia reperfusion injury are unclear.

Applications

It seems possible that the administration of *Nigella sativa* might protect the liver against the ischemia reperfusion injury which can occur due to a liver surgery, trauma or sepsis. Future studies will be required to verify the effectiveness of this substance.

Terminology

Reperfusion injury refers to damage to tissue caused when blood supply returns to the tissue after a period of ischemia. The absence of oxygen and nutrients from blood creates a condition in which the restoration of circulation results in inflammation and oxidative damage through the induction of oxidative stress rather than restoration of normal function.

Peer review

In the present study authors investigated the effects of *Nigella sativa*, an annual flowering plant, on hepatic ischemia-reperfusion injury. The late effects of *Nigella sativa* should be evaluated and survival experiments would be helpful.

REFERENCES

1 **Shin T**, Kuboki S, Huber N, Eismann T, Galloway E, Schuster R, Blanchard J, Pritts TA, Lentsch AB. Activation of peroxisome proliferator-activated receptor-gamma during hepatic ischemia is age-dependent. *J Surg Res* 2008; **147**: 200-205

2 **van Gulik TM**, de Graaf W, Dinant S, Busch OR, Gouma DJ. Vascular occlusion techniques during liver resection. *Dig Surg* 2007; **24**: 274-281

3 **He XS**, Ma Y, Wu LW, Wu JL, Hu RD, Chen GH, Huang JF. Dynamical changing patterns of glycogen and enzyme histochemical activities in rat liver graft undergoing warm ischemia injury. *World J Gastroenterol* 2005; **11**: 2662-2665

4 **Hassan-Khabbar S**, Cottart CH, Wendum D, Vibert F, Clot JP, Savouret JF, Conti M, Nivet-Antoine V. Postischemic treatment by trans-resveratrol in rat liver ischemia-reperfusion: a possible strategy in liver surgery. *Liver Transpl* 2008; **14**: 451-459

5 **Montalvo-Jave EE**, Escalante-Tattersfield T, Ortega-Salgado JA, Pina E, Geller DA. Factors in the pathophysiology of the liver ischemia-reperfusion injury. *J Surg Res* 2008; **147**: 153-159

6 **Polat KY**, Aydinli B, Polat O, Aydin U, Yazici P, Ozturk G, Gundogdu C, Kiziltunc A. The protective effect of aprotinin and alpha-tocopherol on ischemia-reperfusion injury of the rat liver. *Transplant Proc* 2008; **40**: 63-68

7 **Pulitano C**, Aldrighetti L. The protective role of steroids in ischemia-reperfusion injury of the liver. *Curr Pharm Des* 2008; **14**: 496-503

8 **Shen SQ**, Zhang Y, Xiang JJ, Xiong CL. Protective effect of curcumin against liver warm ischemia/reperfusion injury in rat model is associated with regulation of heat shock protein and antioxidant enzymes. *World J Gastroenterol* 2007; **13**: 1953-1961

9 **Khalife KH**, Lupidi G. Nonenzymatic reduction of thymoquinone in physiological conditions. *Free Radic Res* 2007; **41**: 153-161

10 **Kanter M**, Coskun O, Uysal H. The antioxidative and antihistaminic effect of Nigella sativa and its major constituent, thymoquinone on ethanol-induced gastric mucosal damage. *Arch Toxicol* 2006; **80**: 217-224

11 **Morsi NM**. Antimicrobial effect of crude extracts of Nigella sativa on multiple antibiotics-resistant bacteria. *Acta Microbiol Pol* 2000; **49**: 63-74

12 **Bayrak O**, Bavbek N, Karatas OF, Bayrak R, Catal F, Cimentepe E, Akbas A, Yildirim E, Unal D, Akcay A. Nigella sativa protects against ischaemia/reperfusion injury in rat kidneys. *Nephrol Dial Transplant* 2008; **23**: 2206-2212

13 **Hosseinzadeh H**, Parvardeh S, Asl MN, Sadeghnia HR, Ziaee T. Effect of thymoquinone and Nigella sativa seeds oil on lipid peroxidation level during global cerebral ischemia-reperfusion injury in rat hippocampus. *Phytomedicine* 2007; **14**: 621-627

14 **El-Abhar HS**, Abdallah DM, Saleh S. Gastroprotective activity of Nigella sativa oil and its constituent, thymoquinone, against gastric mucosal injury induced by ischaemia/reperfusion in rats. *J Ethnopharmacol* 2003; **84**: 251-258

15 **Abdel-Fattah AM**, Matsumoto K, Watanabe H. Antinociceptive effects of Nigella sativa oil and its major component, thymoquinone, in mice. *Eur J Pharmacol* 2000; **400**: 89-97

16 **Daba MH**, Abdel-Rahman MS. Hepatoprotective activity of thymoquinone in isolated rat hepatocytes. *Toxicol Lett* 1998; **95**: 23-29

17 **Mansour MA**, Ginawi OT, El-Hadiyah T, El-Khatib AS, Al-Shabanah OA, Al-Sawaf HA. Effects of volatile oil constituents of Nigella sativa on carbon tetrachloride-induced hepatotoxicity in mice: evidence for antioxidant effects of thymoquinone. *Res Commun Mol Pathol Pharmacol* 2001; **110**: 239-251

18 **Mansour MA**, Nagi MN, El-Khatib AS, Al-Bekairi AM. Effects of thymoquinone on antioxidant enzyme activities, lipid peroxidation and DT-diaphorase in different tissues of mice: a possible mechanism of action. *Cell Biochem Funct* 2002; **20**: 143-151

19 **Turkdogan MK**, Ozbek H, Yener Z, Tuncer I, Uygan I, Ceylan E. The role of Urtica dioica and Nigella sativa in the prevention of carbon tetrachloride-induced hepatotoxicity in rats. *Phytother Res* 2003; **17**: 942-946

20 **Boskabady MH**, Javan H, Sajady M, Rakhshandeh H. The possible prophylactic effect of Nigella sativa seed extract in asthmatic patients. *Fundam Clin Pharmacol* 2007; **21**: 559-566

21 **Kalus U**, Pruss A, Bystron J, Jurecka M, Smekalova A, Lichius JJ, Kiesewetter H. Effect of Nigella sativa (black seed) on subjective feeling in patients with allergic diseases. *Phytother Res* 2003; **17**: 1209-1214

22 **Dirjomuljono M**, Kristyono I, Tjandrawinata RR, Nofiarny D. Symptomatic treatment of acute tonsillo-pharyngitis patients with a combination of Nigella sativa and Phyllanthus niruri extract. *Int J Clin Pharmacol Ther* 2008; **46**: 295-306

23 **Akhondian J**, Parsa A, Rakhshande H. The effect of Nigella sativa L. (black cumin seed) on intractable pediatric seizures. *Med Sci Monit* 2007; **13**: CR555-CR559

24 **Kanter M**, Coskun O, Budancamanak M. Hepatoprotective effects of Nigella sativa L and Urtica dioica L on lipid peroxidation, antioxidant enzyme systems and liver enzymes in carbon tetrachloride-treated rats. *World J Gastroenterol* 2005; **11**: 6684-6688

25 **El Shenawy NS**, Soliman MF, Reyad SI. The effect of antioxidant properties of aqueous garlic extract and Nigella sativa as anti-schistosomiasis agents in mice. *Rev Inst Med Trop Sao Paulo* 2008; **50**: 29-36

26 **Erel O**. A novel automated method to measure total antioxidant response against potent free radical reactions. *Clin Biochem* 2004; **37**: 112-119

27 **Erel O**. A new automated colorimetric method for measuring total oxidant status. *Clin Biochem* 2005; **38**: 1103-1111

28 **Bolukbas C**, Bolukbas FF, Horoz M, Aslan M, Celik H, Erel O. Increased oxidative stress associated with the severity of the liver disease in various forms of hepatitis B virus infection. *BMC Infect Dis* 2005; **5**: 95
29 **Wei H**, Frenkel K. Relationship of oxidative events and DNA oxidation in SENCAR mice to in vivo promoting activity of phorbol ester-type tumor promoters. *Carcinogenesis* 1993; **14**: 1195-1201
30 **Goth L**. A simple method for determination of serum catalase activity and revision of reference range. *Clin Chim Acta* 1991; **196**: 143-151
31 **Sener G**, Tosun O, Sehirli AO, Kacmaz A, Arbak S, Ersoy Y, Ayanoglu-Dulger G. Melatonin and N-acetylcysteine have beneficial effects during hepatic ischemia and reperfusion. *Life Sci* 2003; **72**: 2707-2718
32 **Chouker A**, Martignoni A, Schauer RJ, Dugas M, Schachtner T, Kaufmann I, Setzer F, Rau HG, Lohe F, Jauch KW, Peter K, Thiel M. Alpha-gluthathione S-transferase as an early marker of hepatic ischemia/reperfusion injury after liver resection. *World J Surg* 2005; **29**: 528-534
33 **Tarpey MM**, Wink DA, Grisham MB. Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations. *Am J Physiol Regul Integr Comp Physiol* 2004; **286**: R431-R444
34 **Cetinkaya A**, Bulbuloglu E, Kurutas EB, Kantarceken B. N-acetylcysteine ameliorates methotrexate-induced oxidative liver damage in rats. *Med Sci Monit* 2006; **12**: BR274-BR278

S- Editor Li DL **L- Editor** Rippe RA **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5210

World J Gastroenterol 2008 September 7; 14(33): 5210-5216
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Efficacy and safety of gemcitabine-oxaliplatin combined with huachansu in patients with advanced gallbladder carcinoma

Tian-Jie Qin, Xin-Han Zhao, Jun Yun, Ling-Xiao Zhang, Zhi-Ping Ruan, Bo-Rong Pan

Tian-Jie Qin, Xin-Han Zhao, Ling-Xiao Zhang, Zhi-Ping Ruan, Department of Medical Oncology, First Affiliated Hospital of Medical School of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China
Bo-Rong Pan, Outpatient Department of Oncology, Cancer Institute, Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China
Jun Yun, Department of the Third General Surgery, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China
Author contributions: Qin TJ and Zhao XH contributed equally to this work; Qin TJ, Zhao XH and Pan BR designed the research; Qin TJ, Zhao XH, Yun J, Zhang LX, Ruan ZP performed the research; Qin TJ and Zhao XH analyzed the data; and Qin TJ, Zhao XH and Pan BR wrote the paper.
Correspondence to: Dr. Xin-Han Zhao, Department of Medical Oncology, First Affiliated Hospital of Medical School of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China. zhaoxinhan@mail.xjtu.edu.cn
Telephone: +86-29-85324136 Fax: +86-29-82655472
Received: June 19, 2008 Revised: August 14, 2008
Accepted: August 21, 2008
Published online: September 7, 2008

Abstract

AIM: To evaluate the efficacy and safety of gemcitabine-oxaliplatin (GEMOX) combined with huachansu (cinobufagin) injection treatment in patients with locally advanced or metastatic gallbladder carcinoma (GBC), and to assess the quality of life (QOL) of such patients.

METHODS: Twenty-five patients with locally advanced or metastatic GBC were treated with intravenous gemcitabine (1000 mg/m^2) over 30 min on days 1 and 8, 2 h infusion of oxaliplatin (120 mg/m^2) on day 1, and 2-3 h infusion of huachansu (20 mL/m^2) on days -3-11, every 3-4 wk. Treatment was continued until occurrence of unacceptable toxicity or disease progression. QOL of patients was assessed by the EORTC QLQ-C30 at baseline, at the end of the first, third and sixth chemotherapy cycles, and 1 mo after the treatment.

RESULTS: Among the 25 patients with a median age of 64 years (range 42-78 years), 23 were evaluable in the study. A total of 137 cycles of therapy were performed and the median cycle was 5 (range 1-8) per patient. Out of the 23 patients whose response could be evaluated, 8 partial responses (PR) were observed (34.8%), while 7 patients (30.4%) demonstrated a stable disease (SD). The disease control rate was 65.2%. Progression of cancer was observed in 8 (34.8%) patients. The median progression-free and overall survival time was 5.8 mo (95% CI: 4.5-7.1 mo) and 10.5 mo, respectively. The therapy was well tolerated, with moderate myelosuppression as the main toxicity. Anemia grade 2 was seen in 16.0%, neutropenia grade 3 in 8.0% and thrombocytopenia grade 3 in 24.0% of patients, respectively. Non-hematologic toxicity ranged from mild to moderate. No death occurred due to toxicity. The QOL of patients was improved after chemotherapy, and the scores of QOL were increased by 10 to 20 points.

CONCLUSION: GEMOX combined with huachansu (cinobufagin) injection is well tolerated, effective, thus improving the QOL of patients with advanced GBC.

Key words: Gallbladder carcinoma; Gemcitabine; Oxaliplatin; Huachansu injection; Quality of life

Peer reviewer: Nahum Méndez-Sánchez, PhD, Department of Gastroenterology and Liver Unit, Medica Sur Clinic & Foundation, Puente de Piedra 150, Col. Toriello Guerra, Mexico City 14050, Mexico

Qin TJ, Zhao XH, Yun J, Zhang LX, Ruan ZP, Pan BR. Efficacy and safety of gemcitabine-oxaliplatin combined with huachansu in patients with advanced gallbladder carcinoma. *World J Gastroenterol* 2008; 14(33): 5210-5216 Available from: URL: http://www.wjgnet.com/1007-9327/14/5210.asp DOI: http://dx.doi.org/10.3748/wjg.14.5210

INTRODUCTION

There is a marked worldwide geographic variation in gallbladder carcinoma (GBC) incidence, which correlates with the prevalence of cholelithiasis, and the highest prevalence of GBC is in Israel, Mexico, Chile, Japan, and America[1,2]. The incidence and mortality of GBC in China has had a tendency to increase in recent years[3].

The early symptoms of GBC are similar to those of other gallbladder diseases, such as gallstones or infection.

But, no characteristic symptoms could be observed at its early stage. In fact, early gallbladder cancer is discovered often when the gallbladder is removed as a treatment for gallstones. Otherwise, gallbladder cancer is often at its advanced stage at the time when it is diagnosed, and has a postoperative 5-year survival rate of less than 5%[4] with a high relapse rate.

Conventional surgery is considered the most effective treatment for GBC. But, many cases are inoperable at the time of its diagnosis. Chemotherapy has recently shown its effect on gallbladder cancer. The most commonly used chemotherapeutic agent is 5-fluorouracil (5-FU), which is often used alone or in combination with leucovorin. Several small trials of combined regimens for GBC, using 5-FU, cisplatin, mitomycin and/or leucovorin, have been reported to have mixed results. Other chemotherapeutic agents that are now in clinical trials include capecitabine, oxaliplatin[5], gemcitabine[6-8], erlotinib[9], *etc*. In addition, more and more people have paid their close attention to Chinese medicines for the prevention and treatment of cancer. Huachansu (cinobufagin) is just a widely used antitumor agent of traditional Chinese medicine in China[10,11].

Because the clinical data have demonstrated the effectiveness of gemcitabine on pancreatic cancer, and gallbladder shares a common embryological origin with the exocrine pancreas, we used gemcitabine-oxaliplatin (GEMOX) combined with huachansu injection (cinobufagin) in treatment of advanced GBC.

Since the prognosis of GBC patients is usually poor, it is important to maintain their health-related quality of life (HRQOL). The QOL of patients has been an important endpoint in assessment of GBC treatment. However, to our knowledge, only a few studies discussing QOL of patients with GBC, and there is no report on GEMOX and huachansu regimen for GBC patients. The aim of this study was to evaluate the efficacy and safety of GEMOX plus huachansu treatment of GBC, and to assess the QOL of GBC patients.

MATERIALS AND METHODS

Patients and eligibility criteria

From January 2003 to July 2005, of the 25 enrolled patients (10 males and 15 females) with locally advanced or metastatic GBC, 23 were assessable. The patients were required to have histologically confirmed diagnosis, measurable computed tomography (CT) scan or magnetic resonance imaging (MRI), Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0-2, age < 78 years, a life expectancy > 3 mo. No patient underwent anticancer procedures within 1 mo before the present study. The laboratory criteria were leukocyte count $\geqslant 4.0 \times 10^9$/L, neutrophils $\geqslant 1.5 \times 10^9$/L, platelet count $\geqslant 100 \times 10^9$/L, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) $\leqslant$ 2.5 × upper limit of normal (ULN), serum creatinine < 1.5 × ULN, serum bilirubin value < 2.5 × ULN. Basic CA19-9 level was recorded. Patients with active infections, unstable cardiovascular conditions, brain metastases, other malignancy or serious medical illnesses were excluded from this study. The pre-treatment characteristics are listed in Table 1.

Table 1 Characteristics of patients with locally advanced or metastatic GBC before treatment

Characteristic	Data
Total No. of patients (evaluable for response)	25 (23)
Sex (female/male)	15/10
Median age (range)	64 (42-78)
Nevin tumor stage	*n* (%)
Ⅲ	4 (16.0)
Ⅳ	12 (48.0)
Ⅴ	9 (36.0)
ECOG performance status at baseline	
0	3 (12.0)
1	17 (68.0)
2	5 (20.0)
Site of metastasis	
Liver	7 (28.0)
Lung	2 (8.0)
Lymph nodes	12 (48.0)
Peritoneum	3 (12.0)
Bone	2 (8.0)
Recurrence after surgical resection	5 (20.0)
Pre-treatment requiring stent or percutaneous transhepatic drainage because of obstructive jaundice,	4 (16.0)
Pre-treatment chemotherapy	4 (16.0) 5-FU-based chemotherapy
Liver and renal functions	
Total bilirubin (6-20.5 μmol/L[1])	
Median	18
Range	7-58
Gamma-glutamyltransferase (183.4-833.5 nkat/L[1])	
Median	1033.5
Range	283.4-2583.9
Aspartate aminotransferase/Alanine aminotransferase (0-666.8 nkat/L[1])	
Median	950.2
Range	383.4-1600.3
Blood urea nitrogen (3.2-7.0 mmol/L[1])	
Median	6.2
Range	3.6-7.8
Serum creatinine (44-140 μmol/L[1])	
Median	78
Range	48-121
CA19-9 (0-39 kU/L[1])	
Median	496
Range	7-10463

[1]Normal level.

Tumor size was assessed by CT scan or MRI within 3 wk prior to the first cycle, and baseline biological analysis was performed within 1 wk. A physical examination and complete blood cell count were performed 2-3 d before each cycle. Blood count was obtained each week to determine the level of myelosuppression. After every three cycles, a full clinical evaluation including performance status and physical examination was performed.

Treatment protocol

All patients received intravenous gemcitabine (1000 mg/m^2) over 30 min on days 1 and 8, 2 h oxaliplatin infusion (120 mg/m^2) on day 1 (oxaliplatin was discontinued if specific cumulative peripheral sensory neuropathy of NCI CTC grade 3 occurred), and 2-3 h huachansu infusion (20 mL/m^2) (0.5 g/mL) in 500 mL of 50 g/L glucose solution on days -3-11. Treatment was repeated every 3-4 wk until limiting toxicity or disease progression occurred, or further treatments were refused by patients. Biliary bypass or stenting was required in four patients before the treatment.

Follow-up

After two cycles of chemotherapy, patients were followed up every 4-6 wk till February 2007.

Assessment of efficacy

Tumor response was evaluated every three cycles by CT scan or MRI, using standard RECIST criteria[12]. Complete response (CR) was defined as a disappearance of all signs and symptoms of disease. Partial response (PR) was defined as a decrease > 30% of the sum of the largest diameters of target (measurable) lesions without appearance of new lesions or progression of non-target (evaluable) lesions. To be assigned a response status, changes in tumor measurement were confirmed by a repeated assessment performed no less than 4 wk after the criteria for response were first met. Stable disease (SD) was defined as no sufficient shrinkage to qualify partial response or less than a 20% increase in the sum of the largest diameters of target lesions without appearance of new lesions or progression of non-target lesions. Progressive disease (PD) was defined as a 20% increase in the sum of the largest diameters of target lesions or as appearance of new lesions or as progression of non-target lesions. Disease control was defined as the absence of tumor progression (i.e. complete and partial response and SD) for at least 2 mo. Progression-free survival (PFS) was determined from the first day of treatment until clinical progression or tumor progression assessed by CT scan. Overall survival (OS) was determined from the first day of treatment until the date of death. OS and PFS were analyzed using the Kaplan-Meier method. Toxicity was evaluated at each cycle according to the NCI CTC version 2.0.

Assessment of QOL

The validated traditional Chinese version of the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire-Core 30 (EORTC QLQ-C30) was used to measure the patients' QOL. Patients completed the EORTC QLQ-C30 at baseline, at the end of the first, third and sixth chemotherapeutic cycles, and 1 mo after completion of chemotherapy.

The QLQ-C30 is a 30-item standardized measure that includes a global QOL/ overall health status scale, five functioning scales (physical, role, emotional, cognitive, and social), three multi-item symptom scales (fatigue, pain, nausea and vomiting) and six single items (dyspnea, sleep disturbance, appetite loss, diarrhea, constipation, and financial impact). The raw scores obtained from the EORTC questionnaire were converted to scores ranging from 0 to 100 using linear transformation according to the scoring procedure. Higher scores on the functioning scales, and the overall health status indicate a higher functioning level and a better QOL. Higher scores on the symptom scales or single item scales represent a higher level of symptoms or problems.

QOL scores were determined in our study in the following domains: physical, role, emotional, cognitive and social function, global QOL/overall health status, fatigue, nausea and vomiting, and pain.

Statistical analysis

The objective response rate, progression-free survival, overall survival, toxicity and QOL were observed, and the 95% confidence interval (CI) was calculated using the method of Clopper and Pearson. Overall survival, progression-free survival, death or last follow-up was made using the Kaplan-Meier method using SPSS version 12.0 software. The life table method was used to evaluate the 1-year survival rate. Data were expressed as mean ± SD. QOL score analysis was done using the paired *t*-test for comparison between baseline and 1 mo after completing chemotherapy. $P < 0.05$ was considered statistically significant, whereas a mean difference of 10 or more points in QOL scales represents a clinically significant/relevant difference[13].

RESULTS

Response and survival

A total of 25 patients (10 males and 15 females) were included this trial and 23 were assessable. Twenty-one patients (84.0%) received GEMOX plus huachansu injection as the first-line and 4 (16.0%) as the second-line chemotherapy. A total of 137 cycles of therapy were performed, the median cycle was 5 (range 1-8) per patient. Up to February 2007, two patients were still alive. The median follow-up time was 11.5 (range, 2-33) mo. The one-year survival rate was 39.1%. There was no complete response. However, out of the 23 patients whose response could be evaluated, 8 (34.8%) went into PR, 7 (30.4%) demonstrated SD, 8 (34.8%) had progression of the disease. The disease control rate was 65.2%. The median progression-free and overall survival was 5.8 mo (95% CI: 4.5-7.1 mo) and 10.5 mo, respectively (Figure 1).

Four patients (16.0%) required biliary tract decompression by endoscopic or percutaneous stenting because of obstructive jaundice before chemotherapy.

Basic CA19-9 levels were recorded in all patients (median 496; range 7-10 463 kU/L), and 15 patients (60%) had elevated baseline levels. Serum CA19-9 levels were measured every 3-4 wk. The relative changes of CA19-9 during chemotherapy was negatively correlated with the baseline and progression-free survival ($P = 0.03$).

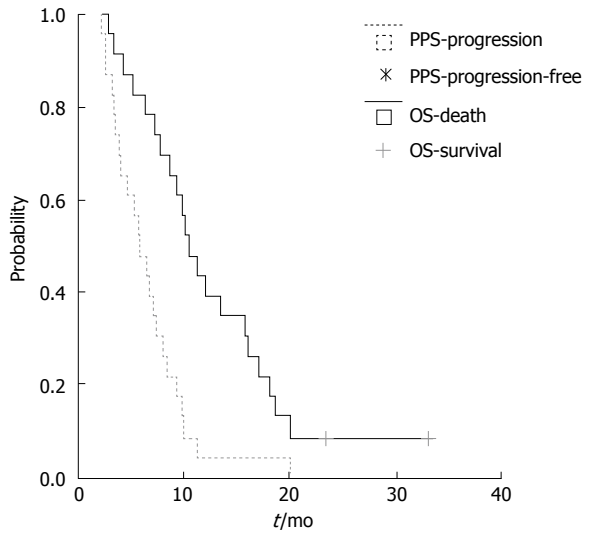


Figure 1 Kaplan-Meier curve of OS and PFS for patients treated with GEMOX plus huachansu. The median progression-free and median overall survival was 5.8 mo and 10.5 mo, respectively.

Toxicity

All patients were evaluable for toxicity. Gemcitabine in combination with oxaliplatin induced neutropenia, thrombocytopenia, nausea/vomiting, and peripheral neuropathy due their toxicity. It was observed by us that gemcitabine-based therapy was associated with a higher rate of thrombocytopenia among Chinese patients with cancer. Myelosuppression was the most frequent side-effect in our study. Anemia grade 2, neutropenia grade 3 and thrombocytopenia grade 3 were observed in 16%, 8% and 24% of patients, respectively. The nadir of thrombocytes appeared on days 6-12 [(51.0 ± 19.4) × 10^9/L, mean ± SD]. Recombinant human interleukin-11 (IL-11) was used to treat chemotherapy-induced thrombocytopenia. The platelet count returned to normal [(126.0 ± 18.2) × 10^9/L] after 3-8 d of IL-11 treatment at a dose of 25-50 μg/kg per day (Figure 2).

The symptoms induced by grade 1 non-hematologic toxicity included nausea/emesis (20.0%), diarrhea (4.0%), pain (8.0%), and peripheral neuropathy (64.0%). Sixteen patients (64.0%) felt anaesthesia of hands and feet, which was mild and did not interfere with their functions.

GEMOX combined with huachansu injection regimen was well tolerated (Table 2) with moderate myelosuppression as the main toxicity. No treatment-related death occurred.

QOL

The completion rate of QOL questionnaire declined from 96.0% at baseline to 73.9%, 1 mo after completion of chemotherapy. The mean score and standard deviation for the EORTC QLQ-C30 are presented in Table 3. An improvement of more than 10 points was found in the global QOL, physical, cognitive and emotional functioning, whereas symptoms such as pain decreased more than 18 points after chemotherapy. One month after the completion of treatment, physical functioning (PF) increased over baseline levels by approximately 12 points, and the global QOL score increased from 52.3 ± 20.0 at baseline to 65.3 ± 22.4, 1 mo after completion of chemotherapy. As compared with baseline, the patients reported fatigue that was worse immediately after the first cycle. However, fatigue then improved from the end of the first cycle to 1 mo after treatment. The five functional scales, global QOL, and multi-item symptom scales are shown in Figure 3. One month after treatment, the trend to improve the functioning, global QOL and symptoms was significant.

Table 2 Combined chemotherapy-related toxicities to patients *n* (%)

Toxicity	NCI-CTC grade			
	1	2	3	4
Leukopenia	9 (36.0)	5 (20.0)	2 (8.0)	0
Neutropenia	6 (24.0)	7 (28.0)	2 (8.0)	0
Anemia	2 (8.0)	4 (16.0)	0	0
Thrombocytopenia	7 (28.0)	5 (20.0)	6 (24.0)	2 (8.0)
Diarrhea	1 (4.0)	0	0	0
Nausea/emesis	5 (20.0)	0	0	0
Peripheral neuropathy	16 (64.0)	0	0	0
Pain	2 (8.0)	0	0	0

NCI-CTC: National Cancer Institute Common Toxicity Criteria. The combined treatment induced myelosuppression.

Table 3 Transformed scores for EORTC QLQ-C30 at different time points in patients treated with GEMOX and huachansu injection (mean ± SD)

Domain/Item	Baseline	First cycle	Third cycle	Sixth cycle	1 mo
Functioning					
Physical	65.4 ± 17.0	57.0 ± 20.3	64.4 ± 21.0	70.6 ± 18.3	77.5 ± 16.4[a]
Role	57.1 ± 26.3	55.0 ± 24.3	58.5 ± 26.2	60.4 ± 25.4	63.4 ± 27.2
Emotional	54.3 ± 33.0	47.2 ± 36.8	59.4 ± 39.1	70.5 ± 24.2	69.4 ± 30.1[a]
Cognitive	66.1 ± 24.6	68.1 ± 30.2	77.4 ± 26.3	79.1 ± 24.0	79.5 ± 21.7[a]
Social	47.2 ± 30.4	39.4 ± 33.6	44.1 ± 29.0	41.1 ± 30.7	48.2 ± 32.6
Global QOL	52.3 ± 20.0	41.4 ± 24.3	50.6 ± 29.7	55.4 ± 30.0	65.3 ± 22.4[a]
symptoms					
Fatigue	43.3 ± 31.2	51.2 ± 33.4	40.1 ± 27.4	42.1 ± 22.0	38.2 ± 28.6
Nausea and vomiting	7.6 ± 6.2	9.0 ± 5.1	7.4 ± 4.3	5.4 ± 2.5	3.2 ± 2.1[a]
Pain	44.5 ± 21.6	28.0 ± 18.3	25.5 ± 12.4	24.3 ± 9.1	26.0 ± 10.4[a]

Quality of life was assessed at baseline, at the end of the first, third and sixth chemotherapeutic cycles, and 1 mo after completion of chemotherapy. Scores of EORTC QLQ-C30 were converted to linear transformation ranging from 0 to 100. Higher scales for the functional and global QOL represent a better function. In contrast, higher scales for symptoms represent worse symptoms. [a]$P < 0.05$ *vs* baseline.

DISCUSSION

Due to the high mortality rate of GBC in China, we made this study to test the efficacy and safety of GEMOX combined with huachansu injection (cinobufagin) in patients with locally advanced or metastatic GBC and to assess the patients' QOL.

GBC, though rare, has a very poor prognosis. There is no generally accepted standard chemotherapy for

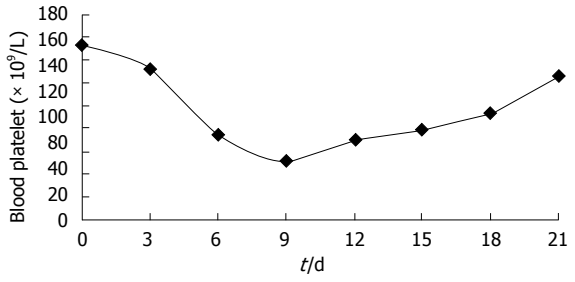


Figure 2 Peripheral blood platelet count after combined chemotherapy. The nadir of thrombocytes appeared on days 6-12.

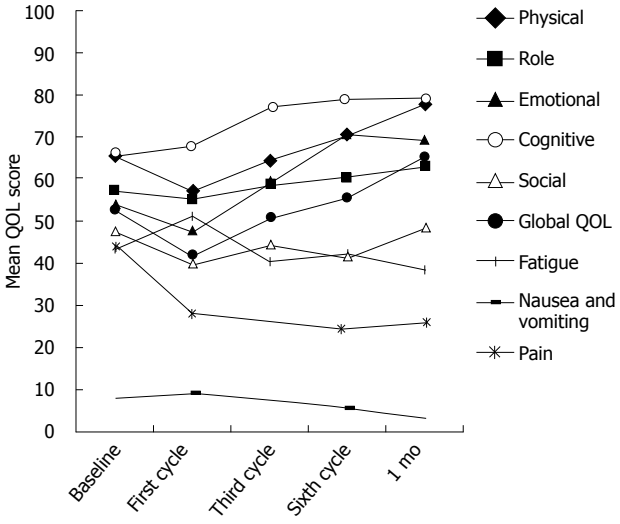


Figure 3 Functioning and symptom scales of the EORTC QLQ-30. First, third and sixth cycles: At the end of the first, third and sixth chemotherapeutic cycles; 1 mo: 1 mo after completion of chemotherapy. An improvement of more than 10 points was found in global QOL, physical and cognitive as well as emotional functioning, whereas symptoms such as pain were decreased by more than 18 points after completion of chemotherapy.

advanced, non-resectable cancer of the gallbladder or biliary tree. The median survival time of advanced GBC patients who receive best supportive care is approximately 6 mo[14].

Some new chemotherapeutic agents have been used in treatment of patients with advanced biliary tract cancer recently, such as gemcitabine, 5-fluorouracil[15-17], capecitabine[18], cisplatin[19,20], oxaliplatin or carboplatin. It was reported that gemcitabine is active against pancreatic and advanced biliary tract adenocarcinoma, and able to induce a response rate of 8.0%-60.0%[21,22].

Gallardo[23] conducted a phase Ⅱ trial with gemcitabine (1 000 mg/m^2 over 30 min weekly for 3 wk followed by a week of rest) in patients with locally advanced or metastatic GBC, and found that the overall response rate was 36.0%. The cancer remained stable in 6 (25.0%) patients, and progressed in 10 (40.0%) patients. The median survival time was 30 wk. In another phase Ⅱ trial with 24-h gemcitabine infusion weekly in patients with advanced gallbladder and biliary tract carcinoma, 18 patients were evaluated for response. One partial response was observed (6.0%), the disease control rate was 61.0%, the median time of tumor progression was 3.6 mo and the median overall survival time was 7.5 mo[24].

Andre *et al*[25] used GEMOX regimen to treat patients with advanced biliary tract adenocarcinoma, and found that the objective response was 36%, the median PFS time was 5.7 mo, the overall survival time was 15.4 mo in group A (PS 0-2), while the objective response was 22.0%, the median PFS time was 3.9 mo and the median OS time was 7.6 mo in group B (PS > 2).

Huachansu, one of the most widely studied traditional Chinese medicine, is a water soluble extract from *Bufo* toad skin, and can be sued in treatment of cancer, especially liver and pancreatic cancer. Some studies demonstrated that huachansu injection can improve the QOL of patients and has been used as a treatment of cancer in China[26-28]. It was reported that digitalis-like cinobufagin can protect prostate cancer cells from proliferation. The protein expression of active caspase 3 in LNCaP, DU145, and PC3 cells was increased after treated with combined cinobufagin and EGTA, the expression of Fas was increased, the expression of Bax was down-regulated in nuclei, and the protein expression of cytosolic cytochrom C was also increased after treatment with cinobufagin in these cell lines[29]. It has been shown that bufalin or cinobufagin increases Ca^{2+} and apoptosis in cancer cells, caspase 3 activities in DU145 and PC3 cells, and caspase 9 activities in LNCaP cells after a 24 h culture[30].

QOL is a multidimensional concept including physical, emotional, social, and daily-life functioning as well as disease symptoms and treatment from the patient's perspective. Improving QOL and disease-associated symptoms is increasingly important for patients with advanced GBC. QOL is now regarded as a biologically and clinically meaningful outcome that is as important as disease-free, and overall survival with regard to anticancer treatment. Huachansu injection was given 3 d before GEMOX in our study, and its effectiveness on pain relief appeared 1-2 d later (9/14 patients). The overall life quality of patients entered this trial improved 10 to 20 points. The patients undergoing GEMOX and huachansu regimen achieved better QOL outcomes. The partial response rate was 34.8%, and the disease control rate was 65.2%. The median progression-free and median overall survival was 5.8 mo and 10.5 mo, respectively. The patients tolerated the treatment well with moderate myelosuppression as the main toxicity. No treatment-related deaths occurred.

In conclusion, combined GEMOX and huachansu injection regimen can be used in treatment of GBC patients, especially those with advanced or metastatic GBC.

COMMENTS

Background

The difficulty in early diagnosis of gallbladder carcinoma (GBC) is its poor specificity in clinical and ambiguous early symptoms, thus affecting its prognosis. Some patients are found having GBC only when other diseases are diagnosed and treated. Surgery is the only curative treatment for gallbladder

cancer. However, because of frequent local and distant recurrence, radical surgery at its advanced stage is often unsuccessful. Thus, chemotherapy for patients with advanced GBC seems to be a better choice of treatment. However, no standard chemotherapy for GBC has yet been established.

Research frontiers

The increasing number of papers on chemotherapy for GBC emphasizes the need of a new standard beyond 5-FU. At present, clinical studies on the treatment of GBC with new chemotherapeutic agents are underway. Further clinical trials, especially large multi-institutional RCTs (phase Ⅲ studies) using novel agents such as gemcitabine, should be performed in order to establish a standard treatment for GBC.

Innovations and breakthroughs

In China, huachansu used in treatment of patients with lung cancer and hepatocellular carcinoma has achieved rather good results in suppressing the growth of cancer, alleviating of pain and fatigue, and improving the function of patients' immune system. Our findings provide certain possible evidence that combined gemcitabine-oxaliplatin (GEMOX) and huachansu chemotherapy may improve the survival and QOL of GBC patients.

Applications

The results of this study indicate that combined GEMOX and huachansu chemotherapy is an effective regimen for metastatic GBC. The patients can tolerate it well. Toxicities are mostly hematological and easily manageable. If this combined regimen could be applied in clinic practice, the patients would have a longer survival time.

Terminology

The World Health Organization (WHO) defines QOL as" an individual's perception of his or her position in life in the context of culture and value system in which he or she lives in relation to his or her goal, expectation, standard and concern. It is a broad-ranging concept affected in a complex way by the person's physical health, psychological state, independence, social relationships, and his or her relationship to salient feature of his or her environment." Studies showed that QOL is associated to the survival of cancer patients.

Peer review

This is an interesting study. The authors evaluated the efficacy and safety of combined GEMOX and huachansu injection (cinobufagin) treatment in patients with locally advanced or metastatic gallbladder carcinoma, and assessed the patients' QOL. The study was well designed.

REFERENCES

1 **Yang DZ**, He J, Zhang JC, Wang ZR. Expression of angiostatin cDNA in human gallbladder carcinoma cell line GBC-SD and its effect on endothelial proliferation and growth. *World J Gastroenterol* 2006; **12**: 2762-2766

2 **Eckel F**, Schmid RM. Chemotherapy in advanced biliary tract carcinoma: a pooled analysis of clinical trials. *Br J Cancer* 2007; **96**: 896-902

3 **Xiao WD**, Peng CH, Zhou GW, Wu WD, Shen BY, Yan JQ, Yang WP, Li HW. Surgical treatment for Nevin stage IV and V gallbladder carcinoma: report of 70 cases. *Hepatobiliary Pancreat Dis Int* 2005; **4**: 589-592

4 **Levy AD**, Murakata LA, Rohrmann CA Jr. Gallbladder carcinoma: radiologic-pathologic correlation. *Radiographics* 2001; **21**: 295-314; questionnaire, 549-555

5 **Androulakis N**, Aravantinos G, Syrigos K, Polyzos A, Ziras N, Tselepatiotis E, Samonis G, Kentepozidis N, Giassas S, Vamvakas L, Georgoulias V. Oxaliplatin as first-line treatment in inoperable biliary tract carcinoma: a multicenter phase II study. *Oncology* 2006; **70**: 280-284

6 **Eng C**, Ramanathan RK, Wong MK, Remick SC, Dai L, Wade-Oliver KT, Mani S, Kindler HL. A Phase II trial of fixed dose rate gemcitabine in patients with advanced biliary tree carcinoma. *Am J Clin Oncol* 2004; **27**: 565-569

7 **Park JS**, Oh SY, Kim SH, Kwon HC, Kim JS, Jin-Kim H, Kim YH. Single-agent gemcitabine in the treatment of advanced biliary tract cancers: a phase II study. *Jpn J Clin Oncol* 2005; **35**: 68-73

8 **Tsavaris N**, Kosmas C, Gouveris P, Gennatas K, Polyzos A, Mouratidou D, Tsipras H, Margaris H, Papastratis G, Tzima E, Papadoniou N, Karatzas G, Papalambros E. Weekly gemcitabine for the treatment of biliary tract and gallbladder cancer. *Invest New Drugs* 2004; **22**: 193-198

9 **Philip PA**, Mahoney MR, Allmer C, Thomas J, Pitot HC, Kim G, Donehower RC, Fitch T, Picus J, Erlichman C. Phase II study of erlotinib in patients with advanced biliary cancer. *J Clin Oncol* 2006; **24**: 3069-3074

10 **Zhang P**, Cui Z, Liu Y, Wang D, Liu N, Yoshikawa M. Quality evaluation of traditional Chinese drug toad venom from different origins through a simultaneous determination of bufogenins and indole alkaloids by HPLC. *Chem Pharm Bull* (Tokyo) 2005; **53**: 1582-1586

11 **Dai LP**, Wang ZM, Gao HM, Jiang X, Ding GZ. [Determination of bufothionine in skin of Bufo bufo gargarizans and Huachansu injection] *Zhongguo Zhongyao Zazhi* 2007; **32**: 224-226

12 **Therasse P**, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000; **92**: 205-216

13 **Osoba D**, Rodrigues G, Myles J, Zee B, Pater J. Interpreting the significance of changes in health-related quality-of-life scores. *J Clin Oncol* 1998; **16**: 139-144

14 **Nehls O**, Oettle H, Hartmann JT, Hofheinz RD, Hass HG, Horger MS, Koppenhofer U, Hochhaus A, Stieler J, Trojan J, Gregor M, Klump B. Capecitabine plus oxaliplatin as first-line treatment in patients with advanced biliary system adenocarcinoma: a prospective multicentre phase II trial. *Br J Cancer* 2008; **98**: 309-315

15 **Murad AM**, Guimaraes RC, Aragao BC, Rodrigues VH, Scalabrini-Neto AO, Padua CA, Moore FC. Phase II trial of the use of gemcitabine and 5-fluorouracil in the treatment of advanced pancreatic and biliary tract cancer. *Am J Clin Oncol* 2003; **26**: 151-154

16 **Knox JJ**, Hedley D, Oza A, Siu LL, Pond GR, Moore MJ. Gemcitabine concurrent with continuous infusional 5-fluorouracil in advanced biliary cancers: a review of the Princess Margaret Hospital experience. *Ann Oncol* 2004; **15**: 770-774

17 **Hsu C**, Shen YC, Yang CH, Yeh KH, Lu YS, Hsu CH, Liu HT, Li CC, Chen JS, Wu CY, Cheng AL. Weekly gemcitabine plus 24-h infusion of high-dose 5-fluorouracil/leucovorin for locally advanced or metastatic carcinoma of the biliary tract. *Br J Cancer* 2004; **90**: 1715-1719

18 **Knox JJ**, Hedley D, Oza A, Feld R, Siu LL, Chen E, Nematollahi M, Pond GR, Zhang J, Moore MJ. Combining gemcitabine and capecitabine in patients with advanced biliary cancer: a phase II trial. *J Clin Oncol* 2005; **23**: 2332-2338

19 **Thongprasert S**, Napapan S, Charoentum C, Moonprakan S. Phase II study of gemcitabine and cisplatin as first-line chemotherapy in inoperable biliary tract carcinoma. *Ann Oncol* 2005; **16**: 279-281

20 **Doval DC**, Sekhon JS, Gupta SK, Fuloria J, Shukla VK, Gupta S, Awasthy BS. A phase II study of gemcitabine and cisplatin in chemotherapy-naive, unresectable gall bladder cancer. *Br J Cancer* 2004; **90**: 1516-1520

21 **Scheithauer W**. Review of gemcitabine in biliary tract carcinoma. *Semin Oncol* 2002; **29**: 40-45

22 **Eckel F**, Schmelz R, Erdmann J, Mayr M, Lersch C. Phase II trial of a 24-hour infusion of gemcitabine in previously untreated patients with advanced pancreatic adenocarcinoma. *Cancer Invest* 2003; **21**: 690-694

23 **Gallardo JO**, Rubio B, Fodor M, Orlandi L, Yanez M, Gamargo C, Ahumada M. A phase II study of gemcitabine in gallbladder carcinoma. *Ann Oncol* 2001; **12**: 1403-1406

24 **von Delius S**, Lersch C, Schulte-Frohlinde E, Mayr M, Schmid RM, Eckel F. Phase II trial of weekly 24-hour infusion of gemcitabine in patients with advanced gallbladder and

biliary tract carcinoma. *BMC Cancer* 2005; **5**: 61

25 **Andre T**, Tournigand C, Rosmorduc O, Provent S, Maindrault-Goebel F, Avenin D, Selle F, Paye F, Hannoun L, Houry S, Gayet B, Lotz JP, de Gramont A, Louvet C. Gemcitabine combined with oxaliplatin (GEMOX) in advanced biliary tract adenocarcinoma: a GERCOR study. *Ann Oncol* 2004; **15**: 1339-1343

26 **Ye M**, Qu G, Guo H, Guo D. Specific 12 beta-hydroxylation of cinobufagin by filamentous fungi. *Appl Environ Microbiol* 2004; **70**: 3521-3527

27 **Ma XC**, Xin XL, Liu KX, Han J, Guo DA. Microbial transformation of cinobufagin by Syncephalastrum racemosum. *J Nat Prod* 2008; **71**: 1268-1270

28 **Ye M**, Guo H, Guo H, Han J, Guo D. Simultaneous determination of cytotoxic bufadienolides in the Chinese medicine ChanSu by high-performance liquid chromatography coupled with photodiode array and mass spectrometry detections. *J Chromatogr B Analyt Technol Biomed Life Sci* 2006; **838**: 86-95

29 **He X**, Tang J, Qiao A, Wang G, Jiang M, Liu RH, Yao X. Cytotoxic biotransformed products from cinobufagin by Mucor spinosus and Aspergillus Niger. *Steroids* 2006; **71**: 392-402

30 **Yeh JY**, Huang WJ, Kan SF, Wang PS. Effects of bufalin and cinobufagin on the proliferation of androgen dependent and independent prostate cancer cells. *Prostate* 2003; **54**: 112-124

S- Editor Li DL **L- Editor** Wang XL **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5217

World J Gastroenterol 2008 September 7; 14(33): 5217-5222
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Efflux pump gene *hefA* of *Helicobacter pylori* plays an important role in multidrug resistance

Zhi-Qiang Liu, Peng-Yuan Zheng, Ping-Chang Yang

Zhi-Qiang Liu, Peng-Yuan Zheng, Department of Gastroenterology, the Second Affiliated Hospital of Zhengzhou University, Zhengzhou 450014, Henan Province, China
Ping-Chang Yang, Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario L8N 4A6, Canada
Author contributions: Liu ZQ, Zheng PY contributed equally to this work; Liu ZQ, Zheng PY, Yang PC designed the research; Liu ZQ, Zheng PY, Yang PC performed the research; Zheng PY contributed to the new reagents/analytic tools; Zheng PY, Yang PC analyzed the data; Liu ZQ, Zheng PY wrote the paper.
Correspondence to: Dr. Peng-Yuan Zheng, Department of Gastroenterology, the Second Affiliated Hospital of Zhengzhou University, 2 Jingba Road, Zhengzhou 450014, Henan Province, China. medp7123@yahoo.com
Telephone: +86-371-65261035 Fax: +86-371-63934118
Received: April 26, 2008 Revised: August 18, 2008
Accepted: August 25, 2008
Published online: September 7, 2008

Abstract

AIM: To determine whether efflux systems contribute to multidrug resistance of *H pylori*.

METHODS: A chloramphenicol-induced multidrug resistance model of six susceptible *H pylori* strains (5 isolates and *H pylori* NCTC11637) was developed. Multidrug-resistant (MDR) strains were selected and the minimal inhibitory concentration (MIC) of erythromycin, metronidazole, penicillin G, tetracycline, and ciprofloxacin in multidrug resistant strains and their parent strains was determined by agar dilution tests. The level of mRNA expression of *hefA* was assessed by fluorescence real-time quantitative PCR. A *H pylori* LZ1026 knockout mutant (Δ*H pylori* LZ1026) for (putative) efflux protein was constructed by inserting the kanamycin resistance cassette from pEGFP-N2 into *hefA*, and its susceptibility profiles to 10 antibiotics were evaluated.

RESULTS: The MIC of six multidrug-resistant strains (including 5 clinical isolates and *H pylori* NCTC11637) increased significantly (≥ 4-fold) compared with their parent strains. The expression level of *hefA* gene was significantly higher in the MDR strains than in their parent strains (P = 0.033). A *H pylori* LZ1026 mutant was successfully constructed and the Δ*H pylori* LZ1026 was more susceptible to four of the 10 antibiotics. All the 20 strains displayed transcripts for *hefA* that confirmed the *in vitro* expression of these genes.

CONCLUSION: The efflux pump gene *hefA* plays an important role in multidrug resistance of *H pylori*.

Key words: Efflux pump; *Helicobacter pylori*; Multidrug resistance; Fluorescence real-time quantitative PCR; Knockout mutant

Peer reviewer: Tadashi Shimoyama, MD, Hirosaki University, 5 Zaifu-cho, Hirosaki 036-8562, Japan

Liu ZQ, Zheng PY, Yang PC. Efflux pump gene *hefA* of *Helicobacter pylori* plays an important role in multidrug resistance. *World J Gastroenterol* 2008; 14(33): 5217-5222 Available from: URL: http://www.wjgnet.com/1007-9327/14/5217.asp DOI: http://dx.doi.org/10.3748/wjg.14.5217

INTRODUCTION

H pylori is a Gram-negative bacterium that colonizes human gastric mucosa[1] and plays an important role in the pathogenesis of chronic gastritis, peptic ulcer, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma[2,3]. Successful treatment usually requires two or three kinds of antibiotics in combination with a proton pump inhibitor. In recent years, the eradication rate of *H pylori* for standard therapies has decreased due to the increasing antimicrobial resistance[4,5]. Possible mechanisms of intrinsic drug resistance involve decreased drug uptake or increased drug efflux[6]. Efflux of compounds is a phenomenon commonly observed in bacteria[7-9]. Through this process, organisms are protected from possible toxic effects of metabolite accumulation or external compounds, and compound efflux results in a decreased susceptibility to a variety of antibiotics. Five families of multidrug efflux transporters have been described[10]. One of them, widespread in Gram-negative bacteria, is the resistance-nodulation-division (RND) family of efflux systems[11-13]. The RND family of efflux systems has three components: inner membrane efflux proteins (IEPs) which act with the other two components, a periplasmic efflux protein (PEP) which facilitates interaction with the other two components, and an outer membrane efflux protein (OEP) which

is the TolC (the outer membrane efflux protein in *Escherichia coli*) or a TolC homolog[14].

Bina JE and coworkers[15] have identified three RND efflux systems, namely *hefABC*, *hefDEF*, and *hefGHI*. Each of them is consisted a translocase, an accessory protein, and a TolC homolog. *hefA*, *hefD*, and *hefG* are the TolC homolog encoding the outer membrane efflux protein. In their study, the *hefGHI* operon was expressed only *in vivo*. Nevertheless, they could not establish a role of these efflux systems in antibiotic resistance. An unfortunate choice of compounds and a methodology that may not be sensitive enough to relatively small differences in susceptibilities between the mutant and parental strains may have impacted their study. van Amsterdam *et al*[16] identified 27 putative translocases in the *H pylori* 26695 genome, but only four putative *H pylori* OEPs or TolC homologs. Parallel translocases may function in a limited number of OEPs[17]. Thus, inactivation of a TolC-like protein may affect the functions of multiple translocases. They evaluated the susceptibility profiles of the inactivation of the four TolC homologs after insertion, but could not quantitate the operon expression levels of the efflux systems in multidrug resistant strains of *H pylori*. In view of the biological difference of the *H pylori* strains in Asia, Europe and North America[18], the present study was to determine whether efflux systems of *H pylori* contribute to antimicrobial susceptibility. A chloramphenicol-induced multidrug resistance model of 6 susceptible *H pylori* strains (5 isolates and *H pylori* NCTC11637) was developed by detecting the minimal inhibitory concentration (MIC) of the strains to metronidazole, tetracycline, erythromycin, penicillin G, and ciprofloxacin. The levels of mRNA expression of *hefA* in multidrug-resistance (MDR) strains and their parent strains were assessed by fluorescence real-time quantitative PCR. To further evaluate the role of *hefA* in multidrug resistance of *H pylori*, a clinical isolate of *H pylori* LZ1026 was selected, which represent the Chinese strains. A *H pylori* LZ1026 knockout mutant was constructed and its susceptibility profiles to 10 kinds of antibiotics were evaluated.

MATERIALS AND METHODS

Reagents

Chemical reagents were purchased from TaKaRa Biotechnology Co. Ltd (Dalian, China). Restriction enzymes, polymerases, T4 DNA ligase, Quant SYBR Green PCR Kits and other molecular biology reagents were purchased from Biosail Biotechnology Co. Ltd (Beijing, China), and used following their manufacturers' instructions. Amoxicillin, cefotaxime, ceftazidime, polymyxin B, nalidixic acid, clarithromycin, norfloxacin, gentamicin, metronidazole, tetracycline, erythromycin, chloramphenicol, and ciprofloxacin, penicillin G were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Nucleic acid sequencing and synthesis were performed in SaiBaiSheng Biotechnology Co. Ltd (Beijing, China).

Bacterial strains, plasmid and culture conditions

H pylori LZ1026 and 20 other clinical isolates of *H pylori* were obtained from routine cultures of clinical gastric biopsies from patients with peptic ulcer or chronic active gastritis at the Second Affiliated Hospital of Zhengzhou University (Zhengzhou, China). *H pylori* NCTC11637 obtained from HeNan Key Laboratory of Molecular Medicine was used as a reference. Isolates were cultured on Brucella agar medium plates containing 7% lysed sheep blood at 37℃ under microaerobic conditions (50 mL/L O_2, 100 mL/L CO_2, 850 mL/L N_2) for 48-72 h. Identification of *H pylori* isolates was based on the results of Gram staining, cell morphology, and positive reaction for catalase, oxidase and urease activity. Putative multidrug efflux knockout mutant of *H pylori* LZ1026 was grown on sheep blood agar plates containing 25 μg of kanamycin (KAN) per mL and 100 μg of ampicillin (AMP) per mL. The vectors pBluescript Ⅱ SK (-)and pEGFP-N2 were from HeNan Key Laboratory of Molecular Medicine. *E.coli* DH5α was used for subcloning the hefA operon. *E.coli* were grown in Luria broth. For cloning in *E.coli*, 100 mg/mL AMP was used.

Induction of multiple antibiotic resistances of clinical isolates

Susceptible strains were isolated from gastric biopsy samples using multiple antibiotic resistance induction tests. Induction of chloramphenicol resistance of susceptible isolates was performed by selecting resistant colonies that arose in the agar plates containing 1/2 × MIC chloramphenicol. The resistant colonies were incubated for 48-72 h under microaerobic conditions to increase the concentration of chloramphenicol by one fold up to 128 × MIC. The strains were further incubated on fresh plates with no chloramphenicol for 4 generations, and then transfered onto plates containing 4 × MIC chloramphenicol[19].

Induced colonies were maintained on plates containing 4 × MIC of erythromycin, metronidazole, tetracycline, penicillin G or ciprofloxacin, respectively. The colonies were incubated for 48-72 h under microaerobic conditions. A four-fold increase in MIC of the multidrug resistant strains was considered significant[20]. Six multidrug resistant strains were selected.

Assessment of susceptibilities to antimicrobials

H pylori cells grown for 48 h on sheep blood agar plates were resuspended in phosphate-buffered saline. Suspensions of *H pylori* were adjusted to an optical density of 0.1 at 625 nm , and 1 μL of these suspensions containing approximately 10^5 CFU/mL, was spread on horse blood agar plates containing approximately 5 × 10^4 bacteria within 5 mm[21]. MICs of erythromycin, metronidazole, penicillin G, tetracycline, and ciprofloxacin in multidrug resistant strains and their parent isolates were determined with the conventional two-fold agar dilution tests. MICs of the kinds of 10 antibiotics against the *hefA* mutant strain of *H pylori* LZ1026 and its wild strain were also detected[22].

Real-time RT-PCR

RNA was isolated using the total RNA kit (SBS Genetech Co., Ltd Beijing, China) and reverse transcribed into cDNA. Complete removal of DNA was verified by direct PCR with the RNA as a template (Figure 1). *hefA*, *versus gyrB* (a housekeeping gene encoding for gyrase B), was utilized to study the relative expression of the *hefA* gene in 6 multidrug resistant strains, and their parent strains: *H pylori* 03154, *H pylori* 12025, *H pylori* 12021, *H pylori* 11032, *H pylori* 03174, NCTC11637. cDNA of *hefA* and *gyrB* was amplified using a 5700 sequence detector real time PCR machine (Perkin Elmer Company) in the presence of Real Master Mix (SYBR Green). The gene-specific primers used were designed based on the sequence alignments of the genes from *H pylori* 11637 in GenBank. The sequences of *hefA* (accession No: AF059041) are F: (5'-ACGCCTCGAGTAAAAGCG CAAGGGAATTTG-3') and R: (5'-ACGCTCTAG ATTCGCTAATTGGCCTAGCAT-3'). The PCR primers were predicted to amplify a 162-bp amplicon. Expression of the housekeeping gene *gyrB* (accession No: AB084049) was assessed in parallel with the primer pair *gyrB*: F: (5'-TTACTACGACTTATCCTGGGGCTA GCGCTG-3') and R: (5'-CCCCATCAATTTCCACAT TCTCCGC-3'). The PCR primers were predicted to amplify a 267-bp amplicon.

To plot standard curves for real-time PCR, cDNAs of the *hefA* and *gyrB* genes were cloned into the pMD19-T vector system (TaKaRa Biotechnology). The vector containing cDNAs of the *hefA* or *gyrB* genes with known molar concentrations was utilized. Single reactions were prepared for each cDNA along with each serial of dilution using the SYBR Green Master Mix (Stratagene). Each PCR also included a reverse transcription negative control to confirm the absence of genomic DNA, and a non template negative control to check the primer-dimer. Each reaction consisted of 20 μL, containing 2 μL of cDNA and 5 pmol of each primer. The cycling conditions were 1 cycle of denaturation at 95℃ for 1 min, followed by 35 three-segment cycles of amplification at 95℃ for 30 s, at 55℃ for 1 min, and at 68℃ for 30 s during which the fluorescence was automatically measured, and one three-segment cycle of product melting at 95℃ for 1 min, at 55℃ for 30 s, and at 95℃ for 30 s. The baseline adjustment method of the Gene Amp 5700 SDS (Perkin-Elmer) software was used to determine Ct (the threshold cycle) in each reaction. A melting curve was plotted for each primer pair to verify the presence of one gene-specific peak, and the absence of primer dimer. All samples were amplified in three independent reactions, and the mean was used for further analysis[23-25].

To assess whether the differences were actually statistically significant, an analysis of paired-samples *t* - test was utilized to study the degree of statistical significance of *hefA* gene expression between MDR and parent strains.

Mutagenesis of putative efflux operon

All standard DNA techniques, transformation of *E.coli*, and DNA analysis procedures were performed as previously described[26-28]. Plasmid DNA was isolated using a TIANprep Mini plasmid kit (TianGen Biotech Co. Ltd, Beijing, China). *H pylori* LZ1026 strain was isolated from a patient with peptic ulcer, and its internal portion (bp 277 to 1 574) of the *hefA* gene (reference from *H pylori* 11637 in GenBank accession number AF059041) was amplified. A restriction fragment, approximately 1298 bp, was purified and ligated into XbaI and XhoI-digested vector pBluescript Ⅱ SK(-). *H pylori* LZ1026 knockout mutant for (putative) efflux protein was constructed by inserting the KAN resistance cassette from vector pEGFP-N2 into *hefA*, resulting in an 859 bp deletion. This suicide plasmid was then introduced into *H pylori* LZ1026 by natural transformation, and transformants were selected by plating them on a selective medium containing 25 mg of kanamycin/mL. Insertion of the KAN resistance cassette at the desired location(s) in the *H pylori* putative efflux gene was confirmed by PCR. Each clone was sequenced by Biosia Biotechnology Company (Shanghai, China).

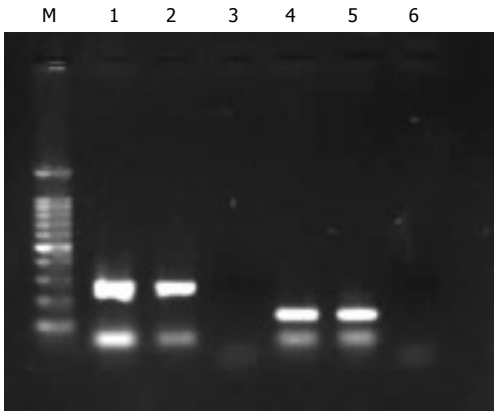


Figure 1 RT- PCR products of the *hefA* and *gyrB* genes. M: 100 bp DNA Marker; lanes 1 and 2: PCR products of *gyrB*; lanes 4 and 5: PCR products of *hefA*; lanes 3 and 6: PCR products with RNA as a template.

RESULTS

Phenotypic induction of hefA-mediated resistance of H pylori

To obtain the multidrug-resistant strains, a chloramphenicol-induced model was developed. Following one-fold concentration increase of chloramphenicol, the MICs of chloramphenicol-induced strains resistant to metronidazole, tetracycline, erythromycin, penicillin G, and ciprofloxacin were detected. The MICs of 6 multidrug-resistant strains (including 5 clinical isolates and *H pylori* NCTC11637) were significantly increased (≥ 4-fold) compared with their parent strains (Table 1).

Expression of hefA in multidrug resistant strains (MDR) and their parent isolates (PT)

The expression of *hefA* in multidrug resistant strains and their parent isolates was assessed by relative real-time RT-PCR. Each relative expression value was the mean of three replicas. The relative expression of *hefA versus gyrB*

Table 1 Chloramphenicol-induced multidrug resistance profiles of *H pylori* strains

Strains	Exptl condition	Agar dilution test MIC (μg/mL)				
		MTZ	ERY	CIP	TET	PEN
03154	Before induction	0.125	0.125	0.25	0.25	0.125
	After induction (fold)	8×	4×	4×	8×	16×
12025	Before induction	2	0.063	0.125	0.03	0.063
	After induction (fold)	16×	2×	8×	4×	16×
12021	Before induction	4	0.125	0.25	0.125	0.063
	After induction (fold)	16	1×	16×	8×	8×
11032	Before induction	1	0.063	0.063	0.063	0.25
	After induction (fold)	8×	4×	4×	16×	16×
03174	Before induction	0.5	0.125	0.25	0.125	0.125
	After induction (fold)	8×	1×	8×	4×	32×
11637	Before induction	0.5	0.063	0.125	0.03	0.25
	After induction (fold)	4×	2×	4×	8×	16×

MTZ: Metronidazole; ERY: Erythromycin; CIP: Ciprofloxacin; TET: Tetracycline; PEN: Penicillin G.

Table 2 Relative expression of *hefA* in PT and MDR strains

Strains	PT (*hefA*/*gyrB*)	MDR (*hefA*/*gyrB*)
03154	1.87	2.67
12025	0.32	2.84
12021	1.22	8.94
11032	3.84	5.04
03174	4.16	6.03
11637	4.41	9.56
Total (mean ± SD)	2.6356 ± 1.7245	5.8466 ± 2.9370

PT: Parent isolates; MDR: Multidrug resistant strains.

in 5 clinical isolates and *H pylori* 11637 was significantly higher in MDR (5.8466 ± 2.9370) than in PT (2.6356 ± 1.7245) (Table 2). The results reveal that the relative expression of *hefA* was higher in the induced multidrug resistant strains than in their wild isolates. The difference in *hefA* expression was statistically significant (P =0.033) (Figure 2).

Active efflux systems in H pylori

To further evaluate the role of efflux pump gene *hefA* of *H pylori* in multidrug resistance, a *H pylori* LZ1026 mutant was successfully constructed, and its disrupted *hefA* gene was uniquely different from that of the wild strain. A *hefA* gene fragment (about 1147 bp) of *H pylori* LZ1026 was sequenced and submitted to the GenBank (accession No. EU271683). Susceptibilities to wild-type strain of *H pylori* (*H pylori* LZ1026) and its isogenic mutant with putative RND outer membrane efflux protein were determined. The *hefA* (*H pilori* 0605, numbers refer to the loci in *H pylori* 26695) was genetically inactivated. MICs were determined by agar dilution tests. The *H pylori* mutant increased its susceptibility. The MIC of four of the 10 tested kinds of antibiotics (amoxicillin, cefotaxime, ceftazidime, polymyxin B, ciprofloxacin, nalidixic acid, clarithromycin, norfloxacin, chloramphenicol, and gentamicin,) was decreased at least 8-fold in the *H pylori* LZ1026 mutant, 8-fold in clarithromycin, 32-fold in cefotaxime, 16-fold in chloramphenicol, 8-fold in gentamicin. The MICs were decreased after treatment with different antibiotics, which is consistent with the hypothesis that the product of hefA participates in multidrug efflux.

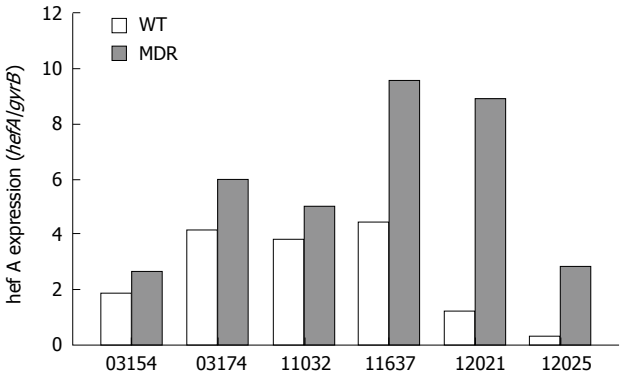


Figure 2 Expression of the *hefA* gene in PT and MDR strains.

Transcribed gene encoding TolC homolog in vitro

Transcription of the gene *hefA* encoding a homolog of *E.coli* TolC outer membrane protein was assessed by RT-PCR with cDNA obtained from 20 different *H pylori* isolates. All the 20 strains displayed transcripts of *hefA* that confirmed the *in vitro* expression of these genes (data not shown).

DISCUSSION

Treatment of *H pylori* infection usually requires administration of two or more kinds of antibiotics concurrently. However, it may ultimately not be sufficient due to increased antibiotic resistance. Indeed, bacterial resistance to antibiotics hampers treatment of *H pylori* infections. Besides chromosomally-encoded drug resistance, intrinsic resistance to toxic compounds through increased export might be of importance in multidrug resistance of *H pylori*. Efflux systems have been identified in *H pylori*[15,16], yet the possibility that these systems are implicated in multidrug resistance has not been established previously[15]. In this study, the *hefA* gene was chosen from three TolC homologs: *hefA*, *hefD* and *hefG*, which encode the outer membrane efflux protein in *H pylori*. It was demonstrated earlier that the *hefD* and *hefG* genes are only poorly expressed *in vitro* and only RND efflux systems are known to have a wide variety of antibiotic substrates[15,22].

In the present study, because it was laborious to select the multidrug resistant strains from clinical isolates of *H pylori*, we developed a chloramphenicol-induced multidrug resistant model of *H pylori*, for the first time to our knowledge. Using this model, six multidrug resistant strains were elicited successfully, and their MICs of metronidazole, tetracycline, erythromycin, penicillin G, and ciprofloxacin were significantly increased (≥ 4-fold) compared with their parent strains, except that the MIC of erythromycin was mildly increased in 4 wild strains, indicating a difference in substrate specificity with other bacteria[29,30]. In contrast, they could only dampen the

function of a group of efflux systems by constructing mutant strain. But, they could not quantitate the operon expression levels in the efflux systems[16]. In this study, the relative real-time RT-PCR was introduced to assess the expression level of the *hefA* gene encoding the outer membrane efflux protein in 5 clinical isolates and *H pylori*11637, suggesting that the relative expression of *hefA* is higher in induced multidrug resistant strains than in their wild susceptible isolates. This is consistent with the hypothesis that the expression of RND efflux systems in many bacteria is regulated by environmental stimuli, including the presence of antibiotics and antimicrobial compounds, growth stage, and stress factors[7-9]. By inserting the KAN resistance cassette into *hefA*, we also constructed a *H pylori* mutant strain (Δ*H pylori* LZ1026). By detecting the susceptibility to 10 kinds of antimicrobials with a more sensitive method of two-fold agar dilution tests, we showed that similar to other gram-negative organisms, the *H pylori hefA* mutant increased its susceptibility to antibiotics, which is consistent with the findings in a previous study[22], confirming that high expression of the *hefA* plays an important role in MDR of *H pylori*. Since *H pylori* contain an active multidrug efflux mechanism, compound efflux needs to be taken into account when determining resistance mechanisms in *H pylori*.

ACKNOWLEDGMENTS

The authors thank Zhi-Gang Huang for excellent technical assistance and providing plasmid pBluescript Ⅱ SK(-) and pEGFP-N2. This research was accomplished at the HeNan Key Laboratory of Molecular Medicine.

COMMENTS

Background

In recent years, the eradication rates of *H pylori* for standard therapies have decreased due to increasing antimicrobial resistance. Multidrug resistant *H pylori* strains have already been discovered. Possible mechanisms underlying multidrug resistant *H pylori* strains are involved in decreased drug uptake or increased drug efflux. Efflux of compounds is a phenomenon commonly observed in bacteria, and compound efflux decreases the susceptibility of *H pylori* strains to a variety of antibiotics. The contribution of efflux proteins to antibiotic resistance of *H pylori* strains is not well established.

Research frontiers

With the increasing antimicrobial resistance to *H pylori*, multidrug resistant *H pylori* strains have been discovered. Researches on the mechanisms of antibiotic resistance of *H pylori* have becoming a medical research hotspot. The mechanism underlying multidrug resistance of *H pylori* is still unclear.

Innovations and breakthroughs

The contribution of efflux proteins to antibiotic resistance of *H pylori*, strains is not well established. In view of the biological difference of *H pylori* strains in Asia, Europe and North America, *H pylori* strains were isolated from Chinese people in the present study. The study was to determine whether efflux systems in *H pylori* strains contribute to their antimicrobial susceptibility. A chloramphenicol-induced multidrug resistant model of *H pylori* strains was developed. The level of mRNA expression of *hefA* in multidrug-resistant (MDR) strains and their parent strains were assessed by fluorescence real-time quantitative PCR. A *H pylori* LZ1026 knockout mutant was constructed and its susceptibility profiles to 10 kinds of antibiotics were evaluated.

Applications

This study confirmed that high expression of the *hefA* plays an important role in MDR of *H pylori*. Since *H pylori* contain an active multidrug efflux mechanism, compound efflux needs to be taken into account when determining resistance mechanisms in *H pylori*.

Terminology

Efflux pump is chromosomally located or acquired by bacteria, which can either be activated by environmental signals or by a mutation in a regulatory gene. Two major categories exist: one is energized by proton motive force and the other is dependent on ATP. The pumps may have limited or broad substrates, the so-called multiple drug resistant pumps themselves form a number of related families.

Peer review

The authors studied the significance of efflux pump gene hefA in multidrug resistance of *H pylori*. The study is interesting and well designed.

REFERENCES

1 **Moayyedi P**, Hunt RH. Helicobacter pylori public health implications. *Helicobacter* 2004; **9** Suppl 1: 67-72
2 **Marshall BJ**, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984; **1**: 1311-1315
3 **Crowe SE**. Helicobacter infection, chronic inflammation, and the development of malignancy. *Curr Opin Gastroenterol* 2005; **21**: 32-38
4 **Egan BJ**, Katicic M, O'Connor HJ, O'Morain CA. Treatment of Helicobacter pylori. *Helicobacter* 2007; **12** Suppl 1: 31-37
5 **Malfertheiner P**, Megraud F, O'Morain C, Bazzoli F, El-Omar E, Graham D, Hunt R, Rokkas T, Vakil N, Kuipers EJ. Current concepts in the management of Helicobacter pylori infection: the Maastricht III Consensus Report. *Gut* 2007; **56**: 772-781
6 **Nikaido H**. Antibiotic resistance caused by gram-negative multidrug efflux pumps. *Clin Infect Dis* 1998; **27** Suppl 1: S32-S41
7 **Borges-Walmsley MI**, Walmsley AR. The structure and function of drug pumps. *Trends Microbiol* 2001; **9**: 71-79
8 **Putman M**, van Veen HW, Konings WN. Molecular properties of bacterial multidrug transporters. *Microbiol Mol Biol Rev* 2000; **64**: 672-693
9 **Dastidar V**, Mao W, Lomovskaya O, Zgurskaya HI. Drug-induced conformational changes in multidrug efflux transporter AcrB from Haemophilus influenzae. *J Bacteriol* 2007; **189**: 5550-5558
10 **Paulsen IT**, Chen J, Nelson KE, Saier MH Jr. Comparative genomics of microbial drug efflux systems. *J Mol Microbiol Biotechnol* 2001; **3**: 145-150
11 **Saier MH Jr**, Tam R, Reizer A, Reizer J. Two novel families of bacterial membrane proteins concerned with nodulation, cell division and transport. *Mol Microbiol* 1994; **11**: 841-847
12 **Liu J**, Takiff HE, Nikaido H. Active efflux of fluoroquinolones in Mycobacterium smegmatis mediated by LfrA, a multidrug efflux pump. *J Bacteriol* 1996; **178**: 3791-3795
13 **Baucheron S**, Imberechts H, Chaslus-Dancla E, Cloeckaert A. The AcrB multidrug transporter plays a major role in high-level fluoroquinolone resistance in Salmonella enterica serovar typhimurium phage type DT204. *Microb Drug Resist* 2002; **8**: 281-289
14 **Johnson JM**, Church GM. Alignment and structure prediction of divergent protein families: periplasmic and outer membrane proteins of bacterial efflux pumps. *J Mol Biol* 1999; **287**: 695-715
15 **Bina JE**, Alm RA, Uria-Nickelsen M, Thomas SR, Trust TJ, Hancock RE. Helicobacter pylori uptake and efflux: basis for intrinsic susceptibility to antibiotics in vitro. *Antimicrob Agents Chemother* 2000; **44**: 248-254
16 **van Amsterdam K**, Bart A, van der Ende A. A Helicobacter pylori TolC efflux pump confers resistance to metronidazole. *Antimicrob Agents Chemother* 2005; **49**: 1477-1482
17 **Koronakis V**. TolC--the bacterial exit duct for proteins and drugs. *FEBS Lett* 2003; **555**: 66-71
18 **Monteiro MA**, Zheng P, Ho B, Yokota S, Amano K, Pan Z, Berg DE, Chan KH, MacLean LL, Perry MB. Expression

of histo-blood group antigens by lipopolysaccharides of Helicobacter pylori strains from asian hosts: the propensity to express type 1 blood-group antigens. *Glycobiology* 2000; **10**: 701-713

19 **Michea-Hamzehpour M**, Kahr A, Pechere JC. In vitro stepwise selection of resistance to quinolones, beta-lactams and amikacin in nosocomial gram-negative bacilli. *Infection* 1994; **22** Suppl 2: S105-S110

20 **Wierzbowski AK**, Boyd D, Mulvey M, Hoban DJ, Zhanel GG. Expression of the mef(E) gene encoding the macrolide efflux pump protein increases in Streptococcus pneumoniae with increasing resistance to macrolides. *Antimicrob Agents Chemother* 2005; **49**: 4635-4640

21 **Jeannot K**, Sobel ML, El Garch F, Poole K, Plesiat P. Induction of the MexXY efflux pump in Pseudomonas aeruginosa is dependent on drug-ribosome interaction. *J Bacteriol* 2005; **187**: 5341-5346

22 **Kutschke A**, de Jonge BL. Compound efflux in Helicobacter pylori. *Antimicrob Agents Chemother* 2005; **49**: 3009-3010

23 **Aellen S**, Que YA, Guignard B, Haenni M, Moreillon P. Detection of live and antibiotic-killed bacteria by quantitative real-time PCR of specific fragments of rRNA. *Antimicrob Agents Chemother* 2006; **50**: 1913-1920

24 **Nygard AB**, Jorgensen CB, Cirera S, Fredholm M. Selection of reference genes for gene expression studies in pig tissues using SYBR green qPCR. *BMC Mol Biol* 2007; **8**: 67

25 **Nayak AK**, Rose JB. Detection of Helicobacter pylori in sewage and water using a new quantitative PCR method with SYBR green. *J Appl Microbiol* 2007; **103**: 1931-1941

26 **Lee DH**, Oh DC, Oh YS, Malinverni JC, Kukor JJ, Kahng HY. Cloning and characterization of monofunctional catalase from photosynthetic bacterium Rhodospirillum rubrum S1. *J Microbiol Biotechnol* 2007; **17**: 1460-1468

27 **Yuan JP**, Li T, Shi XD, Hu BY, Yang GZ, Tong SQ, Guo XK. Deletion of Helicobacter pylori vacuolating cytotoxin gene by introduction of directed mutagenesis. *World J Gastroenterol* 2003; **9**: 2251-2257

28 **Jo C**, Jo SA. A simple method to construct T-vectors using XcmI cassettes amplified by nonspecific PCR. *Plasmid* 2001; **45**: 37-40

29 **Burse A**, Weingart H, Ullrich MS. NorM, an Erwinia amylovora multidrug efflux pump involved in in vitro competition with other epiphytic bacteria. *Appl Environ Microbiol* 2004; **70**: 693-703

30 **Fehlner-Gardiner CC**, Valvano MA. Cloning and characterization of the Burkholderia vietnamiensis norM gene encoding a multi-drug efflux protein. *FEMS Microbiol Lett* 2002; **215**: 279-283

S- Editor Li DL **L- Editor** Wang XL **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5223

World J Gastroenterol 2008 September 7; 14(33): 5223-5225
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Combined endoscopic and ursodeoxycholic acid treatment of biliary cast syndrome in a non-transplant patient

Panagiotis Katsinelos, Jannis Kountouras, Grigoris Chatzimavroudis, Christos Zavos, Ioannis Pilpilidis, George Paroutoglou

Panagiotis Katsinelos, Grigoris Chatzimavroudis, Ioannis Pilpilidis, George Paroutoglou, Department of Endoscopy and Motility Unit, Central Hospital, Thessaloniki 54635, Greece
Jannis Kountouras, Christos Zavos, Department of Gastroenterology, Second Medical Clinic, Aristotle University of Thessaloniki, Ippokration Hospital, Thessaloniki 54635, Greece
Author contributions: Katsinelos P, Kountouras J, Chatzimavroudis G, Zavos C, Pilpilidis I, Paroutoglou G contributed equally to the work; Katsinelos P, Kountouras J, Chatzimavroudis G and Zavos C wrote the paper; Pilpilidis I and Paroutoglou G selected the bibliography.
Correspondence to: Dr. Panagiotis Katsinelos, Department of Endoscopy and Motility Unit, Central Hospital, Ethnikis Aminis 41, Thessaloniki 54635, Greece. gchatzim@med.auth.gr
Telephone: +30-2310-963341 Fax: +30-2310-210401
Received: May 23, 2008 Revised: July 12, 2008
Accepted: July 19, 2008
Published online: September 7, 2008

Abstract

A 76-year-old diabetic man underwent cholecystectomy for gangrenous calculous cholecystitis. His postoperative course was complicated by the development of Candida albicans esophagitis necessitating antifungal therapy, and total parenteral nutrition (TPN) for 15 d. Seven weeks after cholecystectomy, he presented with cholangitis. Endoscopic retrograde cholangiopancreatography (ERCP) demonstrated extrahepatic filling defects. Despite endoscopic extraction of a biliary cast, cholestasis remained unchanged. Oral administration of ursodeoxycholic acid (UDCA), 750 mg/d, resulted in normalization of liver function tests. We, therefore, propose for the first time, combined endoscopic plus UDCA treatment for the management of biliary cast syndrome.

Key words: Biliary cast; Ursodeoxycholic acid; Endoscopic retrograde cholangiopancreatography

Peer reviewer: Laura Llado, PhD, Liver Transplant Unit-Dept of Surgery, Hospital U Bellvitge, Feixa Llarga S/N, Hospitalet LL (Barcelona) 08907, Spain

Katsinelos P, Kountouras J, Chatzimavroudis G, Zavos C, Pilpilidis I, Paroutoglou G. Combined endoscopic and ursodeoxycholic acid treatment of biliary cast syndrome in a non-transplant patient. *World J Gastroenterol* 2008; 14(33): 5223-5225 Available from: URL: http://www.wjgnet.com/1007-9327/14/5223.asp DOI: http://dx.doi.org/10.3748/wjg.14.5223

INTRODUCTION

Though uncommon, biliary casts are most frequently described in liver transplant patients[1-4]. The etiology of cast development is not fully known, but it is likely to be multifactorial with biliary sludge being a prerequisite for cast formation. Bile duct damage and ischemia, hemolysis, cholangitis, fasting, total parenteral nutrition (TPN) and recent surgery are thought to be implicated in cast, pathogenesis *via* sludge development[3,4].

We describe a diabetic man who developed a biliary cast after cholecystectomy. The patient underwent endoscopic retrograde cholangiopancreatography (ERCP) with extraction of an extrahepatic biliary cast. But, his cholestasis remained unchanged. Oral administration of ursodeoxycholic acid (UDCA) resulted in normalization of liver function tests.

CASE REPORT

A 76-year-old diabetic patient underwent emergent cholecystectomy for gangrenous calculous cholecystitis. He had a protracted postoperative course complicated with Candida esophagitis treated with 200 mg fluconazole, twice per day and TPN for 15 d during which dyslipidemia was present. His cholesterol level was 503 mg/dL (< 200 mg/dL) and triglyceride was 967 mg/dL (< 200 mg/dL). Seven weeks later, he presented with abdominal pain, chills and a fever of 38.5℃. On admission, clinical examination revealed right upper quadrant tenderness and jaundice. Laboratory data (normal values in parentheses) showed 7.8 mg/dL total bilirubin (< 1.2 mg/dL), 986 IU/L alkaline phosphatase (< 40 IU/L), 519 IU/L γ-glutamyl transpeptidase (< 40 IU/L), 189 IU/L ALT (< 40 IU/L), 167 IU/L AST (< 40 IU/L), 484 mg/dL cholesterol, 878 mg/dL

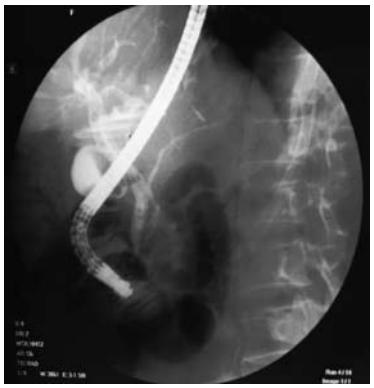


Figure 1 ERCP image revealing the dilated biliary tract with a tubular filling defect in the common bile duct until the junction of right and left hepatic ducts.

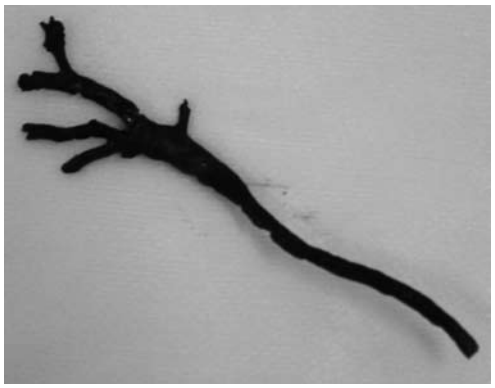


Figure 2 Biliary cast replicating the extrahepatic ductal system including the cystic duct stump.

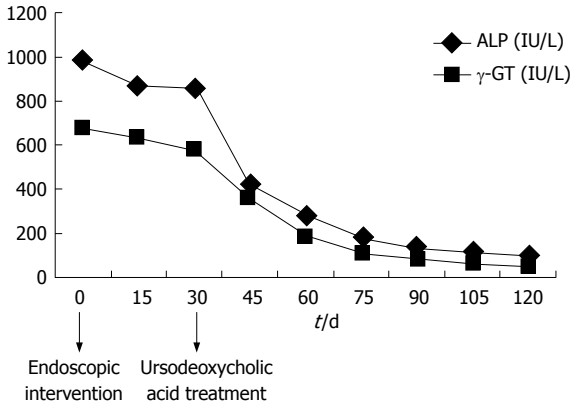


Figure 3 Effect of UDCA on cholestatic enzymes.

triglycerides and 18 000 cells/mm^3 white blood cell count (< 10 000 cells/mm^3).

Abdominal ultrasound and computed tomography demonstrated biliary tract dilatation without apparent lesion. At ERCP, a common bile duct tubular filling defect was found (Figure 1). Endoscopic sphincterotomy was performed, followed by basket retrieval of a cast of extrahepatic biliary tract (Figure 2). No residual cast in the visualized intrahepatic bile ducts was demonstrated. Cholangitis resolved, but cholestasis remained unchanged in the next month. UDCA (750 mg/d) was administered and a gradual improvement in cholestasis was observed with normalization of cholestatic enzymes after a 3-mo treatment period (Figure 3). After a 6-mo treatment period, a repeated ERCP showed a normal biliary tract. The patient's condition was excellent with normal liver function tests 6 mo after cessation of UDCA treatment when we wrote this paper.

DISCUSSION

The biliary cast syndrome describes the presence of casts causing obstruction with its resultant sequelae of biliary infection, hepatocyte damage secondary to bile stasis and ductal damage, all contributing to cholangiopathy. The diagnosis is suspected in liver-transplant patients presenting with abnormal liver-associated enzymes of a cholestatic pattern, evidence of ductal dilatation on abdominal imaging studies or clinical evidence of obstruction, such as cholangitis or jaundice[1-4] and is usually confirmed by ERCP.

To the best of our knowledge, there are only three reports of a total biliary cast in the non-transplant situation. D'Haens *et al*[5] described an elderly lady with extensive cast formation that developed after initial recovery from emergency cholecystectomy. Despite multiple attempts at endoscopic removal, post-mortem findings confirmed the presence of diffuse bile casts and acute suppurative cholangitis. The authors suggest that, in the absence of a gallbladder, the increased bile pigment load may have initiated biliary cast formation. In the second report, Byrne *et al*[6] described a patient with a history of traumatic head injury requiring admission to the intensive care unit, who developed a biliary cast. Despite endoscopic and surgical attempts at cast removal from large bile ducts, liver transplantation was ultimately performed for biliary cast formation. In a recent report, Gleeson *et al*[7] described 2 patients with biliary cast syndrome in a non liver transplant setting. In the first case, biliary cast syndrome developed in a patient with no preexisting liver pathology. But, probably hepatic infarction induced by antiphospholipid antibody syndrome triggered the ischemic event leading to extrahepatic cast formation. In the second case, the etiology of cast formation was multifactorial, including fasting-related gallbladder hypocontractility, parenteral nutrition, biliary infection and possible ischemia due to hypotension-induced sepsis.

Biochemical analysis of biliary casts in liver-transplant patients showed that bilirubin seemed to be the main component (10%-50%) of casts and comprised conjugated free and oxidized varieties. Bile acids comprised 10%-15% of the cast sample and cholesterol represented about 5%-10%[4]. The chemical composition of biliary casts in non-transplant patients is unknown due to its rarity. However, in both reported non-transplant patients[5,6] and in our case, the biliary cast developed after difficult abdominal surgery in two patients, and severe head injury in one patient. They all had a long postoperative course with TPN. Our patient had similar characteristics (fasting, parenteral nutrition, biliary infection) with the second patient in Gleeson *et al*'s report[7], while the underlying mechanism of biliary cast syndrome in the first patient was hepatic ischemia due

to antiphospholipid antibody syndrome. The similarities and differences of all reported non transplant patients with biliary cast syndrome underscore its multifactorial pathogenesis. It is known that sick patients, particularly abdominal surgical and neurosurgical patients, in intensive care units have been shown to develop biliary sludge frequently and rapidly[8,9]. Fasting promotes gallbladder hypocontractility due to insufficient secretion of cholecystokinin and may, in part, explain the abdominal surgical patient group preference[8,9]. Other risk factors for sludge formation include TPN, pregnancy and acquired immunodeficiency syndrome[10,11].

The persistent cholestasis of our patient, despite removal of the extrahepatic biliary cast, is explained by the fact that development of cast was extensive and included the small intrahepatic ducts resulting in obstructive cholangiopathy. It is known that prolonged obstruction of the intra-and/or extrahepatic biliary tree due to stones or sludge, predisposes to caliber irregularities of the biliary system and also toxic effects of bile on hepatocytes with cholangitis further to contribute to cholestatic liver damage[10].

We suppose that the mechanism by which the UDCA resolved our patient's cholestasis is the dissolution of biliary cast of small intrahepatic bile ducts because its main component is cholesterol. The presence of diabetes associated with a high level of cholesterol and combined with long-postoperative TPN contributes to hypersaturation of bile with cholesterol, and initiates biliary cast formation. Stone or sludge dissolution by UDCA is a complex physiochemical process, affecting mainly the cholesterol and phospholipid ionophase and enhancing cholesterol solubilization[12,13]. It would be interesting if chromatography had been performed to reveal the components of our patient's biliary cast and confirm our hypothesis.

The beneficial effect of UDCA on biliary cast-associated cholestatic liver damage may be related to its hepatocytoprotective action, improvement in biliary acid secretion and immunomodulatory properties which reduce immune-related liver damage[14,15]. Mechanisms underlying the beneficial therapeutic effects of UDCA on cholestatic liver diseases include: (1) protection of injured cholangiocytes against toxic effects of bile acids, (2) stimulation of impaired biliary secretion, (3) stimulation of detoxification of hydrophobic bile acids, and (4) inhibition of apoptosis of hepatocytes. However, it is not clear which of these mechanisms by which UDCA exerts its beneficial effects on cholestatic liver diseases. Most likely, such effects depend on the specific cholestatic liver disease and its stage[16]. Knowing that biliary infection, bile duct damage and ischemia, fasting, hemolysis and abdominal surgery are all factors that may be implicated in biliary cast syndrome[17], it would be interesting to use UDCA as a prophylactic in patients at risk of developing biliary cast syndrome. However, the rarity of this complication makes such a study practically impossible.

In conclusion, administration of UDCA, if cholestasis remains unchanged after endoscopic removal of extrahepatic biliary cast, is a very promising treatment modality for biliary cast syndrome.

REFERENCES

1 **Starzl TE**, Putnam CW, Hansbrough JF, Porter KA, Reid HA. Biliary complications after liver transplantation: with special reference to the biliary cast syndrome and techniques of secondary duct repair. *Surgery* 1977; **81**: 212-221

2 **Chen CL**, Wang KL, Chuang JH, Lin JN, Chu MF, Chang CH. Biliary sludge-cast formation following liver transplantation. *Hepatogastroenterology* 1988; **35**: 22-24

3 **Tung BY**, Kimmey MB. Biliary complications of orthotopic liver transplantation. *Dig Dis* 1999; **17**: 133-144

4 **Shah JN**, Haigh WG, Lee SP, Lucey MR, Brensinger CM, Kochman ML, Long WB, Olthoff K, Shaked A, Ginsberg GG. Biliary casts after orthotopic liver transplantation: clinical factors, treatment, biochemical analysis. *Am J Gastroenterol* 2003; **98**: 1861-1867

5 **D'Haens GR**, Ruchim MA, Goldberg MJ, Baker AL. Massive intra-hepatic and extra-hepatic bile cast formation after cholecystectomy. *Gastrointest Endosc* 1993; **39**: 579-581

6 **Byrne MF**, Chong HI, O'Donovan D, Sheehan KM, Leader MB, Kay E, McCormick PA, Broe P, Murray FE. Idiopathic cholangiopathy in a biliary cast syndrome necessitating liver transplantation following head trauma. *Eur J Gastroenterol Hepatol* 2003; **15**: 415-417

7 **Gleeson FC**, Czaja AJ, Baron TH. Successful endoscopic management of biliary cast syndrome in nonliver transplant patients. *J Clin Gastroenterol* 2008; **42**: 752-755

8 **Murray FE**, Stinchcombe SJ, Hawkey CJ. Development of biliary sludge in patients on intensive care unit: results of a prospective ultrasonographic study. *Gut* 1992; **33**: 1123-1125

9 **Bolondi L**, Gaiani S, Testa S, Labo G. Gall bladder sludge formation during prolonged fasting after gastrointestinal tract surgery. *Gut* 1985; **26**: 734-738

10 **Horton JD**, Bilhartz LE. Gallstone disease and its complications. In: Feldman M, Friedman LS, Sleisenger MH. Gastrointestinal and Liver Disease. 7th ed. Philadelphia: Saunders, 2002: 1065-1090

11 **Angelico M**, Della Guardia P. Review article: hepatobiliary complications associated with total parenteral nutrition. *Aliment Pharmacol Ther* 2000; **14** Suppl 2: 54-57

12 **Tint GS**, Salen G, Colalillo A, Graber D, Verga D, Speck J, Shefer S. Ursodeoxycholic acid: a safe and effective agent for dissolving cholesterol gallstones. *Ann Intern Med* 1982; **97**: 351-356

13 **Shiffman ML**, Kaplan GD, Brinkman-Kaplan V, Vickers FF. Prophylaxis against gallstone formation with ursodeoxycholic acid in patients participating in a very-low-calorie diet program. *Ann Intern Med* 1995; **122**: 899-905

14 **Kowdley KV**. Ursodeoxycholic acid therapy in hepatobiliary disease. *Am J Med* 2000; **108**: 481-486

15 **Hempfling W**, Dilger K, Beuers U. Systematic review: ursodeoxycholic acid--adverse effects and drug interactions. *Aliment Pharmacol Ther* 2003; **18**: 963-972

16 **Paumgartner G**, Beuers U. Mechanisms of action and therapeutic efficacy of ursodeoxycholic acid in cholestatic liver disease. *Clin Liver Dis* 2004; **8**: 67-81, vi

17 **Parry SD**, Muiesan P. Cholangiopathy and the biliary cast syndrome. *Eur J Gastroenterol Hepatol* 2003; **15**: 341-343

S- Editor Li DL **L- Editor** Wang XL **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5226

World J Gastroenterol 2008 September 7; 14(33): 5226-5227
World Journal of Gastroenterology ISSN 1007-9327

LETTERS TO THE EDITOR

Management of constipation in the elderly: Emerging therapeutic strategies

Shailendra Kapoor

Shailendra Kapoor, Department of Family Medicine, University of Illinois at Chicago, Chicago, IL 60612, United States
Author contributions: Kapoor S contributed all to this paper.
Correspondence to: Shailendra Kapoor, MD, Department of Family Medicine, University of Illinois at Chicago, Chicago IL-60612, United States. shailendrakapoor@yahoo.com
Telephone: +1-312-3441704 Fax: +1-312-1441464
Received: June 9, 2008 Revised: August 14, 2008
Accepted: August 21, 2008
Published online: September 7, 2008

Abstract

A number of new, novel strategies for managing constipation in the elderly have emerged over the past few years. Prucalopride is one such new agent that is highly efficacious in managing chronic constipation. In fact, Camilleri *et al* in a recent study reported that the average number of bowel movements increased by at least one in nearly 47% of the patients who were administered a dose of 4 mg. Lubiprostone is another new agent recently approved by the FDA that shows efficacy in managing the symptoms of constipation. Neostigmine has also been successfully used for the management of recalcitrant constipation. Most of these studies have used subcutaneous neostigmine. Symbiotic yogurt containing components, such as Bifidobacterium and fructoligosaccharide, have also been recently shown to be highly effective in improving symptoms of constipation. Elderly patients especially those in hospices and nursing homes are often on opiods for pain management. Constipation secondary to opioid use is extremely common in nursing homes. Subcutaneous methylnaltrexone has recently been shown to be highly effective in the management of opioid-related constipation, and was recently approved by the FDA. Sacral nerve stimulation is another emerging strategy. A recent analysis by Mowatt *et al* supports the efficacy of this technique. Botulinum toxin is another agent that has already been successfully used for the management of chronic, refractory constipation in children and may be very effective for elderly constipation. Further larger studies are needed to confirm the findings noted in these studies. Constipation is clearly a major issue in the elderly and these new, emerging strategies will hopefully improve the quality of life and relieve the symptoms of constipation in this population.

Key words: Constipation; Prucalopride; Lubiprostone; Neostigmine; Methylnaltrexone

Peer reviewer: Wallace F Berman, MD, Professor, Box 3009 DUMC, Durham NC 27710, United States

Kapoor S. Management of constipation in the elderly: Emerging therapeutic strategies. *World J Gastroenterol* 2008; 14(33): 5226-5227 Available from: URL: http://www.wjgnet.com/1007-9327/14/5226.asp DOI: http://dx.doi.org/10.3748/wjg.14.5226

TO THE EDITOR

The recent article by McCrea *et al* about the pathophysiology of constipation was highly enlightening[1]. Interestingly, a number of new, novel strategies for managing constipation in the elderly have emerged over the past few years.

Prucalopride is one such new agent that is highly efficacious in managing chronic constipation. In fact, Camilleri *et al* in a recent study reported that the average number of bowel movements increased by at least one in nearly 47% of the patients who were administered a dose of 4 mg[2]. Lubiprostone is another new agent recently approved by the FDA that shows efficacy in managing the symptoms of constipation. Lubiprostone is most efficacious when administered in a daily dose of 16 mg[3]. Neostigmine has also been successfully used for the management of recalcitrant constipation. Most of these studies have used subcutaneous neostigmine administered in cancer patients[4]. Symbiotic yogurt containing components, such as Bifidobacterium and fructoligosaccharide, have also been recently shown to be highly effective in improving symptoms of constipation[5].

Elderly patients especially those in hospices and nursing homes are often on opioids for pain management. Constipation secondary to opioid use is extremely common in nursing homes. Subcutaneous methylnaltrexone has recently been shown to be highly effective in the management of opioid related constipation and was recently approved by the FDA[6,7]. Sacral nerve stimulation is another emerging strategy[8]. A recent analysis by Mowatt *et al* supports the efficacy of this technique[9]. Botulinum toxin is another agent that

has already been successfully used for the management of chronic, refractory constipation in children and may be effective in elderly constipation[10].

Further larger studies are needed to confirm the findings noted in these studies. Constipation is clearly a major issue in the elderly, and these new, emerging strategies will hopefully improve the quality of life and relieve the symptoms of constipation in this population.

REFERENCES

1 **McCrea GL**, Miaskowski C, Stotts NA, Macera L, Varma MG. Pathophysiology of constipation in the older adult. *World J Gastroenterol* 2008; **14**: 2631-2638

2 **Camilleri M**, Kerstens R, Rykx A, Vandeplassche L. A placebo-controlled trial of prucalopride for severe chronic constipation. *N Engl J Med* 2008; **358**: 2344-2354

3 **Johanson JF**, Drossman DA, Panas R, Wahle A, Ueno R. Clinical trial: phase 2 study of lubiprostone for irritable bowel syndrome with constipation. *Aliment Pharmacol Ther* 2008; **27**: 685-696

4 **Rubiales AS**, Hernansanz S, Gutierrez C, Del Valle ML, Flores LA. Neostigmine for refractory constipation in advanced cancer patients. *J Pain Symptom Manage* 2006; **32**: 204-205

5 **De Paula JA**, Carmuega E, Weill R. Effect of the ingestion of a symbiotic yogurt on the bowel habits of women with functional constipation. *Acta Gastroenterol Latinoam* 2008; **38**: 16-25

6 **Portenoy RK**, Thomas J, Moehl Boatwright ML, Tran D, Galasso FL, Stambler N, Von Gunten CF, Israel RJ. Subcutaneous methylnaltrexone for the treatment of opioid-induced constipation in patients with advanced illness: a double-blind, randomized, parallel group, dose-ranging study. *J Pain Symptom Manage* 2008; **35**: 458-468

7 **Traynor K**. Relistor approved for opioid-related constipation. *Am J Health Syst Pharm* 2008; **65**: 996

8 **Lee KJ**, Kim JH, Cho SW. Short-term effects of magnetic sacral dermatome stimulation for idiopathic slow transit constipation: sham-controlled, cross-over pilot study. *J Gastroenterol Hepatol* 2006; **21**: 47-53

9 **Mowatt G**, Glazener C, Jarrett M. Sacral nerve stimulation for fecal incontinence and constipation in adults: a short version Cochrane review. *Neurourol Urodyn* 2008; **27**: 155-161

10 **Irani K**, Rodriguez L, Doody DP, Goldstein AM. Botulinum toxin for the treatment of chronic constipation in children with internal anal sphincter dysfunction. *Pediatr Surg Int* 2008; **24**: 779-783

S- Editor Li DL **L- Editor** Ma JY **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2008 September 7; 14(33): 5228
World Journal of Gastroenterology ISSN 1007-9327

ACKNOWLEDGMENTS

Acknowledgments to Reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

Frank A Anania, Professor
Emory University School of Medicine, Division of Division Digestive Diseases, 615 Michael Street, Room 255 Whitehead Biomedical Research Building, Atlanta, GA 30322, United States

Marc Basson, MD, PhD, MBA, Chief of Surgery
John D Dingell VA Medical Center, 4646 John R. Street, Detroit, MI 48301, United States

Carla W Brady, MD, MHS
Duke University Medical Center, Division of Gastroenterology, DUMC Box 3913, Durham, NC 27705, United States

Dr. Giuseppe Chiarioni
Gastroenterological Rehabilitation Division of the University of Verona, Valeggio sul Mincio Hospital, Azienda Ospedale di Valeggio s/M, Valeggio s/M 37067, Italy

William Dickey
Altnagelvin Hospital, Londonderry, BT47 6SB, Northern Ireland, United Kingdom

Peter Raymond Gibson, Professor
Department of Medicine, Box Hill Hospital, Box Hill, Victoria 3128, Australia

Julia B Greer, MD, MPH
Department of Gastroenterology, Hepatology and Nutrition, University of Pittsburgh Medical Center, M2, Presbyterian University Hospital, 200 Lothrop Street, Pittsburgh, Pa 15213, United States

Kazuhiro Hanazaki, MD, Professor and Chairman
Department of Surgery, Kochi Medical School, Kochi University, Kohasu, Okohcho, Nankoku, Kochi 783-8505, Japan

Yik-Hong Ho, Professor
Department of Surgery, School of Medicine, James Cook University, Townsville 4811, Australia

Dr. Pietro Invernizzi
Division of Internal Medicine, Department of Medicine, Surgery, Dentistry, San Paolo School of Medicine, University of Milan, Via Di Rudinfi 8, 20142 Milan, Italy

Roger Jones, Professor
Department of General Practice and Primary Care, King's College London, 5 Lambeth Walk, London SE11 6SP, United Kingdom

Paul Y Kwo, Professor
Gastroenterology and Hepatology Division, Indiana University School of Medicine, 975 West Walnut, IB 327, Indianapolis, Indiana 46202-5121, United States

Walter E Longo, Professor
Department of Surgery, Yale University School of Medicine, 205 Cedar Street, New Haven 06510, United States

Morito Monden, Professor
Department of Surgery and Clinical Oncology, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita 565-0871, Japan

Peter L Moses, MD, FACG, AGAF, Professor
University of Vermont College of Medicine Section of Gastroenterology & Hepatology,111 Colchester Avenue, Smith 237B, MCHV, Burlington, VT 05401, United States

Shotaro Nakamura, MD
Department of Medicine and Clinical Science, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan

James Neuberger, Professor
Liver Unit, Queen Elizabeth Hospital, Birmingham B15 2TH, United Kingdom

Ramesh Roop Rai, MD, DM (Gastro.), Professor & Head
Department of Gastroenterology & Hepatology, S.M.S. Medical College & Hospital, Jaipur 302019, (Rajasthan), India

Dr. Bernardino Rampone
Department of General Surgery and Surgical Oncology, University of Siena, viale Bracci, Siena 53100, Italy

Ian C Roberts-Thomson, Professor
Department of Gastroenterology and Hepatology, The Queen Elizabeth Hospital, 28 Woodville Road, Woodville South 5011, Australia

Francis Seow-Choen, Professor
Seow-Choen Colorectal Centre, Mt Elizabeth Medical Centre, Singapore, 3 Mt Elizabeth Medical Centre #09-10 , 228510, Singapore

Mitsuo Shimada, Professor
Department of Digestive and Pediatric Surgery, Tokushima University, Kuramoto 3-18-15, Tokushima 770-8503, Japan

Wing-Kin Syn, MD
Division of Gastroenterology, GSRB-1, Suite 1073, DUMC 3256, 595 LaSalle Street, Durham, NC27710, United States

Seyed A Taghavi, Associate Professor
Department of Internal Medicine, Nemazee Hospital, No.23, 59th Alley, Ghasrodasht St., Shiraz 71838-95453, Iran

George Y Wu, Professor
Department of Medicine, Division of Gastroenterology-Hepatology, University of Connecticut Health Center, 263 Farmington Ave, Farmington, CT 06030, United States

Shu Zheng, Professor
Scientific Director of Cancer Institute, Zhejiang University, Secondary Affiliated Hospital, Zhejiang University, 88# Jiefang Road, Hangzhou 310009, Zhejiang Province, China

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2008 September 7; 14(33): 5229
World Journal of Gastroenterology ISSN 1007-9327

Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
January 24-25, Frankfurt, Germany
Falk Workshop: Perspectives in Liver
Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
E-mail: education@asge.org

June 4-7, Helsinki, Finland
The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
ESGAR 2008 19th Annual Meeting and Postgraduate Course
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden
16th International Congress of the European Association for Endoscopic Surgery
E-mail: info@eaes-eur.org

June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
10th World Congress on Gastrointestinal Cancer
Imedex and ESMO
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland
Joint Meeting of the European Pancreatic Club (EPC)
and the International Association of Pancreatology (IAP)
E-mail: office@epc-iap2008.org
www.e-p-c.org
www.pancreatology.org

June 26-28, Bratislava, Slovakia
5th Central European Gastroenterology Meeting
www.ceurgem2008.cz

July 9-12, Paris, France
ILTS 14th Annual International Congress
www.ilts.org

September 10-13, Budapest, Hungary
11th World Congress of the International Society for Diseases of the Esophagus
E-mail: isde@isde.net

September 13-16, New Delhi, India
Asia Pacific Digestive Week
E-mail: apdw@apdw2008.net

III FALK GASTRO-CONFERENCE
September 17, Mainz, Germany
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
Falk Symposium 166:
GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
Falk Symposium 167:
Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
Third Annual Meeting
European Society of Coloproctology
www.escp.eu.com

October 8-11, Istanbul, Turkey
18th World Congress of the International Association of Surgeons,
Gastroenterologists and Oncologists
E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria
16th United European Gastroenterology Week
www.negf.org
www.acv.at

October 22-25, Minnesota, USA
Anstralian Gastroenterology Week 2008
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia
71st Annual Colon and Rectal Surgery Conference
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA
59th AASLD Annual Meeting and Postgraduate Course
The Liver Meeting
Information: www.aasld.org

November 6-9, Lucerne, Switzerland
Neurogastroenterology & Motility Joint International Meeting 2008
E-mail: ngm2008@mci-group.com
www.ngm2008.com

November 12, Santiago de Chile, Chile
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt
1st Hepatology and Gastroenterology Post Graduate Course
www.egyptgastrohep.com

December 7-9, Seoul, Korea
6th International Meeting
Hepatocellular Carcinoma: Eastern and Western Experiences
E-mail: sglee@amc.seoul.kr

INFORMATION FOR ALL
FALK FOUNDATION e.V.
E-mail: symposia@falkfoundation.de
www.falkfoundation.de

Advanced Courses - European Institute of Telesurgery EITS - 2008
Strasbourg, France
January 18-19, March 28-29, June 6-7, October 3-4

N.O.T.E.S
April 3-5, November 27-29
Laparoscopic Digestive Surgery

June 27-28, November 7-8
Laparoscopic Colorectal Surgery

July 3-5
Interventional GI Endoscopy Techniques
Contact address for all courses:
E-mail: info@eits.fr

International Gastroenterological Congresses 2009
March 23-26, Glasgow, Scotland
Meeting of the British Society of Gastroenterology (BSG)
E-mail: bsg@mailbox.ulcc.ac.uk

May 17-20, Denver, Colorado, USA
Digestive Disease Week 2009

November 21-25, London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2008 September 7; 14(33): 5230-5232
World Journal of Gastroenterology ISSN 1007-9327

Instructions to authors

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol* ISSN 1007-9327 CN 14-1219/R) is a weekly open access peer-reviewed journal supported by an editorial board consisting of 1208 experts in gastroenterology and hepatology from 60 countries. The aim of the journal is to deliver the most clinically relevant original and commentary articles to readers, and to make the full text publicly available to all clinicians, scientists, patients and biomedical students on an unrestricted platform, so that they can access and learn about the most recent key advances in the field.

In addition to the open access nature, another key characteristic of *WJG* is its reading guidance for each article which includes background, research frontier, related reports, breakthroughs, applications, terminology, and comments of peer reviewers for the general readers.

WJG publishes articles on esophageal, gastrointestinal, hepatobiliary and pancreatic tumors, and other esophageal, gastrointestinal, hepatic-biliary and pancreatic diseases in relation to epidermiology, immunology, microbiology, motility & nerve-gut interaction, endocrinology, nutrition & obesity, endoscopy, imaging and advanced hi-technology.

The main goal of *WJG* is to publish high quality commentary articles contributed by leading experts in gastroenterology and hepatology and original articles that combine the clinical practice and advanced basic research, to provide an interactive platform for clinicians and researchers in internal medicine, surgery, infectious diseases, traditional Chinese medicine, oncology, integrated Chinese and Western medicine, imaging, endoscopy, interventional therapy, pathology and other basic medical specialities, and thus eventually improving the clinical practice and healthcare for patients.

Indexed and abstracted in

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, Index Medicus, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology* and *Hepatology*, CAB Abstracts and Global Health. ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

Published by

The WJG Press

SUBMISSION OF MANUSCRIPTS

Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of The WJG Press, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the International Committee of Medical Journal Editors to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now available, to our knowledge, is http://www. clinicaltrials.gov sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

Authors should retain one copy of the text, tables, photographs and illustrations because rejected manuscripts will not be returned to the author(s) and the editors will not be responsible for loss or damage to photographs and illustrations sustained during mailing.

Online submissions

Manuscripts should be submitted through the Online Submission System at: http://wjg.wjgnet.com/wjg. Authors are highly recommended to consult the ONLINE INSTRUCTIONS TO AUTHORS (http://www.wjgnet.com/wjg/help/instructions.jsp) before attempting to submit online. For assistance, authors encountering problems with the Online Submission System may send an email describing the problem to submission@wjgnet.com, or by telephone: +86-10-85381892. If you submit your manuscript online, do not make a postal contribution. Repeated online submission for the same manuscript is strictly prohibited.

MANUSCRIPT PREPARATION

All contributions should be written in English. All articles must be submitted using word-processing software. All submissions must be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Style should conform to our house format. Required information for each of the manuscript sections is as follows:

Title page

Full manuscript title, running title, all author(s) name(s), affiliations, institution(s) and/or department(s) where the work was carried out; author contributions; disclosure of any financial support for the research; and the name, full address, telephone and fax numbers and email address of the corresponding author should be included. Titles should be concise and informative (remove all unnecessary words), emphasize what is new, and avoid abbreviations. A short running title of less than 40 letters should be provided. List the author(s)' name(s) as follows: initial and/or first name, middle name or initial(s), and full family name.

Author controbutions: The format of this section should be like this: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed research; Wang CL, Zou CC, Hong F and Wu XM performed research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed data; and Wang CL, Liang L and Fu JF wrote the paper.

Peer reviewers: All articles received are subject to peer review. Normally, three experts are invited for each article. Decision for acceptance is made only when at least two experts recommend an article for publication. Reviewers for accepted manuscripts are acknowledged in each manuscript, and reviewers of articles which were not accepted will be acknowledged at the end of each issue. To ensure the quality of the articles published in *WJG*, reviewers of accepted manuscripts will be announced by publishing the name, title/position and institution of the reviewer in the footnote accompanying the printed article. For example, reviewers: Professor Jing-Yuan Fang, Shanghai Institute of Digestive Disease, Shanghai, Affiliated Renji Hospital, Medical Faculty, Shanghai Jiaotong University, Shanghai, China; Professor Xin-Wei Han, Department of Radiology, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan Province, China; and Professor Anren Kuang, Department of Nuclear Medicine, Huaxi Hospital, Sichuan University, Chengdu, Sichuan Province, China.

Abstract

An informative, structured abstract of no more than 350 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections: AIM: Only the purpose should be included. METHODS: The materials, techniques, instruments and equipment, and the experimental procedures should be included. RESULTS: The observed and experimental results, including data, effects, outcome, *etc.* should be included. Authors should present *P* value where necessary, and also include any significant data. CONCLUSION: Accurate view and the value of the results should be included.

The format for structured abstracts can be found at: http://www.wjgnet.com/wjg/help/11.doc.

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles, rapid communication

and case reports, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the body text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, should be found at: http://www.wjgnet.com/wjg/help/instructions.jsp.

Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: …*etc.* It is our principle to publish high resolution-figures for the printed and E-versions.

Tables

Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of P values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscripts and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

Coding system

The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability[1,2]". If references are cited directly in the text, they should be put together within the text, for example, "From references[19,22-24], we know that..."

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

PMID requirement

PMID roots in the abstract serial number indexed by PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed). The author should supply the PMID for journal citation. For those references that have not been indexed by PubMed, a printed copy of the first page of the full reference should be submitted.

The accuracy of the information for journal citations is very important. Using the reference testing system, the authors and editor should check the authors name, title, journal title, publication date, volume number, start page, and end page. We will interlink all references with PubMed in an ASP file so that the readers can immediately access the abstract of the citations online.

DOI requirement

A CrossRef DOI® (Digital Object Identifier) name is a unique string created to identify a piece of scholarly content in the online environment. The author should supply the DOIs for journal citation(doi:10.3748/wjg.13.6458). This link (http://www.crossref.org/SimpleTextQuery/) allows you to retrieve Digital Object Identifiers (DOIs) for journal articles, books, and chapters by simply cutting and pasting the reference list into the box. You may use the form with any reference style, although the tool works most reliably if references are formatted in a standard style such as shown in this example: Assimakopoulos SF, Scopa CD, Vagianos CE. Pathophysiology of increased intestinal permeability in obstructive jaundice. *World J Gastroenterol* 2007; 13(48): 6458-6464

The accuracy of the information of journal citations is very important. We will interlink all references with DOI in ASP file so that readers can access the abstracts of cited articles online immediately.

Style for journal references

Authors: the name of the first author should be typed in bold-faced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

Style for book references

Authors: the name of the first author should be typed in bold-faced letters. The surname of all authors should be typed with the initial letter capitalized, followed by their abbreviated middle and first initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR) Book title. Publication number. Publication place: Publication press, Year: start page and end page.

Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Inappropriate references

Authors should always cite references that are relevant to their article, and avoid any inappropriate references. Inappropriate references include those linked with a hyphen when the difference between the two numbers is greater than five. For example, [1-6], [2-14] and [1, 3, 4-10, 22] are all considered inappropriate references. Authors should not cite their own unrelated published articles.

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/wjg/help/15.doc.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H pylori*, *E coli*, *etc.*

SUBMISSION OF THE REVISED MANUSCRIPTS AFTER ACCEPTED

Please revise your article according to the revision policies of *WJG*. The revised version including manuscript and high-resolution image figures (if any) should be copied on a floppy or compact disk. The author should send the revised manuscript, along with printed high-resolution color or black and white photos, copyright transfer letter, and responses to the reviewers by courier (such as EMS/DHL).

Editorial Office

World Journal of Gastroenterology
Editorial Department: Room 903
Ocean International Center, Building D
No. 62 Dongsihuan Zhonglu
Chaoyang District, Beijing 100025, China
E-mail: wjg@wjgnet.com
http://www.wjgnet.com
Telephone: +86-10-59080039
Fax: +86-10-85381893

Language evaluation

The language of a manuscript will be graded before it is sent for revision. (1) Grade A: priority publishing; (2) Grade B: minor language polishing; (3) Grade C: a great deal of language polishing needed; (4) Grade D: rejected. Revised articles should reach Grade A or B.

Copyright assignment form

Please download a Copyright assignment form from http://www.wjgnet.com/wjg/help/9.doc.

Responses to reviewers

Please revise your article according to the comments/suggestions provided by the reviewers. The format for responses to the reviewers' comments can be found at: http://www.wjgnet.com/wjg/help/10.doc.

Proof of financial support

For paper supported by a foundation, authors should provide a copy of the document and serial number of the foundation.

Links to documents related to the manuscript

WJG will be initiating a platform to promote dynamic interactions between the editors, peer reviewers, readers and authors. After a manuscript is published online, links to the PDF version of the submitted manuscript, the peer-reviewers' report and the revised manuscript will be put on-line. Readers can make comments on the peer reviewer's report, authors' responses to peer reviewers, and the revised manuscript. We hope that authors will benefit from this feedback and be able to revise the manuscript accordingly in a timely manner.

Science news releases

Authors of accepted manuscripts are suggested to write a science news item to promote their articles. The news will be released rapidly at EurekAlert/AAAS (http://www.eurekalert.org). The title for news items should be less than 90 characters; the summary should be less than 75 words; and main body less than 500 words. Science news items should be lawful, ethical, and strictly based on your original content with an attractive title and interesting pictures.

Publication fee

Authors of accepted articles must pay a publication fee.
EDITORIAL, TOPIC HIGHLIGHTS, BOOK REVIEWS and LETTERS TO THE EDITOR are published free of charge.

ISSN 1007-9327
CN 14-1219/R

World Journal of Gastroenterology®

Indexed and Abstracted in:

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, *Index Medicus*, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology and Hepatology*, CAB Abstracts and Global Health.
ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

Volume 14 Number 34
September 14, 2008
World J Gastroenterol
2008 September 14; 14(34): 5233-5360

Online Submissions
wjg.wjgnet.com
www.wjgnet.com
Printed on Acid-free Paper
世界胃肠病学杂志

A Weekly Journal of Gastroenterology and Hepatology

World Journal of Gastroenterology®

Editorial Board

2007-2009

Published by The WJG Press and Baishideng
Room 903, Ocean International Center, Building D
No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Fax: +86-10-8538-1893 E-mail: wjg@wjgnet.com http://www.wjgnet.com

The World Journal of Gastroenterology Editorial Board consists of 1208 members, representing a team of worldwide experts in gastroenterology and hepatology. They are from 60 countries, including Albania (1), Argentina (4), Australia (39), Austria (10), Belarus (1), Belgium (15), Brazil (2), Bulgaria (1), Canada (28), Chile (1), China (60), Croatia (2), Cuba (1), Czech (2), Denmark (7), Egypt (4), Estonia (1), Finland (4), France (44), Germany (108), Greece (9), Hungary (2), Iceland (1), India (12), Iran (3), Ireland (4), Israel (8), Italy (96), Japan (176), Lebanon (3), Lithuania (1), Macedonia (1), Malaysia (3), Mexico (6), Monaco (1), Morocco (1), The Netherlands (26), New Zealand (1), Nigeria (1), Norway (3), Pakistan (2), Peru (1), Poland (6), Portugal (1), Russia (3), Saudi Arabia (2), Serbia (1), Singapore (4), Slovakia (2), Slovenia (1), South Africa (2), South Korea (14), Spain (38), Sweden (15), Switzerland (13), Turkey (8), United Arab Emirates (1), United Kingdom (83), United States (316) and Uruguay (2).

Andrew V Biankin, *Sydney*
Filip Braet, *Sydney*
Andrew D Clouston, *Sydney*
Graham Cooksley, *Queensland*
Darrell HG Crawford, *Brisbane*
Adrian G Cummins, *Woodville South*
Guy D Eslick, *Sydney*
Michael A Fink, *Melbourne*
Robert JL Fraser, *Daw Park*
Peter Raymond Gibson, *Victoria*
Jacob George, *Westmead*
Mark D Gorrell, *Sydney*
Yik-Hong Ho, *Townsville*
Gerald J Holtmann, *Adelaide*
Michael Horowitz, *Adelaide*
John E Kellow, *Sydney*
Rupert Leong, *Concord*
Geoffrey W McCaughan, *Sydney*
Finlay A Macrae, *Victoria*
Daniel Markovich, *Brisbane*
Phillip S Oates, *Perth*
Jacqui Richmond, *Victoria*
Stephen M Riordan, *Sydney*
Ian C Roberts-Thomson, *Adelaide*
Devanshi Seth, *Camperdown*
Arthur Shulkes, *Melbourne*
Ross C Smith, *Sydney*
Kevin J Spring, *Brisbane*
Huy A Tran, *New South Wales*
Debbie Trinder, *Fremantle*
Martin J Veysey, *Gosford*
Daniel L Worthley, *Bedford*

Austria

Peter Ferenci, *Vienna*
Valentin Fuhrmann, *Vienna*
Alfred Gangl, *Vienna*
Christoph Gasche, *Vienna*
Kurt Lenz, *Linz*
Markus Peck-Radosavljevic, *Vienna*
Rudolf E Stauber, *Auenbruggerplatz*
Herbert Tilg, *Innsbruck*
Michael Trauner, *Graz*
Harald Vogelsang, *Vienna*
Guenter Weiss, *Innsbruck*

Belarus

Yury K Marakhouski, *Minsk*

Belgium

Rudi Beyaert, *Gent*
Bart Rik De Geest, *Leuven*
Inge I Depoortere, *Leuven*
Olivier Detry, *Liège*
Benedicte Y De Winter, *Antwerp*
Karel Geboes, *Leuven*
Thierry Gustot, *Brussels*
Yves J Horsmans, *Brussels*
Geert G Leroux-Roels, *Ghent*
Louis Libbrecht, *Leuven*
Etienne M Sokal, *Brussels*
Marc Peeters, *De Pintelaan*
Gert A Van Assche, *Leuven*
Yvan Vandenplas, *Brussels*
Eddie Wisse, *Keerbergen*

Brazil

Heitor Rosa, *Goiania*
Ana Cristina Simões e Silva, *Belo Horizonte*

Bulgaria

Zahariy Krastev, *Sofia*

Canada

Fernando Alvarez, *Québec*
David Armstrong, *Ontario*
Jeffrey P Baker, *Toronto*
Olivier Barbier, *Québec*
Nancy Baxter, *Toronto*
Matthew Bjerknes, *Toronto*
Frank J Burczynski, *Manitoba*
Michael F Byrne, *Vancouver*
Wang-Xue Chen, *Ottawa*
Chantal Guillemette, *Québec*
Samuel S Lee, *Calgary*
Gary A Levy, *Toronto*
Andrew L Mason, *Alberta*
John K Marshall, *Ontario*
Donna-Marie McCafferty, *Calgary*
Thomas I Michalak, *St. John's*
Gerald Y Minuk, *Manitoba*
Paul Moayyedi, *Hamilton*
Kostas Pantopoulos, *Québec*
William G Paterson, *Kingston*
Eldon Shaffer, *Calgary*
Morris Sherman, *Toronto*
Martin Storr, *Calgary*
Elena F Verdu, *Ontario*
John L Wallace, *Calgary*
Eric M Yoshida, *Vancouver*

Chile

Silvana Zanlungo, *Santiago*

China

Henry LY Chan, *Hongkong*
Xiao-Ping Chen, *Wuhan*
Zong-Jie Cui, *Beijing*
Da-Jun Deng, *Beijing*
Er-Dan Dong, *Beijing*
Sheung-Tat Fan, *Hong Kong*
Jin Gu, *Beijing*
Xin-Yuan Guan, *Pokfulam*
De-Wu Han, *Taiyuan*
Ming-Liang He, *Hong Kong*
Wayne HC Hu, *Hong Kong*
Chee-Kin Hui, *Hong Kong*
Ching-Lung Lai, *Hong Kong*
Kam Chuen Lai, *Hong Kong*
James YW Lau, *Hong Kong*
Yuk-Tong Lee, *Hong Kong*
Suet-Yi Leung, *Hong Kong*
Wai-Keung Leung, *Hong Kong*
John M Luk, *Pokfulam*
Chung-Mau Lo, *Hong Kong*
Jing-Yun Ma, *Beijing*
Ronnie Tung Ping Poon, *Hong Kong*
Lun-Xiu Qin, *Shanghai*
Yu-Gang Song, *Guangzhou*
Qin Su, *Beijing*
Wai-Man Wong, *Hong Kong*
Hong Xiao, *Shanghai*
Dong-Liang Yang, *Wuhan*
Winnie Yeo, *Hong Kong*
Yuan Yuan, *Shenyang*
Man-Fung Yuen, *Hong Kong*
Jian-Zhong Zhang, *Beijing*
Xin-Xin Zhang, *Shanghai*
Bo-Jian Zheng, *Hong Kong*
Shu Zheng, *Hangzhou*

Croatia

Tamara Cacev, *Zagreb*
Marko Duvnjak, *Zagreb*

Cuba

Damian C Rodriguez, *Havana*

Czech

Milan Jirsa, *Praha*
Pavel Trunečka, *Prague*

Denmark

Peter Bytzer, *Copenhagen*
Asbjørn M Drewes, *Aalborg*
Hans Gregersen, *Aalborg*
Jens H Henriksen, *Hvidovre*
Claus P Hovendal, *Odense*
Fin S Larsen, *Copenhagen*
Søren Møller, *Hvidovre*

Egypt

Abdel-Rahman El-Zayadi, *Giza*
Amr M Helmy, *Cairo*
Ayman Yosry, *Cairo*

Estonia

Riina Salupere, *Tartu*

Finland

Irma E Jarvela, *Helsinki*
Katri M Kaukinen, *Tampere*
Minna Nyström, *Helsinki*
Pentti Sipponen, *Espoo*

France

Bettaieb Ali, *Dijon*
Corlu Anne, *Rennes*
Denis Ardid, *Clermont-Ferrand*
Charles P Balabaud, *Bordeaux*
Soumeya Bekri, *Rouen*
Jacques Belghiti, *Clichy*
Jacques Bernuau, *Clichy Cedex*
Pierre Brissot, *Rennes*
Patrice P Cacoub, *Paris*
Franck Carbonnel, *Besancon*
Laurent Castera, *Pessac*
Bruno Clément, *Rennes*
Benoit Coffin, *Colombes*
Jacques Cosnes, *Paris*
Thomas Decaens, *Cedex*

Francoise L Fabiani, *Angers*
Gérard Feldmann, *Paris*
Jean Fioramonti, *Toulouse*
Jean-Noël Freund, *Strasbourg*
Jean-Paul Galmiche, *Nantes*
Catherine Guettier, *Villejuif*
Chantal Housset, *Paris*
Juan L Iovanna, *Marseille*
Rene Lambert, *Lyon*
Patrick Marcellin, *Paris*
Philippe Mathurin, *Lille*
Tamara Matysiak–Budnik, *Paris*
Francis Mégraud, *Bordeaux*
Richard Moreau, *Clichy*
Thierry Piche, *Nice*
Raoul Poupon, *Paris*
Jean Rosenbaum, *Bordeaux*
Dominique Marie Roulot, *Bobigny*
Thierry Poynard, *Paris*
Jean-Philippe Salier, *Rouen*
Didier Samuel, *Villejuif*
Jean-Yves Scoazec, *Lyon*
Khalid A Tazi, *Clichy*
Emmanuel Tiret, *Paris*
Baumert F Thomas, *Strasbourg*
Marie-Catherine Vozenin-brotons, *Villejuif*
Jean-Pierre H Zarski, *Grenoble*
Jessica Zucman-Rossi, *Paris*

Germany

Hans-Dieter Allescher, *G-Partenkirchen*
Martin Anlauf, *Kiel*
Rudolf Arnold, *Marburg*
Max G Bachem, *Ulm*
Thomas F Baumert, *Freiburg*
Daniel C Baumgart, *Berlin*
Hubert Blum, *Freiburg*
Thomas Bock, *Tuebingen*
Katja Breitkopf, *Mannheim*
Dunja Bruder, *Braunschweig*
Markus W Büchler, *Heidelberg*
Christa Buechler, *Regensburg*
Reinhard Buettner, *Bonn*
Elke Cario, *Essen*
Uta Dahmen, *Essen*
Christoph F Dietrich, *Bad Mergentheim*
Arno J Dormann, *Koeln*
Rainer J Duchmann, *Berlin*
Volker F Eckardt, *Wiesbaden*
Paul Enck, *Tuebingen*
Fred Fändrich, *Kiel*
Ulrich R Fölsch, *Kiel*
Helmut Friess, *Heidelberg*
Peter R Galle, *Mainz*
Nikolaus Gassler, *Aachen*
Andreas Geier, *Aachen*
Markus Gerhard, *Munich*
Wolfram H Gerlich, *Giessen*
Dieter Glebe, *Giessen*
Burkhard Göke, *Munich*
Florian Graepler, *Tuebingen*
Axel M Gressner, *Aachen*
Veit Gülberg, *Munich*
Rainer Haas, *Munich*
Eckhart G Hahn, *Erlangen*
Stephan Hellmig, *Kiel*
Martin Hennenberg, *Bonn*
Johannes Herkel, *Hamburg*
Klaus R Herrlinger, *Stuttgart*
Eva Herrmann, *Homburg/Saar*
Eberhard Hildt, *Berlin*
Joerg C Hoffmann, *Berlin*
Ferdinand Hofstaedter, *Regensburg*
Werner Hohenberger, *Erlangen*
Jörg C Kalff, *Bonn*
Ralf Jakobs, *Ludwigshafen*
Jutta Keller, *Hamburg*
Andrej Khandoga, *Munich*
Sibylle Koletzko, *München*
Stefan Kubicka, *Hannover*
Joachim Labenz, *Siegen*
Frank Lammert, *Bonn*
Thomas Langmann, *Regensburg*
Christian Liedtke, *Aachen*
Matthias Löhr, *Mannheim*
Christian Maaser, *Muenster*
Ahmed Madisch, *Dresden*
Peter Malfertheiner, *Magdeburg*
Michael P Manns, *Hannover*
Helmut Messmann, *Augsburg*
Stephan Miehlke, *Dresden*
Sabine Mihm, *Göttingen*
Silvio Nadalin, *Essen*
Markus F Neurath, *Mainz*
Johann Ockenga, *Berlin*
Florian Obermeier, *Regensburg*
Gustav Paumgartner, *Munich*
Ulrich KS Peitz, *Magdeburg*
Markus Reiser, *Bochum*
Emil C Reisinger, *Rostock*
Steffen Rickes, *Magdeburg*
Tilman Sauerbruch, *Bonn*
Dieter Saur, *Munich*
Hans Scherubl, *Berlin*
Joerg Schirra, *Munich*
Roland M Schmid, *München*
Volker Schmitz, *Bonn*
Andreas G Schreyer, *Regensburg*
Tobias Schroeder, *Essen*
Henning Schulze-Bergkamen, *Mainz*
Hans Seifert, *Oldenburg*
Norbert Senninger, *Muenster*
Manfred V Singer, *Mannheim*
Gisela Sparmann, *Rostock*
Christian J Steib, *München*
Jurgen M Stein, *Frankfurt*
Ulrike S Stein, *Berlin*
Manfred Stolte, *Bayreuth*
Christian P Strassburg, *Hannover*
Wolfgang R Stremmel, *Heidelberg*
Harald F Teutsch, *Ulm*
Robert Thimme, *Freiburg*
Hans L Tillmann, *Leipzig*
Tung-Yu Tsui, *Regensburg*
Axel Ulsenheimer, *Munich*
Patrick Veit-Haibach, *Essen*
Claudia Veltkamp, *Heidelberg*
Siegfried Wagner, *Deggendorf*
Henning Walczak, *Heidelberg*
Heiner Wedemeyer, *Hannover*
Fritz von Weizsacker, *Berlin*
Jens Werner, *Heidelberg*
Bertram Wiedenmann, *Berlin*
Reiner Wiest, *Regensburg*
Stefan Wirth, *Wuppertal*
Stefan JP Zeuzem, *Homburg*

Greece

Alexandra A Alexopoulou, *Athens*
George N Dalekos, *Larissa*
Christos Dervenis, *Athens*
Melanie Maria Deutsch, *Athens*
Tsianos Epameinondas, *Ioannina*
Elias A Kouroumalis, *Heraklion*
George Papatheodoridis, *Athens*
Spiros Sgouros, *Athens*

Hungary

Peter L Lakatos, *Budapest*
Zsuzsa Szondy, *Debrecen*

Iceland

Hallgrimur Gudjonsson, *Reykjavik*

India

Philip Abraham, *Mumbai*
Rakesh Aggarwal, *Lucknow*
Kunissery A Balasubramanian, *Vellore*
Deepak Kumar Bhasin, *Chandigarh*
Sujit K Bhattacharya, *Kolkata*
Yogesh K Chawla, *Chandigarh*
Radha K Dhiman, *Chandigarh*
Sri Prakash Misra, *Allahabad*
Ramesh Roop Rai, *Jaipur*
Nageshwar D Reddy, *Hyderabad*
Rakesh Kumar Tandon, *New Delhi*

Iran

Seyed-Moayed Alavian, *Tehran*
Reza Malekzadeh, *Tehran*
Seyed A Taghavi, *Shiraz*

Ireland

Billy Bourke, *Dublin*
Ronan A Cahill, *Cork*
Anthony P Moran, *Galway*

Israel

Simon Bar-Meir, *Hashomer*
Abraham R Eliakim, *Haifa*
Zvi Fireman, *Hadera*
Yaron Ilan, *Jerusalem*
Avidan U Neumann, *Ramat-Gan*
Yaron Niv, *Pardesia*
Ran Oren, *Tel Aviv*
Ami D Sperber, *Beer-Sheva*

Italy

Giovanni Addolorato, *Roma*
Luigi E Adinolfi, *Naples*
Domenico Alvaro, *Rome*
Mario Angelico, *Rome*
Vito Annese, *San Giovanni Rotond*
Filippo Ansaldi, *Genoa*
Adolfo F Attili, *Roma*
Giovanni Barbara, *Bologna*
Claudio Bassi, *Verona*
Gabrio Bassotti, *Perugia*
Pier M Battezzati, *Milan*
Stefano Bellentani, *Carpi*
Antonio Benedetti, *Ancona*
Mauro Bernardi, *Bologna*
Livia Biancone, *Rome*
Luigi Bonavina, *Milano*
Flavia Bortolotti, *Padova*
Giuseppe Brisinda, *Rome*
Elisabetta Buscarini, *Crema*
Giovanni Cammarota, *Roma*

Antonino Cavallari, *Bologna*
Giuseppe Chiarioni, *Valeggio*
Michele Cicala, *Rome*
Massimo Colombo, *Milan*
Amedeo Columbano, *Cagliari*
Massimo Conio, *Sanremo*
Dario Conte, *Milano*
Gino R Corazza, *Pavia*
Francesco Costa, *Pisa*
Antonio Craxi, *Palermo*
Silvio Danese, *Milan*
Roberto de Franchis, *Milano*
Roberto De Giorgio, *Bologna*
Maria Stella De Mitri, *Bologna*
Giovanni D De Palma, *Naples*
Fabio Farinati, *Padua*
Giammarco Fava, *Ancona*
Francesco Feo, *Sassari*
Fiorucci Stefano, *Perugia*
Andrea Galli, *Firenze*
Valeria Ghisett, *Turin*
Gianluigi Giannelli, *Bari*
Edoardo G Giannini, *Genoa*
Paolo Gionchetti, *Bologna*
Fabio Grizzi, *Milan*
Salvatore Gruttadauria, *Palermo*
Mario Guslandi, *Milano*
Pietro Invernizzi, *Milan*
Ezio Laconi, *Cagliari*
Giacomo Laffi, *Firenze*
Giovanni Maconi, *Milan*
Lucia Malaguarnera, *Catania*
Emanuele D Mangoni, *Napoli*
Paolo Manzoni, *Torino*
Giulio Marchesini, *Bologna*
Fabio Marra, *Florence*
Marco Marzioni, *Ancona*
Giuseppe Mazzella, *Bologna*
Mario U Mondelli, *Pavia*
Giuseppe Montalto, *Palermo*
Giovanni Monteleone, *Rome*
Giovanni Musso, *Torino*
Gerardo Nardone, *Napoli*
Valerio Nobili, *Rome*
Fabio Pace, *Milano*
Luisi Pagliaro, *Palermo*
Francesco Pallone, *Rome*
Fabrizio R Parente, *Milan*
Maurizio Parola, *Torino*
Francesco Perri, *San Giovanni Rotondo*
Raffaele Pezzilli, *Bologna*
Alberto Pilotto, *San Giovanni Rotondo*
Alberto Piperno, *Monza*
Mario Pirisi, *Novara*
Anna C Piscaglia, *Roma*
Paolo Del Poggio, *Treviglio*
Gabriele B Porro, *Milano*
Piero Portincasa, *Bari*
Cosimo Prantera, *Roma*
Bernardino Rampone, *Siena*
Oliviero Riggio, *Rome*
Claudio Romano, *Messina*
Marco Romano, *Napoli*
Gerardo Rosati, *Potenza*
Mario Del Tacca, *Pisa*
Gloria Taliani, *Rome*
Pier A Testoni, *Milan*
Enrico Roda, *Bologna*
Domenico Sansonno, *Bari*
Vincenzo Savarino, *Genova*
Vincenzo Stanghellini, *Bologna*
Giovanni Tarantino, *Naples*
Roberto Testa, *Genoa*
Dino Vaira, *Bologna*
Anna Linda Zignego, *Florence*

Japan

Kyoichi Adachi, *Izumo*
Yasushi Adachi, *Sapporo*
Taiji Akamatsu, *Matsumoto*
Sk Md Fazle Akbar, *Ehime*
Takafumi Ando, *Nagoya*
Akira Andoh, *Otsu*
Taku Aoki, *Tokyo*
Masahiro Arai, *Tokyo*
Tetsuo Arakawa, *Osaka*
Yasuji Arase, *Tokyo*
Masahiro Asaka, *Sapporo*
Hitoshi Asakura, *Tokyo*
Takeshi Azuma, *Fukui*
Yoichi Chida, *Fukuoka*
Takahiro Fujimori, *Tochigi*
Jiro Fujimoto, *Hyogo*
Kazuma Fujimoto, *Saga*
Mitsuhiro Fujishiro, *Tokyo*
Yoshihide Fujiyama, *Otsu*
Hiroyuki Fukui, *Tochigi*
Hiroyuki Hanai, *Hamamatsu*
Naohiko Harada, *Fukuoka*
Makoto Hashizume, *Fukuoka*
Tetsuo Hayakawa, *Nagoya*
Toru Hiyama, *Higashihiroshima*
Kazuhide Higuchi, *Osaka*
Keisuke Hino, *Ube*
Keiji Hirata, *Kitakyushu*
Yuji Iimuro, *Nishinomiya*
Kenji Ikeda, *Tokyo*
Toru Ikegami, *Fukuoka*
Kenichi Ikejima, *Bunkyo-ku*
Fumio Imazeki, *Chiba*
Yutaka Inagaki, *Kanagawa*
Yasuhiro Inokuchi, *Yokohama*
Haruhiro Inoue, *Yokohama*
Masayasu Inoue, *Osaka*
Hiromi Ishibashi, *Nagasaki*
Shunji Ishihara, *Izumo*
Toru Ishikawa, *Niigata*
Kei Ito, *Sendai*
Masayoshi Ito, *Tokyo*
Hiroaki Itoh, *Akita*
Ryuichi Iwakiri, *Saga*
Yoshiaki Iwasaki, *Okayama*
Terumi Kamisawa, *Tokyo*
Hiroshi Kaneko, *Aichi-Gun*
Shuichi Kaneko, *Kanazawa*
Takashi Kanematsu, *Nagasaki*
Mitsuo Katano, *Fukuoka*
Junji Kato, *Sapporo*
Mototsugu Kato, *Sapporo*
Shinzo Kato, *Tokyo*
Norifumi Kawada, *Osaka*
Sunao Kawano, *Osaka*
Mitsuhiro Kida, *Kanagawa*
Yoshikazu Kinoshita, *Izumo*
Tsuneo Kitamura, *Chiba*
Seigo Kitano, *Oita*
Kazuhiko Koike, *Tokyo*
Norihiro Kokudo, *Tokyo*
Satoshi Kondo, *Sapporo*
Shoji Kubo, *Osaka*
Shigeki Kuriyama, *Kagawa*[2]
Katsunori Iijima, *Sendai*
Masato Kusunoki, *Tsu Mie*
Shin Maeda, *Tokyo*
Shigeru Marubashi, *Suita*
Masatoshi Makuuchi, *Tokyo*
Osamu Matsui, *Kanazawa*
Yasuhiro Matsumura, *Chiba*
Yasushi Matsuzaki, *Tsukuba*
Kiyoshi Migita, *Omura*
Kenji Miki, *Tokyo*
Tetsuya Mine, *Kanagawa*
Hiroto Miwa, *Hyogo*
Masashi Mizokami, *Nagoya*
Yoshiaki Mizuguchi, *Tokyo*
Motowo Mizuno, *Hiroshima*
Morito Monden, *Suita*
Hisataka S Moriwaki, *Gifu*
Yasuaki Motomura, *Iizuka*
Yoshiharu Motoo, *Kanazawa*
Naofumi Mukaida, *Kanazawa*
Kazunari Murakami, *Oita*
Kunihiko Murase, *Tusima*
Hiroaki Nagano, *Suita*
Masahito Nagaki, *Gifu*
Masaki Nagaya, *Kawasaki*
Yujl Naito, *Kyoto*
Atsushi Nakajima, *Yokohama*
Hisato Nakajima, *Tokyo*
Hiroki Nakamura, *Yamaguchi*
Shotaro Nakamura, *Fukuoka*
Mikio Nishioka, *Niihama*
Shuji Nomoto, *Nagoya*
Susumu Ohmada, *Maebashi*
Hirohide Ohnishi, *Akita*
Masayuki Ohta, *Oita*
Tetsuo Ohta, *Kanazawa*
Kazuichi Okazaki, *Osaka*
Katsuhisa Omagari, *Nagasaki*
Saburo Onishi, *Nankoku*
Morikazu Onji, *Ehime*
Satoshi Osawa, *Hamamatsu*
Masanobu Oshima, *Kanazawa*
Hiromitsu Saisho, *Chiba*
Hidetsugu Saito, *Tokyo*
Yutaka Saito, *Tokyo*
Isao Sakaida, *Yamaguchi*
Michiie Sakamoto, *Tokyo*
Yasushi Sano, *Chiba*
Hiroki Sasaki, *Tokyo*
Iwao Sasaki, *Sendai*
Motoko Sasaki, *Kanazawa*
Chifumi Sato, *Tokyo*
Shuichi Seki, *Osaka*
Hiroshi Shimada, *Yokohama*
Mitsuo Shimada, *Tokushima*
Tomohiko Shimatan, *Hiroshima*
Hiroaki Shimizu, *Chiba*
Ichiro Shimizu, *Tokushima*
Yukihiro Shimizu, *Kyoto*
Shinji Shimoda, *Fukuoka*
Tooru Shimosegawa, *Sendai*
Tadashi Shimoyama, *Hirosaki*
Ken Shirabe, *Iizuka City*
Yoshio Shirai, *Niigata*
Katsuya Shiraki, *Mie*
Yasushi Shiratori, *Okayama*
Masayuki Sho, *Nara*
Yasuhiko Sugawara, *Tokyo*
Hidekazu Suzuki, *Tokyo*
Minoru Tada, *Tokyo*
Tadatoshi Takayama, *Tokyo*
Tadashi Takeda, *Osaka*
Koji Takeuchi, *Kyoto*
Kiichi Tamada, *Tochigi*
Akira Tanaka, *Kyoto*
Eiji Tanaka, *Matsumoto*
Noriaki Tanaka, *Okayama*
Shinji Tanaka, *Hiroshima*
Hideki Taniguchi, *Yokohama*
Kyuichi Tanikawa, *Kurume*
Akira Terano, *Shimotsugagun*
Hitoshi Togash, *Yamagata*
Shinji Togo, *Yokohama*
Kazunari Tominaga, *Osaka*
Takuji Torimura, *Fukuoka*

Minoru Toyota, *Sapporo*
Akihito Tsubota, *Chiba*
Takato Ueno, *Kurume*
Naomi Uemura, *Tokyo*
Shinichi Wada, *Tochigi*
Hiroyuki Watanabe, *Kanazawa*
Toshio Watanabe, *Osaka*
Yuji Watanabe, *Ehime*
Toshiaki Watanabe, *Tokyo*
Chun-Yang Wen, *Nagasaki*
Satoshi Yamagiwa, *Niigata*
Koji Yamaguchi, *Fukuoka*
Takayuki Yamamoto, *Yokkaichi*
Takashi Yao, *Fukuoka*
Masashi Yoneda, *Tochigi*
Hiroshi Yoshida, *Tokyo*
Masashi Yoshida, *Tokyo*
Norimasa Yoshida, *Kyoto*
Hitoshi Yoshiji, *Nara*
Kentaro Yoshika, *Toyoake*
Yasunobu Yoshikai, *Fukuoka*
Masahide Yoshikawa, *Kashihara*
Katsutoshi Yoshizato, *Higashihiroshima*

Lebanon

Bassam N Abboud, *Beirut*
Ala I Sharara, *Beirut*
Joseph D Boujaoude, *Beirut*

Lithuania

Limas Kupcinskas, *Kaunas*

Macedonia

Vladimir C Serafimoski, *Skopje*

Malaysia

Andrew Seng Boon Chua, *Ipoh*
Khean-Lee Goh, Kuala *Lumpur*
Jayaram Menon, *Sabah*

Mexico

Diego Garcia-Compean, *Monterrey*
Eduardo R Marin-Lopez, *Jesús García*
Nahum Méndez-Sánchez, *Mexico*
Saúl Villa-Treviño, *México*

Monaco

Patrick Rampal, *Monaco*

Morocco

Abdellah Essaid, *Rabat*

The Netherlands

Ulrich Beuers, *Amsterdam*
Gerd Bouma, *Amsterdam*
Lee Bouwman, *Leiden*
J Bart A Crusius, *Amsterdam*
NKH de Boer, *Amsterdam*
Koert P de Jong, *Groningen*
Henrike Hamer, *Maastricht*
Frank Hoentjen, *Haarlem*
Janine K Kruit, *Groningen*
Ernst J Kuipers, *Rotterdam*
CBHW Lamers, *Leiden*
Ton Lisman, *Utrecht*
Yi Liu, *Amsterdam*
Jeroen Maljaars, *Maastricht*
Servaas Morré, *Amsterdam*
Chris JJ Mulder, *Amsterdam*
Michael Müller, *Wageningen*
Amado S Peña, *Amsterdam*
Robert J Porte, *Groningen*
Ingrid B Renes, *Rotterdam*
Andreas Smout, *Utrecht*
Paul E Sijens, *Groningen*
Reinhold W Stockbrugger, *Maastricht*
Luc JW van der Laan, *Rotterdam*
Karel van Erpecum, *Utrecht*
Gerard P VanBerge-Henegouwen,*Utrecht*

New Zealand

Ian D Wallace, *Auckland*

Nigeria

Samuel B Olaleye, *Ibadan*

Norway

Trond Berg, *Oslo*
Tom H Karlsen, *Oslo*
Helge L Waldum, *Trondheim*

Pakistan

Muhammad S Khokhar, *Lahore*
Syed MW Jafri, *Karachi*

Peru

Hector H Garcia, *Lima*

Poland

Tomasz Brzozowski, *Cracow*
Robert Flisiak, *Bialystok*
Hanna Gregorek, *Warsaw*
Dariusz M Lebensztejn, *Bialystok*
Wojciech G Polak, *Wroclaw*
Marek Hartleb, *Katowice*

Portugal

Miguel C De Moura, *Lisbon*

Russia

Vladimir T Ivashkin, *Moscow*
Leonid Lazebnik, *Moscow*
Vasiliy I Reshetnyak, *Moscow*

Saudi Arabia

Ibrahim A Al Mofleh, *Riyadh*
Ahmed Helmy, *Riyadh*

Serbia

Dusan M Jovanovic, *Sremska Kamenica*

Singapore

Bow Ho, *Singapore*
Khek-Yu Ho, *Singapore*
Fock Kwong Ming, *Singapore*
Francis Seow-Choen, *Singapore*

Slovakia

Silvia Pastorekova, *Bratislava*
Anton Vavrecka, *Bratislava*

Slovenia

Sasa Markovic, *Ljubljana*

South Africa

Rosemar Joyce Burnett, *Pretoria*
Michael C Kew, *Parktown*

South Korea

Byung Ihn Choi, *Seoul*
Ho Soon Choi, *Seoul*
Marie Yeo, *Suwon*
Sun Pyo Hong, *Gyeonggi-do*
Jae J Kim, *Seoul*
Jin-Hong Kim, *Suwon*
Myung-Hwan Kim, *Seoul*
Chang Hong Lee, *Seoul*
Jong Kyun Lee, *Seoul*
Eun-Yi Moon, *Seoul*
Jae-Gahb Park, *Seoul*
Dong Wan Seo, *Seoul*
Dong Jin Suh, *Seoul*
Byung Chul Yoo, *Seoul*

Spain

Juan G Abraldes, *Barcelona*
Agustin Albillos, *Madrid*
Raul J Andrade, *Málaga*
Luis Aparisi, *Valencia*
Fernando Azpiroz, *Barcelona*
Ramon Bataller, *Barcelona*
Josep M Bordas, *Barcelona*
Xavier Calvet, *Sabadell*
Jordi Camps, *Catalunya*
Andres Cardenas, *Barcelona*
Vicente Carreño, *Madrid*
Jose Castellote, *Barcelona*
Antoni Castells, *Barcelona*
Vicente Felipo, *Valencia*
Juan C Garcia-Pagán, *Barcelona*
Jaime B Genover, *Barcelona*
Javier P Gisbert, *Madrid*
Jaime Guardia, *Barcelona*
Isabel Fabregat, *Barcelona*
Mercedes Fernandez, *Barcelona*
Angel Lanas, *Zaragoza*
Juan-Ramón Larrubia, *Guadalajara*
Laura Lladóa, *Barcelona*
María IT López, *Jaén*
Juan R Malagelada, *Barcelona*
José M Mato, *Derio*
Juan F Medina, *Pamplona*
Miguel A Muñoz-Navas, *Pamplona*
Julian Panes, *Barcelona*
Miguel M Perez, *Valencia*
Miguel Perez-Mateo, *Alicante*

Josep M Pique, *Barcelona*
Jesús M Prieto, *Pamplona*
Sabino Riestra, *Pola De Siero*
Luis Rodrigo, *Oviedo*
Manuel Romero-Gómez, *Sevilla*
Joan Roselló-Catafau, *Barcelona*

Sweden

Einar S Björnsson, *Gothenburg*
Curt Einarsson, *Huddinge*
Per M Hellström, *Stockholm*
Ulf Hindorf, *Lund*
Elisabeth Hultgren-Hörnquist, *Örebro*
Anders E Lehmann, *Mölndal*
Hanns-Ulrich Marschall, *Stockholm*
Lars C Olbe, *Molndal*
Lars A Pahlman, *Uppsala*
Matti Sallberg, *Stockholm*
Magnus Simrén, *Göteborg*
Xiao-Feng Sun, *Linköping*
Ervin Tóth, *Malmö*
Weimin Ye, *Stockholm*
Christer S von Holstein, *Lund*

Switzerland

Chrish Beglinger, *Basel*
Pierre A Clavien, *Zurich*
Jean-Francois Dufour, *Bern*
Franco Fortunato, *Zürich*
Jean L Frossard, *Geneva*
Gerd A Kullak-Ublick, *Zurich*
Pierre Michetti, *Lausanne*
Francesco Negro, *Genève*
Bruno Stieger, *Zurich*
Radu Tutuian, *Zurich*
Stephan R Vavricka, *Zurich*
Gerhard Rogler, *Zurich*
Arthur Zimmermann, *Berne*

Turkey

Yusuf Bayraktar, *Ankara*
Figen Gurakan, *Ankara*
Aydin Karabacakoglu, *Konya*
Serdar Karakose, *Konya*
Hizir Kurtel, *Istanbul*
Osman C Ozdogan, *Istanbul*
Özlem Yilmaz, *Izmir*
Cihan Yurdaydin, *Ankara*

United Arab Emirates

Sherif M Karam, *Al-Ain*

United Kingdom

David H Adams, *Birmingham*
Simon Afford, *Birmingham*
Navneet K Ahluwalia, *Stockport*
Ahmed Alzaraa, *Manchester*
Lesley A Anderson, *Belfast*
Charalambos G Antoniades, *London*
Anthony TR Axon, *Leeds*
Qasim Aziz, *Manchester*
Nicholas M Barnes, *Birmingham*
Jim D Bell, *London*
Mairi Brittan, *London*
Alastair D Burt, *Newcastle*
Simon S Campbell, *Manchester*
Simon R Carding, *Leeds*
Paul J Ciclitira, *London*
Eithne Costello, *Liverpool*
Tatjana Crnogorac-Jurcevic, *London*
Harry Dalton, *Truro*
Amar P Dhillon, *London*
William Dickey, *Londonderry*
James E East, *London*
Emad M El-Omar, *Aberdeen*
Ahmed M Elsharkawy, *Newcastle Upon Tyne*
Annette Fristscher-Ravens, *London*
Elizabeth Furrie, *Dundee*
Daniel R Gaya, *Edinburgh*
Subrata Ghosh, *London*
William Greenhalf, *Liverpool*
Indra N Guha, *Southampton*
Peter C Hayes, *Edinburgh*
Gwo-Tzer Ho, *Edinburgh*
Anthony R Hobson, *Salford*
Lesley A Houghton, *Manchester*
Stefan G Hübscher, *Birmingham*
Robin Hughes, *London*
Pali Hungin, *Stockton*
David P Hurlstone, *Sheffield*
Rajiv Jalan, *London*
Janusz AZ Jankowski, *Oxford*
Brian T Johnston, *Belfast*
David EJ Jones, *Newcastle*
Roger Jones, *London*
Michael A Kamm, *Harrow*
Peter Karayiannis, *London*
Laurens Kruidenier, *Harlow*
Patricia F Lalor, *Birmingham*
Chee Hooi Lim, *Midlands*
Hong-Xiang Liu, *Cambridge*
Yun Ma, *London*
Kenneth E L McColl, *Glasgow*
Stuart AC McDonald, *London*
Dermot P Mcgovern, *Oxford*
Giorgina Mieli-Vergani, *London*
Nikolai V Naoumov, *London*
John P Neoptolemos, *Liverpool*
James Neuberger, *Birmingham*
Philip Noel Newsome, *Birmingham*
Mark S Pearce, *Newcastle Upon Tyne*
Stephen P Pereira, *London*
D Mark Pritchard, *Liverpool*
Sakhawat Rahman, *London*
Stephen E Roberts, *Swansea*
Marco Senzolo, *Padova*
Soraya Shirazi-Beechey, *Liverpool*
Robert Sutton, *Liverpool*
Simon D Taylor-Robinson, *London*
Paris P Tekkis, *London*
Ulrich Thalheimer, *London*
David G Thompson, *Salford*
Nick P Thompson, *Newcastle*
David Tosh, *Bath*
Frank I Tovey, *London*
Chris Tselepis, *Birmingham*
Diego Vergani, *London*
Geoffrey Warhurst, *Salford*
Alastair John Watson, *Liverpool*
Peter J Whorwell, *Manchester*
Roger Williams, *London*
Karen L Wright, *Bath*
Min Zhao, *Foresterhill*

United States

Manal F Abdelmalek, *Durham*
Gary A Abrams, *Birmingham*
Maria T Abreu, *New York*
Reid B Adams, *Virginia*
Golo Ahlenstiel, *Bethesda*
BS Anand, *Houston*
Frank A Anania, *Atlanta*
M Ananthanarayanan, *New York*
Gavin E Arteel, *Louisville*
Jasmohan S Bajaj, *Milwaukee*
Subhas Banerjee, *Palo Alto*
Peter A Banks, *Boston*
Jamie S Barkin, *Miami Beach*
Kim E Barrett, *San Diego*
Marc D Basson, *Detroit*
Anthony J Bauer, *Pittsburgh*
Wallace F Berman, *Durham*
Timothy R Billiar, *Pittsburgh*
Edmund J Bini, *New York*
David G Binion, *Milwaukee*
Jennifer D Black, *Buffalo*
Herbert L Bonkovsky, *Charlotte*
Carla W Brady, *Durham*
Andrea D Branch, *New York*
Robert S Bresalier, *Houston*
Alan L Buchman, *Chicago*
Ronald W Busuttil, *Los Angeles*
Alan Cahill, *Philadelphia*
John M Carethers, *San Diego*
David L Carr-Locke, *Boston*
Maurice A Cerulli, *New York*
Ravi S Chari, *Nashville*
Jiande Chen, *Galveston*
Xian-Ming Chen, *Omaha*
Xin Chen, *San Francisco*
Ramsey Chi-man Cheung, *Palo Alto*
William D Chey, *Ann Arbor*
John Y Chiang, *Rootstown*
Parimal Chowdhury, *Arkansas*
Raymond T Chung, *Boston*
James M Church, *Cleveland*
Ram Chuttani, *Boston*
Mark G Clemens, *Charlotte*
Ana J Coito, *Los Angeles*
Vincent Coghlan, *Beaverton*
David Cronin II, *New Haven*
John Cuppoletti, *Cincinnati*
Mark J Czaja, *New York*
Peter V Danenberg, *Los Angeles*
Kiron M Das, New *Brunswick*
Conor P Delaney, *Cleveland*
Jose L del Pozo, *Rochester*
Sharon DeMorrow, *Temple*
Deborah L Diamond, *Seattle*
Douglas A Drossman, *Chapel Hill*
Katerina Dvorak, *Tucson*
Bijan Eghtesad, *Cleveland*
Hala El-Zimaity, *Houston*
Michelle Embree-Ku, *Providence*
Sukru Emre, New *Haven*
Douglas G Farmer, *Los Angeles*
Alessio Fasano, *Baltimore*
Mark A Feitelson, *Philadelphia*
Ariel E Feldstein, *Cleveland*
Alessandro Fichera, *Chicago*
Robert L Fine, *New York*
Magali Fontaine, *Stanford*
Chris E Forsmark, *Gainesville*
Glenn T Furuta, *Aurora*
Chandrashekhar R Gandhi, *Pittsburgh*
Susan L Gearhart, *Baltimore*
Xupeng Ge, *Boston*
Xin Geng, *New Brunswick*
M Eric Gershwin, *Suite*
Jean-Francois Geschwind, *Baltimore*
Ignacio Gil-Bazo, *New York*
Shannon S Glaser, *Temple*
Ajay Goel, *Dallas*
Richard M Green, *Chicago*
Julia B Greer, *Pittsburgh*

James H Grendell, *New York*
David R Gretch, *Seattle*
Stefano Guandalini, *Chicago*
Anna S Gukovskaya, *Los Angeles*
Sanjeev Gupta, *Bronx*
David J Hackam, *Pittsburgh*
Stephen B Hanauer, *Chicago*
Gavin Harewood, *Rochester*
Margaret M Heitkemper, *Washington*
Alan W Hemming, *Gainesville*
Samuel B Ho, *San Diego*
Peter R Holt, *New York*
Colin W Howden, *Chicago*
Hongjin Huang, *Alameda*
Jamal A Ibdah, *Columbia*
Atif Iqbal, *Omaha*
Hajime Isomoto, *Rochester*
Hartmut Jaeschke, *Tucson*
Dennis M Jensen, *Los Angeles*
Cheng Ji, *Los Angeles*
Leonard R Johnson, *Memphis*
Michael P Jones, *Chicago*
Peter J Kahrilas, *Chicago*
Anthony N Kalloo, *Baltimore*
Marshall M Kaplan, *Boston*
Neil Kaplowitz, *Los Angeles*
Serhan Karvar, *Los Angeles*
Rashmi Kaul, *Tulsa*
Jonathan D Kaunitz, *Los Angeles*
Ali Keshavarzian, *Chicago*
Miran Kim, *Providence*
Joseph B Kirsner, *Chicago*
Leonidas G Koniaris, *Miami*
Burton I Korelitz, *New York*
Robert J Korst, *New York*
Richard A Kozarek, *Seattle*
Alyssa M Krasinskas, *Pittsburgh*
Michael Kremer, *Chapel Hill*
Shiu-Ming Kuo, *Buffalo*
Paul Y Kwo, *Indianapolis*
Daryl Tan Yeung Lau, *Galvesto*
Stephen J Lanspa, *Omaha*
Joel E Lavine, *San Diego*
Bret Lashner, *Cleveland*
Dirk J van Leeuwen, *Lebanon*
Glen A Lehman, *Indianapolis*
Alex B Lentsch, *Cincinnati*
Andreas Leodolter, *La Jolla*
Gene LeSage, *Houston*
Josh Levitsky, *Chicago*
Cynthia Levy, *Gainesville*
Ming Li, *New Orleans*
Zhiping Li, *Baltimore*
Zhe-Xiong Lian, *Davis*
Lenard M Lichtenberger, *Houston*
Gary R Lichtenstein, *Philadelphia*
Otto Schiueh-Tzang Lin, *Seattle*
Martin Lipkin, *New York*
Chen Liu, *Gainesville*
Edward V Loftus, *Rocheste*
Robin G Lorenz, *Birmingham*
Michael R Lucey, *Madison*
James D Luketich, *Pittsburgh*
Guangbin Luo, *Cheveland*
Henry T Lynch, *Omaha*
Patrick M Lynch, *Houston*
John S Macdonald, *New York*
Bruce V MacFadyen, *Augusta*
Willis C Maddrey, *Dallas*
Ashok Malani, *Los Angeles*
Mercedes Susan Mandell, *Aurora*
Peter J Mannon, *Bethesda*
Charles M Mansbach, *Tennessee*
John F Di Mari, *Texas*
John M Mariadason, *Bronx*
Jorge A Marrero, *Ann Arbor*
Paul Martin, *New York*
Paulo Ney Aguiar Martins, *Boston*
Wendy M Mars, *Pittsburgh*
Laura E Matarese, *Pittsburgh*
Richard W McCallum, *Kansas*
Beth A McCormick, *Charlestown*
Lynne V McFarland, *Washington*
Kevin McGrath, *Pittsburgh*
Harihara Mehendale, *Monroe*
Ali Mencin, *New York*
Fanyin Meng, *Ohio*
Stephan Menne, *New York*
Didier Merlin, *Atlanta*
Howard Mertz, *Nashville*
George W Meyer, *Sacramento*
George Michalopoulos, *Pittsburgh*
James M Millis, *Chicago*
Fabrizio Michelassi, *New York*
Albert D Min, *New York*
Pramod K Mistry, *New Haven*
Emiko Mizoguchi, *Boston*
Smruti R Mohanty, *Chicago*
Satdarshan S Monga, *Pittsburgh*
Timothy H Moran, *Baltimore*
Peter L Moses, *Burlington*
Steven F Moss, *Providence*
Andrew J Muir, *Durham*
Milton G Mutchnick, *Detroit*
Masaki Nagaya, *Boston*
Victor Navarro, *Philadelphia*
Laura E Nagy, *Cleveland*
Hiroshi Nakagawa, *Philadelphia*
Douglas B Nelson, *Minneapolis*
Justin H Nguyen, *Florida*
Patrick G Northup, *Charlottesville*
Christopher O'Brien, *Miami*
Robert D Odze, *Boston*
Brant K Oelschlager, *Washington*
Curtis T Okamoto, *Los Angeles*
Stephen JD O'Keefe, *Pittsburgh*
Dimitry Oleynikov, *Omaha*
Stephen J Pandol, *Los Angeles*
Georgios Papachristou, *Pittsburgh*
Pankaj J Pasricha, *Galveston*
Zhiheng Pei, *New York*
Michael A Pezzone, *Pittsburgh*
CS Pitchumoni, *New Brunswiuc*
Paul J Pockros, *La Jolla*
Jay Pravda, *Gainesville*
Massimo Raimondo, *Jacksonville*
GS Raju, *Galveston*
Raymund R Razonable, *Minnesota*
Murray B Resnick, *Providence*
Adrian Reuben, *Charleston*
Douglas K Rex, *Indianapolis*
Victor E Reyes, *Galveston*
Basil Rigas, *New York*
Yehuda Ringel, *Chapel Hill*
Richard A Rippe, *Chapel Hill*
Maribel Rodriguez-Torres, *Santurce*
Marcos Rojkind, *Washington*
Philip Rosenthal, *San Francisco*
Barry Rosser, *Jacksonville Florida*
Hemant K Roy, *Evanston*
Sammy Saab, *Los Angeles*
Shawn D Safford, *Norfolk*
Dushyant V Sahani, *Boston*
Bruce E Sands, *Boston*
James M Scheiman, *Ann Arbor*
Eugene R Schiff, *Miami*
Nicholas J Shaheen, *Chapel Hill*
Vanessa M Shami, *Charlottesville*
Prateek Sharma, *Kansas City*
Harvey L Sharp, *Minneapolis*
Stuart Sherman, *Indianapolis*
Shivendra Shukla, *Columbia*
Alphonse E Sirica, *Virginia*
Shanthi V Sitaraman, *Atlanta*
Stuart J Spechler, *Dallas*
Shanthi Srinivasan, *Atlanta*
Michael Steer, *Boston*
Peter D Stevens, *New York*
Charmaine A Stewart, *Rochester*
Christian D Stone, *Saint Louis*
Gary D Stoner, *Columbus*
R Todd Stravitz, *Richmond*
Liping Su, *Chicago*
Christina Surawicz, *Seattle*
Robert W Summers, *Iowa City*
Wing-Kin Syn, *Durham*
Gyongyi Szabo, *Worcester*
Yvette Taché, *Los Angeles*
Seng-Lai Tan, *Seattle*
Andrzej S Tarnawski, *Orange*
K-M Tchou-Wong, *New York*
Jonathan P Terdiman, *San Francisco*
Neil D Theise, *New York*
Christopher C Thompson, *Boston*
Swan N Thung, *New York*
Michael Torbenson, *Baltimore*
Natalie J Torok, *Sacramento*
RA Travagli, *Baton Rouge*
George Triadafilopoulos, *Stanford*
Chung-Jyi Tsai, *Lexington*
Janet Elizabeth Tuttle-Newhall, *Durham*
Andrew Ukleja, *Florida*
Michael F Vaezi, *Nashville*
Hugo E Vargas, *Scottsdale*
Arnold Wald, *Wisconsin*
Scott A Waldman, *Philadelphia*
Jian-Ying Wang, *Baltimore*
Timothy C Wang, *New York*
Irving Waxman, *Chicago*
Steven A Weinman, *Galveston*
Steven D Wexner, *Weston*
Keith T Wilson, *Baltimore*
Jacqueline L Wolf, *Boston*
Jackie Wood, *Ohio*
George Y Wu, *Farmington*
Jian Wu, *Sacramento*
Samuel Wyllie, *Houston*
Wen Xie, *Pittsburgh*
Vijay Yajnik, *Boston*
Vincent W Yang, *Atlanta*
Francis Y Yao, *San Francisco*
Hal F Yee, *San Francisco*
Xiao-Ming Yin, *Pittsburgh*
Min You, *Tampa*
Zobair M Younossi, *Virginia*
Liqing Yu, *Winston-Salem*
David Yule, *Rochester*
Ruben Zamora, *Pittsburgh*
Michael E Zenilman, *New York*
Zhi Zhong, *Chapel Hill*
Michael A Zimmerman, *Colorado*
Stephen D Zucker, *Cincinnati*

Uruguay

Henry Cohen, *Montevideo*

[1]Passed away on October 20, 2007
[2]Passed away on June 11, 2007

National Journal Award 2005

World Journal of Gastroenterology®

Weekly Established in October 1995

Volume 14 Number 34
September 14, 2008

Contents

Contents

World Journal of Gastroenterology
Volume 14 **Number** 34 **September** 14, 2008

World Journal of Gastroenterology
Volume 14 **Number** 34 **September** 14, 2008

Contents

NAME OF JOURNAL
World Journal of Gastroenterology

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

EDITING
Editorial Board of *World Journal of Gastroenterology,* Room 903, Ocean International Center, Building D, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

PUBLISHING
The WJG Press and Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Ocean International Center, Building D, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

PRINTING
Beijing Kexin Printing House

OVERSEAS DISTRIBUTOR
Beijing Bureau for Distribution of Newspapers and Journals (Code No. 82-261)
China International Book Trading Corporation PO Box 399, Beijing, China (Code No. M4481)

PUBLICATION DATE
September 14, 2008

EDITOR-IN-CHIEF
Lian-Sheng Ma, *Beijing*

SUBSCRIPTION
RMB 50 Yuan for each issue, RMB 2400 Yuan for one year

CSSN
ISSN 1007-9327
CN 14-1219/R

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5233

World J Gastroenterol 2008 September 14; 14(34): 5233-5236
World Journal of Gastroenterology ISSN 1007-9327

EDITORIAL

Heterogeneity of endoscopy negative heartburn: Epidemiology and natural history

Fabio Pace, Valentina Casini, Stefano Pallotta

Fabio Pace, Department of Clinical Sciences "L. Sacco" University Hospital, Milan 20157, Italy
Valentina Casini, Stefano Pallotta, Division of Gastroenterology, "L. Sacco" University Hospital, Milan 20157, Italy
Author contributions: Pace F had the idea and wrote the outline of the manuscript; Casini V and Pallotta S assisted in editorial work.
Correspondence to: Fabio Pace, Professor, Department of Clinical Sciences "L. Sacco" University Hospital, Milan 20157, Italy. fabio.pace@unimi.it
Telephone: +39-2-39042943 Fax: +39-2-39042337
Received: June 5, 2008 Revised: August 13, 2008
Accepted: August 20, 2008
Published online: September 14, 2008

Abstract

It has now become clear that only about 40% or less of patients with heartburn and/or regurgitation have esophagitis, and that the majority of them lack visible distal esophageal mucosa breaks. These subjects are referred to as non-erosive gastroesophageal reflux disease (NERD) patients. It has been estimated that in the Western world at least one tenth of the general population has at least weekly heartburn. This proportion seems to be lower in Asia, while prevalence is rapidly increasing. Although it would be extremely useful to have prospective information regarding the fate of such patients, the natural history of NERD is largely unknown, and very few studies in the literature have addressed this issue. These studies are for the greater part old, not well conducted, and suffer from methodological drawbacks including ill-defined entry criteria. However, a review of these studies indicates that a consistent minority of NERD patients may develop erosive disease at an approximate rate of about 10% per year.

Key words: Gastroesophageal reflux disease; Non-erosive gastroesophageal reflux disease; Esophagitis; Proton pump inhibitor

Peer reviewer: Tomohiko Shimatani, Assistant Professor, Department of General Medicine, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 7348551, Japan

Pace F, Casini V, Pallotta S. Heterogeneity of endoscopy negative heartburn: Epidemiology and natural history. *World J Gastroenterol* 2008; 14(34): 5233-5236 Available from: URL: http://www.wjgnet.com/1007-9327/14/5233.asp DOI: http://dx.doi.org/10.3748/wjg.14.5233

INTRODUCTION

The recently published Montreal Criteria, dealing with a global classification of gastroesophageal reflux disease (GERD), define heartburn as a burning sensation in the retrosternal area (behind the breastbone) (level of agreement = 100%), claim gastroesophageal reflux (GER) as the most common cause of heartburn (level of agreement = 100%), but admit that heartburn can have a number of non-reflux related causes (level of agreement 98%) and that the prevalence of these is unknown[1]. Moreover, these criteria state that the typical reflux syndrome can be diagnosed on the basis of the presence of characteristic symptoms, i.e. heartburn and regurgitation, without diagnostic testing (level of agreement = 100%). The epidemiology of heartburn shows a clear geographical variation; in North America heartburn occurring at least weekly ranges between 13.2% and 27%; it is slightly lower in Europe ranging between 7.7% and 15%, whereas the prevalence remains definitely lower of in Asia (3.1%)[2].

It is now clear that only about 40% of patients with heartburn and/or regurgitation have visible distal esophageal mucosal breaks caused by gastroesophageal reflux[3,4]. The remaining approximately 60% suffer from non-erosive reflux disease (NERD) or, according with the Montreal criteria, a typical reflux syndrome[2], i.e. the presence of heartburn and/or regurgitation without esophageal injury.

This negative etiologic definition is not satisfactory: it has been suggested that this may lead to a rather heterogeneous group of patients, including both patients with and without pathological esophageal acid exposure[5]. Thus, subcategorization of NERD relies primarily on the results of 24-h esophageal pH monitoring. Patients with GER symptoms and abnormal esophageal acid exposure during 24-h esophageal pH monitoring can be classified as NERD; additionally, even patients with a normal esophageal acid exposure but a positive symptom-reflux association may be defined as NERD. The remainder patient may be defined as

having "functional heartburn"[5]. Recently, the Rome Ⅲ Committee added that functional heartburn patients also have to demonstrate a negative response to standard course of proton pump inhibitor (PPI) treatment[6].

Since these definitions appear to be useful only at a research setting, and not at a primary care level, in this review we will describe the epidemiology and natural history of NERD patients solely defined on the basis of their symptoms and the absence of endoscopic injury.

EPIDEMIOLOGY

By far, the best study available up to now is the Kalixanda study[3]. The aim of this study was to estimate the prevalence of, and to identify risk factors for gastroesophageal reflux symptoms and esophagitis in the adult population of two Swedish municipalities, Kalix and Haparanda ("the Kalixanda study"), with roughly 30 000 inhabitants, chosen because the distribution of age and gender in this area was similar to the national average in Sweden. In the two communities, upper endoscopies were provided by both primary and secondary care physicians and by two endoscopy units involved in the study. By using the computerized Swedish national population register, consisting of all citizens in order of date of birth, the adult population living in the two municipalities was identified and defined as the target population (n = 21 610). Subsequently, a systematic sample (every seventh) of the target population (13.9% of the target population) was enrolled as the study population (n = 3000), and one-third of them were submitted to an esophago-gastroduodenoscopy (EGD) on a voluntary basis, and this formed the study population, i.e. 1000 individuals in random order, representing 4.6% of the target population. The primary symptom analysis in this study was based on the presence of troublesome heartburn and/or acid regurgitation over the past 3 mo.

Four hundred subjects (40%, CI = 37.0-43.0) reported at the time of the EGD visit that they had been bothered by troublesome heartburn and/or acid regurgitation over the past 3 mo. There was no statistically significant difference in prevalence between the sexes, except in the oldest age group, where women had more symptoms ($P < 0.01$).

Weekly symptoms were reported by 200 (20%, CI = 17.5-22.5, mean age 52.4, 45% M) and daily symptoms by 59 individuals (5.9%, CI = 4.4-7.4, mean age 52.8, 44.1% M). There was no statistically significant difference in age or gender between these two groups. Erosive esophagitis (EE) was found in 155 subjects (15.5%, CI = 13.2-17.7) with a mean age of 52.6 years and was most prevalent in men (22%) especially in the youngest age group (32%), and most often mild esophagitis (L-A grade A or B in 95.5% of cases) was diagnosed. The esophagus was macroscopically normal in 769 subjects (76.9%, CI = 74.3-79.5) in the EGD study sample. These subjects had a mean age of 53.5 years and 340 of them (44.1%) were men. This group also includes 123 individuals who had a hiatus hernia as the only finding. Overall, a hiatus hernia was observed in 239 individuals (23.9%, CI = 21.2-26.5) with a mean age of 55.6 years, 54.4% being men. Thus, in this study, 40% of subjects reported typical GER symptoms during the last 3 mo (half of them on a weekly basis), and of these 15.5% had esophagitis whereas 76.9% had absence of esophagitis (NERD) at upper endoscopy. Globally, about 10% of the study population had erosive esophagitis (n = 98), whereas almost 27% of the sample had typical GER symptoms but no esophagitis (n = 271); if only cases with weekly symptoms were considered, the rate cuts down to 12.5% (n = 125).

In a preliminary report of an Italian endoscopic study, the Loiano-Monghidoro project, conducted on 892 adult subjects belonging to the general population, the prevalence of esophagitis was 8.2%, and 24.8% of those had no symptoms[4]. The prevalence of at least weekly heartburn in the same population was 21.5%.

Therefore, from these two population studies, we can estimate that in Europe at least one tenth of the general population has at least weekly heartburn.

NATURAL HISTORY

Evaluating the natural history of NERD is useful for a number of reasons[7], this knowledge may help (1) to discern the percentage of the population that will progress from non-erosive to erosive disease and possibly to its complications, such as stricture, Barrett's oesophagus, and esophageal adenocarcinoma, or from exclusively esophageal to supraesophageal manifestations, (2) to define, assess, and validate productivity of risk factors for such complicated forms of the disease, (3) to determine if medical or other therapies are able to positively modify the natural course of the disease, and (4) to determine the need for maintenance therapy to prevent complications and persistent symptoms in such patients.

Until recently, patients with NERD were considered to suffer from a milder disease[8], i.e. requiring less intensive/prolonged treatment and possibly characterized by a better long-term prognosis. This concept was subsequently proven to be incorrect, since the impairment in disease-related quality of life (HRQoL), for example, appears to be similar in GERD patients with or without endoscopic esophagitis and is related in both instances to symptom severity[9]. Also, the symptomatic acute response to PPI drugs in patients with or without endoscopic mucosal damage seems not to be different, and in fact might be worse in NERD[10,11]. Finally, after discontinuation of acute treatment, symptomatic relapse within 6 months appears to affect a similarly high proportion of both GERD groups[12].

We reported one of the first natural history studies of symptomatic GERD patients without endoscopic esophagitis but with a pathological esophageal pH-metry[13]. In that study we showed that 5 of 33 such patients treated with antacids or prokinetic agents developed endoscopic esophagitis within 6 mo, and that the extent of esophageal acid exposure at entry was not

predictive for this complication. In a subsequent study[14], we extended the observation of the original patient group up to a median duration of 10 years. The first interesting observation regarding this patient sample is that almost all patients that we were able to trace (28/29) are affected by GERD symptoms when anti-secretory drugs are discontinued, and therefore the majority (75%) were on such therapy due to GERD symptoms. Secondly, a very high proportion (89%) of our patients in whom repeat endoscopy was performed (n = 18) showed an erosive esophagitis. Thus, a considerable proportion of the original patient cohort indeed showed a *progression* from non-erosive to erosive disease.

Schindlbeck *et al*[15], in a study investigating the fate of GERD patients with and without esophagitis, reported on 16 patients with pH-documented GERD and no esophagitis 3 years after the diagnosis. During this period, four patients (25%) developed reflux esophagitis, while the majority of the patient population, which also included patients with esophagitis at entry, was still taking medications on a daily basis because of their GERD symptoms. Symptoms were rated to be equal or worse than at entry by 70% of patients in the absence of treatment.

In a Finnish study, 57 consecutive referrals with symptoms of GERD were treated by modification of lifestyle/antacids[16]. Initial assessment included endoscopy and esophageal pH recording, and patients were then followed up for a median of 19.5 years. Of the 30 patients with no evidence of erosive esophagitis at presentation, five (17%) developed grade 1 esophagitis according to Savary-Miller classification. In the study by McDougall *et al*[17], 71% of the 17 patients with a pH-metry documented NERD complained of frequent heartburn 3 to 4.5 years after initial diagnosis, 59% were on daily acid suppressive therapy, and 24% of those patients who had repeat endoscopy developed esophagitis. Again, a progression from non-erosive to erosive GERD was observed, at least in a proportion of patients.

More recently, we have performed a study on patients with typical GERD symptoms presenting to our laboratory to undergo 24-h esophageal pH-monitoring. We have analyzed patients (n = 35) with a pathological investigation, defined as a 24-h % of GER exceeding 5.0% of the total recording time, and with a negative upper GI endoscopy. These NERD patients have been interviewed by mean of a structured questionnaire on average three years after the initial diagnosis, in order to assess the presence and severity of GERD symptoms, the therapy (if any) received during this period of follow-up, and the results of any subsequent endoscopic examination performed.

The results of this retrospective survey show that 14% of those NERD patients who underwent repeat endoscopy developed erosive esophagitis during the 3-year follow-up, despite the fact that almost all of them received effective symptomatic treatment, i.e. H2-RA or PPI therapy[18].

Finally, in a recent multicenter trial[19] conducted on 588 patients with NERD and assessing the effectiveness of continuous *vs* on demand PPI maintenance therapy, it was observed that a proportion as high as 5% of patients treated "on-demand" developed erosive changes within 6 mo of study, as compared with 0% in the continuous treatment arm.

A study has been conducted in a cohort of 3894 patients with predominant heartburn, with or without esophagitis, (1717 NERD, 1512 Los Angeles grade A/B and 278 LA grade C/D, and 387 had Barrett's esophagus) under routine clinical care in Germany, Austria, and Switzerland (ProGERD study)[20]. After initial treatment with esomeprazole, they were followed up for two years, regardless of their response. Medical therapy or endoscopy was initiated at the discretion of their primary care physician, in line with routine care. At two years, endoscopy with biopsy was performed according to the protocol. The results were as follows: 25% of patients who had NERD at baseline progressed to LA A/B and 0.6% to LA C/D. At 2 years, 22% of patients had been off medication for at least 3 mo. The conclusions of the authors were that GERD does not seem to be a categorical disease. Progression and regression (the latter likely due to therapy) between grades was observed in this large cohort of patients under routine clinical care.

Another recent study has examined the possible progression in 47 subjects with symptomatic GERD without endoscopic evidence of esophagitis, out of a group of 497 patients undergoing upper GI endoscopy for various reasons[21]; all those patients (47 + 450) were endoscopically assessed annually for 5 years. Esophagitis developed in 36.2% of patients with NERD, as compared with 11.3% in the control group, with a hazard ratio of developing esophagitis in the former group of 3.07. The authors concluded that the condition of symptomatic GERD carries a high risk of developing esophagitis, which increases steadily with time and was more frequent in those NERD patients with hiatus hernia, who smoke and drink alcohol, and who are without *H pylori* infection[21].

All these studies indicate that some patients with NERD may indeed develop erosive disease, at an approximate rate of about 10% per year. If this rate remains stable with time, a substantial proportion of patients with NERD may develop ERD within 10 years, which is a rate close to what we observed in our 10-year follow-up study of NERD patients[14].

These conclusions are in accordance with results of a recently published systematic review of 22 publications on the endoscopic assessment of erosive or non-erosive GERD over periods larger than 12 months[22]. In this review, authors conclude that the observed progression rate from NERD to ERD ranges in the literature from 0% to 30%. The variability may be related to the duration of follow-up and other factors as *H pylori* infection.

CONCLUSION

NERD is a heterogeneous condition, presently defined on the basis of the presence of typical GERD symptoms and the absence of esophageal damage as

judged by upper endoscopy. This definition is for various reasons unsatisfactory. The prevalence of at least weekly heartburn in the general population in Europe can be estimated to range from 10% to 20%.

A consistent proportion of this group will develop an erosive esophagitis (progression), even under routine therapeutic care, with a rate probably around 10% per year within a 10-year frame.

REFERENCES

1 **Vakil N**, van Zanten SV, Kahrilas P, Dent J, Jones R. The Montreal definition and classification of gastroesophageal reflux disease: a global evidence-based consensus. *Am J Gastroenterol* 2006; **101**: 1900-1920; quiz 1943

2 **Dent J**, El-Serag HB, Wallander MA, Johansson S. Epidemiology of gastro-oesophageal reflux disease: a systematic review. *Gut* 2005; **54**: 710-717

3 **Ronkainen J**, Aro P, Storskrubb T, Johansson SE, Lind T, Bolling-Sternevald E, Graffner H, Vieth M, Stolte M, Engstrand L, Talley NJ, Agreus L. High prevalence of gastroesophageal reflux symptoms and esophagitis with or without symptoms in the general adult Swedish population: a Kalixanda study report. *Scand J Gastroenterol* 2005; **40**: 275-285

4 **Zagari RM**, Fuccio L, Wallander MA, Johansson S, Fiocca R, Casanova S, Farahmand BY, Winchester CC, Roda E, Bazzoli F. Gastro-oesophageal reflux symptoms, oesophagitis and Barrett's oesophagus in the general population: Loiano-Monghidoro study. *Gut* 2008; **57**: 1354-1359

5 **Tack J**, Fass R. Review article: approaches to endoscopic-negative reflux disease: part of the GERD spectrum or a unique acid-related disorder? *Aliment Pharmacol Ther* 2004; **19** Suppl 1: 28-34

6 **Galmiche JP**, Clouse RE, Balint A, Cook IJ, Kahrilas PJ, Paterson WG, Smout AJ. Functional esophageal disorders. *Gastroenterology* 2006; **130**: 1459-1465

7 **Locke GR 3rd**. Natural history of nonerosive reflux disease. Is all gastroesophageal reflux disease the same? What is the evidence? *Gastroenterol Clin North Am* 2002; **31**: S59-S66

8 **Quigley EM**, DiBaise JK. Non-erosive reflux disease: the real problem in gastro-oesophageal reflux disease. *Dig Liver Dis* 2001; **33**: 523-527

9 **Glise H**, Hallerback B, Wiklund I. Quality of life: a reflection of symptoms and concerns. *Scand J Gastroenterol Suppl* 1996; **221**: 14-17

10 **Smout AJPM**. Endoscopy-negative acid reflux disease. *Aliment Pharmacol Ther* 1997; **11** Suppl 2: 81-85

11 **Fass R**, Fennerty MB, Vakil N. Nonerosive reflux disease-current concepts and dilemmas. *Am J Gastroenterol* 2001; **96**: 303-314

12 **Carlsson R**, Dent J, Watts R, Riley S, Sheikh R, Hatlebakk J, Haug K, de Groot O, van Oudvorst A, Dalvag A, Junghard O, Wiklund I. Gastro-oesophageal reflux disease in primary care: an international study of different treatment strategies with omeprazole. *Eur J Gastroenterol Hepatol* 1998; **10**: 119-124

13 **Pace F**, Santalucia F, Bianchi Porro G. Natural history of gastro-oesophageal reflux disease without oesophagitis. *Gut* 1991; **32**: 845-848

14 **Pace F**, Bollani S, Molteni P, Bianchi Porro G. Natural history of gastro-oesophageal reflux disease without oesophagitis (NERD)--a reappraisal 10 years on. *Dig Liver Dis* 2004; **36**: 111-115

15 **Schindlbeck NE**, Klauser AG, Berghammer G, Londong W, Mueller-Lissner SA. Three year follow up of patients with gastrooesophageal reflux disease. *Gut* 1992; **33**: 1016-1019

16 **Isolauri J**, Luostarinen M, Isolauri E, Reinikainen P, Viljakka M, Keyrilainen O. Natural course of gastroesophageal reflux disease: 17-22 year follow-up of 60 patients. *Am J Gastroenterol* 1997; **92**: 37-41

17 **McDougall NI**, Johnston BT, Collins JS, McFarland RJ, Love AH. Three- to 4.5-year prospective study of prognostic indicators in gastro-oesophageal reflux disease. *Scand J Gastroenterol* 1998; **33**: 1016-1022

18 **Pace F**, Pallotta S, Molteni P, Zentilin P, Russo L, Savarino V, Bianchi Porro G, Grossi E, Cuomo R. Natural history of NERD in 3 Italian tertiary referral centres after 5 years of follow up. *Gut* 2006; **55** suppl: A62

19 **Bayerdörffer E**, Sipponen P, Bigard M, Weiss W, Mearin F, Rodrigo L, Dominguez-Munoz J, Grundling H, Nauclér E, Svedberg L, Keeling N, Eklund S. Esomeprazole 20 mg continous versus on demand treatment of patients with endoscopy-negative reflux disease (ENRD). *Gut* 2004; **53** (Suppl 4): A106

20 **Labenz J**, Nocon M, Lind T, Leodolter A, Jaspersen D, Meyer-Sabellek W, Stolte M, Vieth M, Willich SN, Malfertheiner P. Prospective Follow-Up data from the ProGERD Study Suggest that GERD Is Not a categorial disease. *Am J Gastroenterol* 2006; **101**: 2457-2462

21 **Kawanishi M**. Will symptomatic gastroesophageal reflux disease develop into reflux esophagitis? *J Gastroenterol* 2006; **41**: 440-443

22 **Fullard M**, Kang JY, Neild P, Poullis A, Maxwell JD. Systematic review: does gastrooesophageal reflux disease progress? *Aliment Pharmacol Ther* 2006; **24**: 33-45

S- Editor Li DL **L- Editor** Mihm S **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5237

World J Gastroenterol 2008 September 14; 14(34): 5237-5244
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Miguel Angel Muñoz-Navas, Profesor, *Series Editors*

Capsule endoscopy in non-steroidal anti-inflammatory drugs-enteropathy and miscellaneous, rare intestinal diseases

Gerard Gay, Michel Delvaux, Muriel Frederic

Gerard Gay, Michel Delvaux, Muriel Frederic, Department of Internal Medicine and Digestive Pathology, Hopitaux de Brabois, CHU of Nancy, Vandoeuvre les Nancy F-54511, France
Correspondence to: Gerard Gay, Professor, Department of Internal Medicine and Digestive Pathology, CHU of Nancy, Hopitaux de Brabois, Allee du Morvan, Vandoeuvre les Nancy F-54511, France. g.gay@chu-nancy.fr
Telephone: +33-383-154366 Fax: +33-383-154012
Received: February 15, 2008 Revised: August 10, 2008
Accepted: August 17, 2008
Published online: September 14, 2008

Abstract

Despite significant advances over the last decade, mucosal lesions of the small bowel are poorly detected by imaging studies such as CT scan, MRI-enteroclysis and contrast-enhanced abdominal ultrasound. Capsule endoscopy (CE) has dramatically changed the diagnostic approach to intestinal diseases. Moreover, the use of CE can be extended to include other conditions. However, it is difficult to assess the positive influence of CE on patient outcomes in conditions involving a small number of patients, or in critically ill and difficult to examine patients. CE has the advantage of diagnosing intestinal lesions and of directing the use of double balloon enteroscopy (DBE) in order to obtain biopsy specimens. Moreover, CE allows repeated assessment in chronic conditions, especially to detect relapse of an infectious disease.

Key words: Capsule endoscopy; Non-steroidal anti-inflammatory drugs-enteropathy; Intestinal diseases

Peer reviewer: Giovanni D De Palma, Professor, Department of Surgery and Advanced Technologies, University of Naples Federico Ⅱ, School of Medicine, Naples 80131, Italy

Gay G, Delvaux M, Frederic M. Capsule endoscopy in non-steroidal anti-inflammatory drugs-enteropathy and miscellaneous, rare intestinal diseases. *World J Gastroenterol* 2008; 14(34): 5237-5244 Available from: URL: http://www.wjgnet.com/1007-9327/14/5237.asp DOI: http://dx.doi.org/10.3748/wjg.14.5237

INTRODUCTION

Since it was introduced by Iddan and Meron in 2000[1], capsule endoscopy (CE) has dramatically changed the diagnostic approach to intestinal diseases. Despite significant advances over the last decade, mucosal lesions of the small bowel are poorly detected by techniques such as CT scan, MRI-enteroclysis and contrast-enhanced abdominal ultrasound[2]. The diagnostic superiority of CE over these methods is related to its ability to provide a complete examination of the small bowel mucosa. On the other hand, the drawback of CE is the inability to obtain biopsies. However, this deficiency has been overcome with the use of double balloon enteroscopy, which permits obtaining biopsies from lesions detected by CE[3].

Therefore, the diagnostic approach to conditions such as obscure GI bleeding, Crohn's disease and coeliac sprue has been dramatically altered by CE[4-6]. Moreover, the use of CE has resulted in significant advances in the understanding and diagnosis of several gastrointestinal diseases including assessment of the effect of medications on the small bowel, intestinal lesions secondary to systemic diseases, and some rare conditions[7].

In this review, the role of CE will be discussed in the following conditions: (Ⅰ) Intestinal consequences of medications such as non-steroidal anti-inflammatory drugs (NSAIDs). (Ⅱ) Rare conditions mainly involving the small bowel. (1) Primary lymphangiectasia such as Waldmann's disease. (2) Common variable immunodeficiency disorder. (3) Familial polyposis syndromes with small bowel involvement. (Ⅲ) Immunological disorders with small bowel involvement. (1) Acute and chronic graft versus host disease. (2) Hypobetalipoproteinaemia. (Ⅳ) General diseases with intestinal lesions, such as vasculitides. (Ⅴ) Infectious intestinal diseases such as Whipple's disease and CMV infection in immunosuppressed patients.

For each of these conditions, the clinical and biological characteristics will be discussed when needed, to understand the role of CE in diagnosis. The typical endoscopic patterns observed with CE will be described, and the use of CE will be integrated in a global diagnostic approach.

CE FOR THE EVALUATION OF INTESTINAL TOXICITY OF MEDICATIONS: THE EXAMPLE OF NSAID-RELATED ENTEROPATHY

CE is a simple and efficient tool to detect intestinal lesions due to NSAIDs

Background: NSAIDs account for 5%-10% of all drug prescriptions in developed countries and about 25% of the reported side effects caused by all classes of medications, including gastroduodenal ulcers seen in 10%-30% patients with intestinal lesions[8]. The prevalence of severe side effects involving the small bowel and colon is 0.89 per 100 patient-years with naproxen, and 0.41 with rofecoxib, corresponding to 39.4% and 42.0%, respectively, of all the severe gastrointestinal side effects[9]. NSAIDs alter the intestinal permeability, about 12 hours after intake and result in mucosal inflammation within 10 days. A variety of endoscopic lesions have been described, ranging from asymptomatic enteropathy to severe lesions such as ulcers, perforation, stenosis (Figure 1), diaphragms and villous atrophy. CE may be able to help detect these lesions and clarify their pathophysiology.

Description of the lesions: One of the most interesting studies was reported by Maiden *et al*[10], who evaluated the number and the type of intestinal lesions induced by NSAIDs in 40 healthy volunteers between the ages of 21 and 61 years, using CE and fecal calprotectin. The fecal calprotectin test and CE were performed before and after a 2-week course of treatment with diclofenac (75 mg) and omeprazole (20 mg twice a day). The CE recordings were read by three independent investigators, and the lesions were regarded as significant if indicated by at least two of them. The lesions were classified into six categories: (1) reddened folds; (2) denuded area with loss of villous architecture; (3) petechiae; (4) mucosal breaks; (5) presence of blood without a lesion being visualized; and (6) other findings, mainly lymphangiectasia and angiodysplasia. Before treatment, lymphangiectasia and arteriovenous malformations were observed in three patients each. After treatment with nonsteroidal anti-inflammatory drugs (NSAIDs), lesions were seen in 27 patients, 15 of whom had more than one lesion: 16 mucosal breaks (40%), including two cases of bleeding; 14 reddened folds (35%); 13 petechiae and red spots (33%); eight denuded areas (20%), including two patients with mucosal breaks; and three patients with blood in the intestinal lumen (8%). After 2 weeks, the fecal calprotectin level increased significantly in 30 subjects (+ 75-82 μg; $P < 0.0001$) but this increase did not correlate with the number of lesions detected by CE. The authors concluded that short-term NSAID treatment was associated with a high level of intestinal inflammation, and an increased frequency of intestinal lesions.

CE helps understanding the pathophysiology of NSAIDs-enteropathy

NSAID-related lesions were located equally in the proximal and distal parts of the small bowel. The study by Maiden *et al*[10] was interesting, as it showed that significant lesions can occur even after a short course of NSAID treatment in healthy individuals. However, the study had several methodological limitations: (1) The interpretation of CE images was often difficult[11] and the clinical relevance of lesions detected by CE was not always established, since such lesions could be found in the absence of any treatment in healthy individuals[12]. (2) Other causes of intestinal lesions such as chronic ischemia were not excluded. (3) A standardized terminology allowing a consistent description of the lesions and comparisons between different studies was lacking[11]. Moreover, DBE provides a direct access to intestinal lesions detected by CE and biopsy specimens can be obtained. Therefore, our understanding of the pathophysiology of early lesions induced by NSAIDs is likely to improve.

Table 1 Intestinal diseases with lesions similar to Crohn's disease, ranked by order of frequency

	Frequency
Crohn's disease	++++
Drug-related injuries (NSAIDs)	+++
Mesenteric ischaemia	++
Coeliac disease (jejunitis)	++
Cryptogenic multifocal ulcerous stenosing enteritis	+
Radiation enteritis	+
Lymphoma, ulcerated cancer	+
Vasculitides (lupus, polyarthritis, PAN)	+
Behçet's disease	+/-
Eosinophilic enteritis	+/-
Infections (CMV, Whipple, yersinia *etc*)	+/-

CE to evaluate the benefit of medications preventing the toxic intestinal effects of NSAIDs

Shunji and Nakamura showed in a randomized trial on 16 patients that rebaminide significantly decreased the prevalence of diclofenac-induced small intestinal mucosal injury[13]. These results need to be confirmed in larger studies. On the other hand, the etiological diagnosis of intestinal lesions observed by CE is often difficult. The distinction of lesions related to NSAIDs or those seen in Crohn's disease is particularly difficult, as recently shown by Voderholzer who reported a rate of misdiagnosed lesions in as many as 25% of the 40 patients with 146 intestinal lesions detected by CE[14].

Table 1 shows how difficult the differential diagnosis is of intestinal erosions and ulcers, since they may be related to a number of pathological conditions and diseases. Therefore, DBE with biopsy of the lesions will often be necessary to obtain a correct diagnosis.

RARE DIGESTIVE DISEASES WITH SMALL BOWEL INVOLVEMENT

Waldmann's disease (Figure 2)

Intestinal lymphangiectasia appears on CE recordings as whitish areas, often diffusely spread over the intestinal mucosa. The endoscopic appearance of

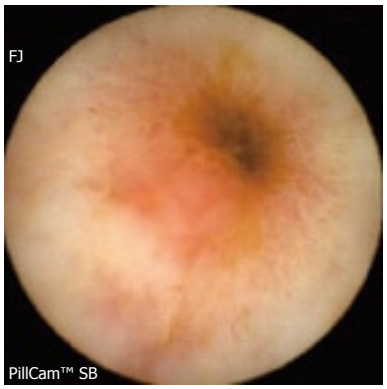


Figure 1 Intestinal stenosis caused by NSAIDs. Capsule endoscopy shows narrowed segment, surrounded by an ulcerated but non-inflammatory mucosa.

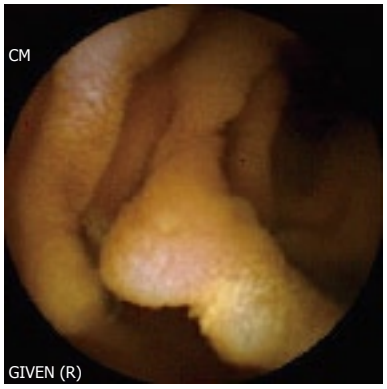


Figure 2 Waldmann's disease. The presence of dilated lymphatic vessels in the submucosa gives a whitish appearance to the mucosa. If lymphangiectasia becomes more prominent, it may protrude into the lumen.

lymphangiectasia is the result of an accumulation of chylomicrons in the dilated lymphatic vessels, varying in size from a few millimeter white spots to large white nodular areas. Lymphangiectasia may be diffuse or may involve a localized segment of the small bowel. Clinically, lymphangiectasia is classified as primary, secondary or functional. Functional lymphangiectasia are not clinically relevant and it is often encountered in patients with functional digestive disorders. Secondary lymphangiectasia is a consequence of intestinal or extra-intestinal diseases causing compression of the gut. By contrast, Waldmann's disease is primary idiopathic lymphangiectasia, which results in an exudative enteropathy. This condition may present as a diffuse disease or may involve only a localized segment of the small bowel. Localized lymphangiectasia can be treated surgically, by resection of the pathological segment. However, diffuse disease requires medical treatment (diet containing medium chain fatty acids). DBE shows thickened mucosal folds without villous atrophy in the duodenum, jejunum and ileum. Small size nodules (spots) may be observed as well as large confluent areas[15]. CT scan may show thickening of the mucosal folds.

Immunoglobulin deficiency: The example of common variable immunodeficiency disorder (CVID, Figure 3)

This condition is characterized by hypogammaglobulinaemia, recurrent bacterial infections, mainly pulmonary, and is frequently associated with autoimmune and neoplastic disorders. Table 2 summarizes the clinical features. CE shows small (millimetre size) nodular lesions spread diffusely over the intestinal mucosa. In some cases, the lesions are polypoidal, of variable sizes[16]. DBE shows similar findings and may also demonstrate areas of atrophic mucosa, in the event of an associated infection. The nodules correspond to hypertrophic lymphoid follicles, without plasma cells.

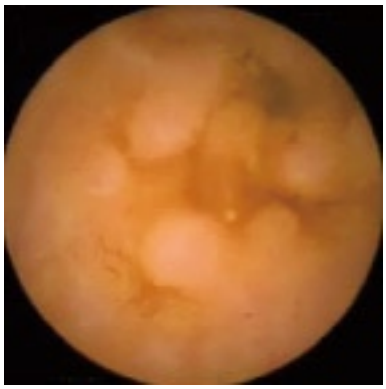


Figure 3 Common variable immunodeficiency disorder. The characteristic lesions include polypoidal and nodular lesions of variable size, which are disseminated over the intestinal mucosa.

Table 2 Common variable immunodeficiency disorder: Clinical manifestations

Gastrointestinal signs	Extra-intestinal signs
Giardia lamblia infection	Recurrent respiratory tract infections
Small bowel bacterial overgrowth	Increased risk of lymphomas
Viral and infectious diarrhoea	Increased risk of gastric cancer
Coeliac disease	
Frequent nodular lymphoid hyperplasia, not premalignant	

In most instances, the lesions (either nodules or polyps) are disseminated throughout the gut. There is an increased risk of intestinal lymphoma in these patients. CE allows regular surveillance of the small bowel. When CE reveals a change in the endoscopic findings, DBE with biopsy should be performed or the patient should refered to a surgeon for intestinal resection.

Familial polyposis syndromes

Background: CE is very effective in detecting intestinal tumours. Intestinal tumours account for 3% to 6% of all digestive neoplasms, less than 2% of which are malignant. The data collected since the introduction of CE, shows a higher frequency of intestinal tumours, between 6.3% and 12.3% in patients investigated for obscure digestive bleeding by CE[17]. Since the use of CE permits earlier diagnosis, the clinical course of intestinal tumours has changed. These tumours are now seen as ulcerated, haemorrhagic lesions, before the occurrence of obstructive signs. Therefore, one may assume that CE can be used for the detection of intestinal tumours in patients with familial polyposis syndromes. There are

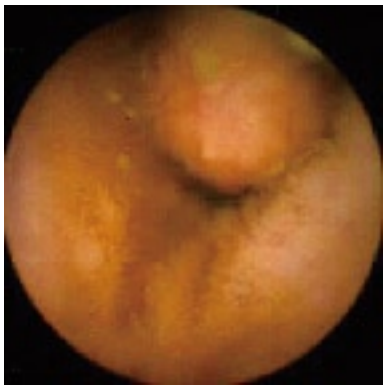


Figure 4 Peutz-Jeghers syndrome. Presence of a large polyp in the ileum, in a young patient with Peutz-Jeghers syndrome. The polyp was ulcerated and required endoscopic resection.

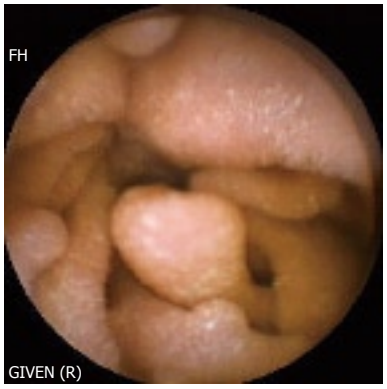


Figure 5 Familial adenomatous polyposis. Multiple polyps of regular shape are present in the ileum.

two conditions that are of particular concern: Peutz-Jeghers syndrome and familial adenomatous polyposis syndrome.

Peutz-Jeghers syndrome (Figure 4): In patients with Peutz-Jeghers syndrome, the prevalence of small bowel cancer is particularly high. Intussusception of the polyps may cause intestinal obstruction, while ulcerated lesions may be responsible for acute or chronic gastrointestinal bleeding. CE can detect polyps in the entire length of the small intestines, with a higher diagnostic yield compared to CT scan and MRI with enteroclysis, particularly for lesions < 1 cm in diameter[18]. The role of CE has recently been established in the initial work-up, as well as for the follow-up of patients with Peutz-Jeghers syndrome[18]. However, it is difficult to determine the size of the polyps on capsule recordings, which is an important factor in selecting patients for removal by DBE. It should be noted that polyps selected for removal are of a large size, and those at high risk of ulceration, malignancy and intussusception.

Familial adenomatous polyposis syndrome (FAP, Figure 5): There is currently no established indication for small bowel CE in the initial work-up of patients with FAP. By contrast, CE may be indicated in FAP patients with multiple duodenal polyps (Spiegelmann Ⅲ or Ⅳ)

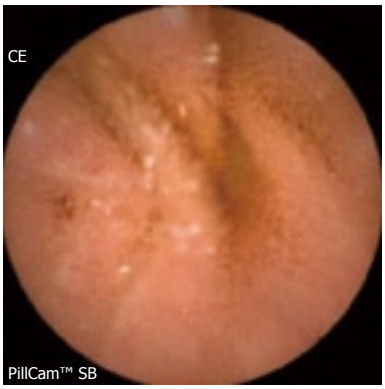


Figure 6 Acute Graft versus Host Disease (GVHD). The lesions correspond to Stage Ⅲ disease, manifesting as diffuse erythema and oedema of the mucosa, with a few erosions.

as these patients have more frequent intestinal polyps[19]. Moreover, polyps larger than 10 mm in diameter should be removed because of the risk of malignancy, either by DBE or intra-operative enteroscopy. On the other hand, examination of the duodenum, in particular the area of the papilla is often difficult with CE because of the rapid transit of the capsule through this segment. Therefore, assessment of the duodenum with a lateral viewing endoscope is the preferred approach.

Capsule endoscopy should be used with caution in familial polyposis syndromes, since many of these patients have undergone multiple abdominal surgeries which may delay capsule progression or even cause its retention.

B-cell lymphoma: Finally, in this section on rare intestinal tumours, it is important to mention the role of CE in the investigation of patients with suspected B-cell lymphoma. CE not only allows the diagnosis of such tumours but also helps to evaluate the extent of the intestinal disease[20]. CE is also useful in evaluating the response to chemotherapy.

IMMUNOLOGICAL DIGESTIVE DISORDERS (PHOTO N° 9 ET 10) (MALADIE DE BORDIGONI)

Acute gastrointestinal graft versus host disease (GVHD) after bone marrow transplantation (Figure 6)

Bone marrow transplantation is widely used for the treatment of a number of haematological diseases. Acute GVHD is a severe complication that requires quick initiation of treatment with immunosuppressive drugs. The small bowel is often involved in acute GVHD, with the development of hematochezia, diarrhoea, abdominal pain, nausea and vomiting. The diagnosis is difficult and GVHD must be distinguished from other conditions sharing a similar clinical picture such as cytomegalovirus enteritis and *Clostridium difficile* infection.

The diagnosis is easier in the presence of multi-system disease, especially when the skin lesions can be biopsied. In patients without skin involvement, an endoscopic

work-up is needed, with esogastroduodenoscopy and ileocolonoscopy. Until recently, esogastroduodenoscopy was regarded as the gold standard for the examination of the duodenum, with findings of mucosal oedema, denuded mucosa, erosions, erythema and bleeding ulcers. These lesions are classified into 4 grades, grade Ⅰ refers to focal erythema, grade Ⅱ diffuse erythema and oedema, grade Ⅲ severe oedema and friable mucosa with erosions, and grade Ⅳ presence of exudate, ulcers and active bleeding[21].

The clinical usefulness of CE in acute GVHD was demonstrated in two studies. Neumann *et al*[22] evaluated 14 patients with clinical signs of acute GVHD after bone marrow transplantation, and observed typical intestinal lesions with histological confirmation on biopsy obtained by endoscopy, performed subsequently. This study also showed that the lesions involved the entire length of the small bowel and were more intense in the ileum compared to in the jejunum. The most important finding was the very high negative predictive value of CE since patients with a negative CE did not develop acute GVHD during the 2-month follow-up period after CE. These findings were confirmed by Aghai *et al*[23] in a study on a larger cohort of patients.

Although it is worth mentioning that CE is well tolerated by such critically ill patients, certain limitations should be noted. In some patients, the capsule may be retained in the stomach. The Rapidview system can be used to monitor the progression of the capsule[24]. In the event of delayed gastric clearance, erythromycin may be administered. Moreover, it should be emphasized that in some patients, CE was normal but these patients had acute GVHD[25].

Despite the limited data available in the literature, CE should now be regarded as an alternative to esogastroduodenoscopy in the workup of patients with suspected GVHD, particularly in critically ill patients requiring quick therapeutic decisions. Indeed, CE appears to be as effective as esogastroduodenoscopy with biopsy for the diagnosis of acute GVHD[25-27].

Hypobetalipoproteinemia (Figure 7)

Background: Hypobetalipoproteinemia is an autosomal dominant disorder caused by a mutation or deletion in the *apoB* gene which produces a truncated apolipoprotein B. The plasma concentrations of the two forms of apolipoprotein B, that is, Apo B-100 synthesized by the liver and Apo-B48 synthesized by the intestine, are undetectable. The biological and clinical picture is similar to that of abetalipoproteinemia. Heterozygous hypobetalipoproteinemia is present in 0.1%-0.8% of the general population, while homozygous hypobetalipoproteinemia is rare. The prognosis of Apo B-related disorders depends mainly on the consequences of the malabsorption syndrome, and on the progression of neurological alterations caused by the deficiency of soluble vitamin E[28].

Diagnostic procedure: Laboratory tests show hypocholesterolemia, acanthocytosis and undetectable ApoB lipoprotein. CT scan shows massive and diffuse infiltration of the liver. CE demonstrates diffuse whitish pattern of the intestinal mucosa with occasional yellow areas seen in the entire length of the small bowel, but without any villous atrophy. Enteroscopy, formerly performed with push enteroscopes and currently with DBE shows a similar pattern as CE and moreover, biopsy specimens can be obtained. These lesions are produced by the accumulation of fat vesicles in the enterocytes in the jejunum and ileum, without causing villous atrophy[29]. Immunohistchemistry shows migration of truncated Apo-B and the absence of normal Apo-B hypobetalipoproteinemia.

INTESTINAL EXPRESSION OF VASCULITIDES

Background

General considerations: Systemic diseases include several very heterogeneous pathological conditions which share some common features: (1) symptoms suggesting the involvement of multiple organs, (2) specific immunological and biological alterations, (3) a tendency to chronicity, and (4) clinical response to immunosuppressive therapy. Systemic diseases are divided into two main categories[30,31]: connective tissue or collagenous diseases with predominant abnormal production or deregulated synthesis of collagen, and vasculitides with predominant inflammation of the blood vessels resulting in abnormal vascular permeability, thrombosis and tissue ischaemia. These conditions share some pathophysiological mechanisms such as abnormal production of proteins or cytokines, and abnormal humoral or cellular immunity that results in muscular inflammation, atrophy and fragmentation. In the end, the vascular endothelial changes become predominant and are responsible for the muscular, neurological and digestive abnormalities.

Digestive manifestations of vasculitides and systemic diseases include a number of clinical conditions (Table 3)[32]. The development of GI symptoms raises three main questions: Do the digestive symptoms correspond to the initial manifestation of the condition or to a new acute phase of the disease? Are the digestive symptoms secondary to a complication of the treatment? Is the observed complication a component of the primary disease? To answer these questions, the clinician may use diagnostic techniques such as CT scan, biological and immunological tests, and CE. The use of CE will be illustrated by a few examples.

Specific characteristics of diseases

Behçet's disease: Behçet's disease is a systemic inflammatory condition, with 10% to 25% patients developing GI manifestations. The symptoms consist mainly of abdominal pain, diarrhoea and acute or chronic bleeding. CE may show erosions and aphthous ulcers in the small bowel, as reported by Fylejk in a series of 20 patients[33]. Intestinal lesions may be present

Table 3 Frequency of digestive manifestations of vasculitides and connective tissue diseases (adapted from Muller-Ladner[32])

Disease	Frequency of digestive manifestations
Primary vasculitides	
Periarteritis nodosa.	30%-50%
Churg-Strauss Syndrome	25%-50%
Behçet's disease	Up to 30%
Takayasu's arteritis	Up to 15%
Wegenre's disease (granulomatosis)	5%-10%
Lymphomatous granulomatosis	1.5%
Horton's disease	1%
Henoch-Schönlein purpura	50%-90%
Secondary vasculitides	
Systemic lupus erythematosus	Up to 50%
Rheumatoid arthritis	Up to 10%
Connective tissue disorders	
Systemic sclerosis	75%-90%
Systemic lupus erythematosus (except vascular lesions)	25%

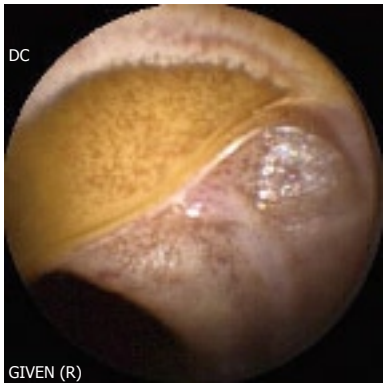


Figure 7 Hypobetalipoproteinemia. The endoscopic picture is characterized by a diffuse whitish pattern of the mucosa due to the accumulation of fat vesicles in the enterocytes. The size of the intestinal villi is normal.

in symptomatic and asymptomatic patients, and do not show a specific endoscopic pattern.

Churg et Strauss disease (Figure 8): Churg et Strauss disease is a systemic disease characterized by eosinophilia and NTE manifestations suggesting an allergenic reaction. The small bowel is involved in nearly 50% patients, presenting with gastrointestinal bleeding, diarrhoea and abdominal pain. Intestinal ulcers are not uncommon and often manifest as bleeding or perforation. Sanchez *et al*[34] have shown that the ulcers detected by CE are deep, involve the jejunum and ileum, and respond well to immunosuppressive treatment. Therefore, CE is also useful in evaluating the response to treatment.

Henoch-Schönlein purpura: The clinical picture of Henoch-Schönlein purpura is characterized by the occurrence of purpuric lesions, abdominal pain, polyarthralgia and renal failure. The disease is a consequence of the deposition of IgA in the blood vessels. CE may reveal intestinal involvement, with

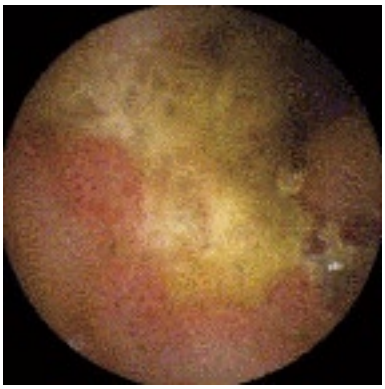


Figure 8 Churg and Strauss disease. A large ulcer is present in the ileum.

findings of oedematous mucosa, multiple ulcers, fibrin deposits and bleeding. Lesions observed by CE are often diffuse, involving the jejunum and ileum. If the clinical picture suggests Henoch-Schönlein purpura, the presence of intestinal lesions at CE should be regarded as diagnostic, and further endoscopic assessment is not required[35].

Antral vascular ectasia associated with vasculitides: Antral vascular ectasia are a common cause of bleeding and anaemia, particularly in patients with liver cirrhosis and portal hypertension. However, portal hypertension is not observed in nearly 70% patients. In these patients, the condition is related to immunological disorders such as systemic sclerosis and rheumatoid arthritis, renal failure and diabetes. Antral vascular ectasia can be diagnosed by CE with a typical endoscopic pattern of "watermelon stomach". Thus, CE is useful not only in diagnosing the cause of anaemia but also for the follow-up of patients treated with endoscopic haemostasis[36].

INFECTIOUS INTESTINAL DISEASES AND VIRAL INFECTIONS IN IMMUNODEFICIENT PATIENTS

Background

The clinical manifestation of intestinal infection is usually an acute episode of diarrhoea, which resolves spontaneously, and endoscopic assessment is not indicated in most patients. By contrast, patients with persistent diarrhoea or malabsorption require further workup. Recently, there has been an increase in the incidence of certain viral infections in patients with immunodeficiency due to HIV infection or those on immunosuppressive therapy. Endoscopy is the main diagnostic procedure as it allows examination of the mucosa, and biopsy specimens can be obtained for identification of the causal virus. Biopsies are frequently performed during esogastroduodenoscopy and ileo-colonoscopy. These procedures have a limited range of examination, since a large part of the small bowel is excluded. CE is a very useful complement to these investigations, as it allows assessment of the intestinal

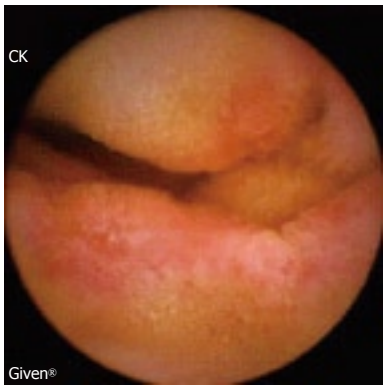


Figure 9 Cytomegalovirus infection in a heart transplant patient. The disease is characterized by the presence of bleeding ulcers and erosions.

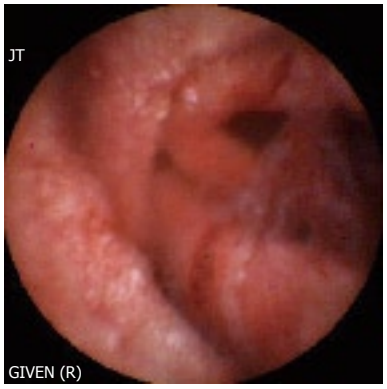


Figure 10 Whipple's disease. The endoscopic picture shows an oedematous and friable mucosa with erosions and serpiginous ulcers. The lesions may involve the entire length of the small bowel.

mucosa with a high diagnostic yield, and can have a direct impact on the management of such patients[37]. Moreover, lesions detected by CE can be subsequently biopsied during DBE.

Infectious diseases in immunodeficient patients (Figure 9)

CE can detect ulcers involving the intestinal mucosa in patients with viral, fungal or bacterial infections. Cytomegalovirus infection can be diagnosed by the presence of one or two large jejunal ulcers in immunodeficient patients, as for example in transplant patients. These ulcers may bleed and are the source of haematochezia when located in the distal ileum or the colon[38]. Fungal infection in HIV patients, such as histoplasmosis is characterized by the presence of multiple deep ulcerations accompanied with submucosal nodules protruding into the lumen. The diagnosis is confirmed by the detection of the pathogenic agent on biopsies obtained during enteroscopy[39]. In patients with tuberculosis, CE may reveal deep ulcers, often appearing as serpiginous. The lesions are difficult to differentiate from those of Crohn's disease. The pathological examination of the biopsies may show a typical granuloma. Studies using CE have shown that the lesions may not be restricted to the ileum but are also seen in the jejunum[40].

Whipple's disease, an example of diffuse intestinal infection (Figure 10)

Whipple's disease is a multisystem infectious condition caused by *Tropheryma whippelii*. GI symptoms are often present and the diagnosis is made by demonstrating the presence of the infectious agent on duodenal biopsy. The most typical endoscopic finding is the presence of an oedematous, friable and haemorrhagic mucosa with multiple erosions and serpiginous ulcers. CE has shown that the intestinal lesions are diffuse, involving the jejunum and ileum. This is explained by the fact that previous investigative techniques allowed only limited access to the intestinal mucosa, i.e. before CE and DBE became available[41]. Moreover, CE is also a useful non-invasive method to determine the response to treatment, which has to be maintained for at least two years, and occasionally five years in order to avoid relapse, especially of the neurological manifestations[42].

CONCLUSION

Since it was introduced in 2000, the use of CE in several clinical situations has been validated. However, the indications for CE can be extended to other diseases, although an assessment of a positive influence of CE on patient outcomes is difficult obtain in conditions which occur either in a small number of patients, or in critically ill and difficult to examine patients. Clearly, CE has the advantage of detecting lesions in the small intestine and directing DBE to the correct location in order to obtain biopsies. Moreover, repeated assessment can be made in chronic conditions, especially to detect the response to treatment.

REFERENCES

1 **Iddan G**, Meron G, Glukhovsky A, Swain P. Wireless capsule endoscopy. *Nature* 2000; **405**: 417
2 **Orjollet-Lecoanet C**, Menard Y, Martins A, Crombe-Ternamian A, Cotton F, Valette PJ. [CT enteroclysis for detection of small bowel tumors] *J Radiol* 2000; **81**: 618-627
3 **Gay G**, Delvaux M, Fassler I. Outcome of capsule endoscopy in determining indication and route for push-and-pull enteroscopy. *Endoscopy* 2006; **38**: 49-58
4 **Triester SL**, Leighton JA, Leontiadis GI, Fleischer DE, Hara AK, Heigh RI, Shiff AD, Sharma VK. A meta-analysis of the yield of capsule endoscopy compared to other diagnostic modalities in patients with obscure gastrointestinal bleeding. *Am J Gastroenterol* 2005; **100**: 2407-2418
5 **Triester SL**, Leighton JA, Leontiadis GI, Gurudu SR, Fleischer DE, Hara AK, Heigh RI, Shiff AD, Sharma VK. A meta-analysis of the yield of capsule endoscopy compared to other diagnostic modalities in patients with non-stricturing small bowel Crohn's disease. *Am J Gastroenterol* 2006; **101**: 954-964
6 **Cellier C**, Green PH, Collin P, Murray J. ICCE consensus for celiac disease. *Endoscopy* 2005; **37**: 1055-1059
7 **Delvaux M**, Gerard Gay. Capsule endoscopy in 2005: facts and perspectives. *Best Pract Res Clin Gastroenterol* 2006; **20**: 23-39
8 **Silverstein FE**, Faich G, Goldstein JL, Simon LS, Pincus T, Whelton A, Makuch R, Eisen G, Agrawal NM, Stenson WF, Burr AM, Zhao WW, Kent JD, Lefkowith JB, Verburg KM, Geis GS. Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis

and rheumatoid arthritis: the CLASS study: A randomized controlled trial. Celecoxib Long-term Arthritis Safety Study. *JAMA* 2000; **284**: 1247-1255

9 **Bombardier C**, Laine L, Reicin A, Shapiro D, Burgos-Vargas R, Davis B, Day R, Ferraz MB, Hawkey CJ, Hochberg MC, Kvien TK, Schnitzer TJ. Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. VIGOR Study Group. *N Engl J Med* 2000; **343**: 1520-1528, 2 p following 1528

10 **Maiden L**, Thjodleifsson B, Theodors A, Gonzalez J, Bjarnason I. A quantitative analysis of NSAID-induced small bowel pathology by capsule enteroscopy. *Gastroenterology* 2005; **128**: 1172-1178

11 **Korman LY**, Delvaux M, Gay G, Hagenmuller F, Keuchel M, Friedman S, Weinstein M, Shetzline M, Cave D, de Franchis R. Capsule endoscopy structured terminology (CEST): proposal of a standardized and structured terminology for reporting capsule endoscopy procedures. *Endoscopy* 2005; **37**: 951-959

12 **Goldstein JL**, Eisen GM, Lewis B, Gralnek IM, Aisenberg J, Bhadra P, Berger MF. Small bowel mucosal injury is reduced in healthy subjects treated with celecoxib compared with ibuprofen plus omeprazole, as assessed by video capsule endoscopy. *Aliment Pharmacol Ther* 2007; **25**: 1211-1222

13 **Nakamura M**, Niwa Y, Ohmiya N, Goto H. Protective effect of rebaminide for dictofenac-induced small intestinal mucosal injury: A cross over randomized, double blind, placebo-controlled study. 6th ICCE Report, 2007: 70

14 **Voderholzer W**, Maiden L, Adler S.N, Thjodleifsson B, Lochs H, Bjarnason I. Inter observer variability of wireless capsule endoscopy (WCE) in patients with Crohn's disease and NSAID enteropathy. 6th ICCE Report, 2007: 13

15 **Toth E**, Keuchel M, Riemann JF. Intestinal lymphangiectasia. In: Keuchel M, Hagenmüller F, Fleischer D. Atlas of video capsule endoscopy. Heildelberg: Springer, 2006: 101-106

16 **Mihaly E**, Nemeth A, Zagoni T, Nemet A, Werling K, Racz I, Tulassay Z. Gastrointestinal manifestations of common variable immunodeficiency diagnosed by video- and capsule endoscopy. *Endoscopy* 2005; **37**: 603-604

17 **Bailey AA**, Debinski HS, Appleyard MN, Remedios ML, Hooper JE, Walsh AJ, Selby WS. Diagnosis and outcome of small bowel tumors found by capsule endoscopy: a three-center Australian experience. *Am J Gastroenterol* 2006; **101**: 2237-2243

18 **Caspari R**, von Falkenhausen M, Krautmacher C, Schild H, Heller J, Sauerbruch T. Comparison of capsule endoscopy and magnetic resonance imaging for the detection of polyps of the small intestine in patients with familial adenomatous polyposis or with Peutz-Jeghers' syndrome. *Endoscopy* 2004; **36**: 1054-1059

19 **Schulmann K**, Hollerbach S, Kraus K, Willert J, Vogel T, Moslein G, Pox C, Reiser M, Reinacher-Schick A, Schmiegel W. Feasibility and diagnostic utility of video capsule endoscopy for the detection of small bowel polyps in patients with hereditary polyposis syndromes. *Am J Gastroenterol* 2005; **100**: 27-37

20 **Flieger D**, Keller R, May A, Ell C, Fischbach W. Capsule endoscopy in gastrointestinal lymphomas. *Endoscopy* 2005; **37**: 1174-1180

21 **Thompson B**, Salzman D, Steinhauer J, Lazenby AJ, Wilcox CM. Prospective endoscopic evaluation for gastrointestinal graft-versus-host disease: determination of the best diagnostic approach. *Bone Marrow Transplant* 2006; **38**: 371-376

22 **Neumann S**, Schoppmeyer K, Lange T, Wiedmann M, Golsong J, Tannapfel A, Mossner J, Niederwieser D, Caca K. Wireless capsule endoscopy for diagnosis of acute intestinal graft-versus-host disease. *Gastrointest Endosc* 2007; **65**: 403-409

23 **Yakoub-Agha I**, Maunoury V, Wacrenier A, Couignoux S, Depil S, Desreumaux P, Bauters F, Colombel JF, Jouet JP. Impact of Small Bowel Exploration Using Video-Capsule Endoscopy in the Management of Acute Gastrointestinal Graft-versus-Host Disease. *Transplantation* 2004; **78**: 1697-1701

24 **Delvaux M**, Gay G. Real-Time Viewing of Capsule Endoscopy Recordings: Principle and Clinical Potential. Techn. *Gastrointest Endosc* 2006; **8**: 160-163

25 **Eisen GM**. Using capsule endoscopy to diagnose graft-versus-host disease: seeing is believing? *Gastrointest Endosc* 2007; **65**: 410-411

26 **Terdiman JP**, Linker CA, Ries CA, Damon LE, Rugo HS, Ostroff JW. The role of endoscopic evaluation in patients with suspected intestinal graft-versus-host disease after allogeneic bone-marrow transplantation. *Endoscopy* 1996; **28**: 680-685

27 **Shapira M**, Adler SN, Jacob H, Resnick IB, Slavin S, Or R. New insights into the pathophysiology of gastrointestinal graft-versus-host disease using capsule endoscopy. *Haematologica* 2005; **90**: 1003-1004

28 **Gay G**, Delmotte JS. Abeta and hypobetalipoproteinemias in Atlas of Enteroscopy. Rossini FP, Gay G (Eds). Milan: Springer, 1998: 119-120

29 **Gay G**, Barth E, Keuchel M. Involvement of the small intestine in systemic disease. In Keuchel L, Hagenmüller F, Fleischer D. Atlas of video capsule endoscopy. Heildelberg: Springer, 2006: 136-144

30 **Lie JT**. Nomenclature and classification of vasculitis: plus ca change, plus c'est la meme chose. *Arthritis Rheum* 1994; **37**: 181-186

31 **Jennette JC**, Falk RJ, Andrassy K, Bacon PA, Churg J, Gross WL, Hagen EC, Hoffman GS, Hunder GG, Kallenberg CG. Nomenclature of systemic vasculitides. Proposal of an international consensus conference. *Arthritis Rheum* 1994; **37**: 187-192

32 **Muller-Ladner U**. Vasculitides of the gastrointestinal tract. *Best Practice Res Gastroenterol* 2001; **15**: 59-82

33 **Fylyk S**, Fylyk SN, Safatle-Riberio AV, Neves F, Goncalves CR, Sipahi AM, Ishiok AS, Sakai P, Galvao-Neto M. Small intestine involvement in Behçet's disease: A capsule endoscopy approach. 6 th ICCE Report, 2007: 19

34 **Sanchez R**, Aparicio JR, Baeza T, Calero Y. Capsule endoscopy diagnosis of intestinal involvement in a patient with Churg-Strauss syndrome. *Gastrointest Endosc* 2006; **63**: 1082-1084

35 **Skogestad E**. Capsule endoscopy in Henoch-Schonlein purpura. *Endoscopy* 2005; **37**: 189

36 **Tang SJ**, Zanati S, Kandel G, Marcon NE, Kortan P. Gastric intestinal vascular ectasia syndrome: findings on capsule endoscopy. *Endoscopy* 2005; **37**: 1244-1247

37 **Keuchel M**, Soares J, Reddy DN. Infectious diseases of the small intestine. In: Keuchel M, Hagenmüller F, Fleischer DE. Atlas of video capsule endoscopy. Heildelberg: Springer, 2006: 126-135

38 **Bramwell NH**, Davies RA, Koshal A, Tse GN, Keon WJ, Walley VM. Fatal gastrointestinal hemorrhage caused by cytomegalovirus duodenitis and ulceration after heart transplantation. *J Heart Transplant* 1987; **6**: 303-706

39 **Gupta R**, Reddy D. Infections diseases of the small intestine. Techn. *Gastrointest Endosc* 2006; **8**: 182-187

40 **Reddy DN**, Sriram PV, Rao GV, Reddy DB. Capsule endoscopy appearances of small-bowel tuberculosis. *Endoscopy* 2003; **35**: 99

41 **Fritscher-Ravens A**, Swain CP, von Herbay A. Refractory Whipple's disease with anaemia: first lessons from capsule endoscopy. *Endoscopy* 2004; **36**: 659-662

42 **Gay G**, Roche JF, Delvaux M. Capsule endoscopy, transit times, and Whipple's disease. *Endoscopy* 2005; **37**: 272-273

S- Editor Zhong XY **L- Editor** Anand BS **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5245

World J Gastroenterol 2008 September 14; 14(34): 5245-5253
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Miguel Angel Muñoz-Navas, Profesor, *Series Editors*

Capsule endoscopy in neoplastic diseases

Marco Pennazio, Emanuele Rondonotti, Roberto de Franchis

Marco Pennazio, Division of Gastroenterology 2, Department of Gastroenterology and Clinical Nutrition, S. Giovanni A.S. Hospital, Via Cavour 31, Turin 10123, Italy
Emanuele Rondonotti, Roberto de Franchis, University of Milan, IRCCS Policlinico, Mangiagalli, Regina Elena Foundation, Gastroenterology and Gastrointestinal Endoscopy Unit, Via Pace 9, Milan 20100, Italy
Correspondence to: Dr. Marco Pennazio, Division of Gastroenterology 2, Department of Gastroenterology and Clinical Nutrition, S. Giovanni A.S. Hospital, Via Cavour 31, Turin 10123, Italy. pennazio.marco@gmail.com
Telephone: +39-11-6333594 Fax: +39-11-6333623
Received: February 15, 2007 Revised: July 26, 2008
Accepted: August 2, 2008
Published online: September 14, 2008

Abstract

Until recently, diagnosis and management of small-bowel tumors were delayed by the difficulty of access to the small bowel and the poor diagnostic capabilities of the available diagnostic techniques. An array of new methods has recently been developed, increasing the possibility of detecting these tumors at an earlier stage. Capsule endoscopy (CE) appears to be an ideal tool to recognize the presence of neoplastic lesions along this organ, since it is non-invasive and enables the entire small bowel to be visualized. High-quality images of the small-bowel mucosa may be captured and small and flat lesions recognized, without exposure to radiation. Recent studies on a large population of patients undergoing CE have reported small-bowel tumor frequency only slightly above that reported in previous surgical series (range, 1.6%-2.4%) and have also confirmed that the main clinical indication to CE in patients with small-bowel tumors is obscure gastrointestinal (GI) bleeding. The majority of tumors identified by CE are malignant; many were unsuspected and not found by other methods. However, it remains difficult to identify pathology and tumor type based on the lesion's endoscopic appearance. Despite its limitations, CE provides crucial information leading in most cases to changes in subsequent patient management. Whether the use of CE in combination with other new diagnostic (MRI or multidetector CT enterography) and therapeutic (Push-and-pull enteroscopy) techniques will lead to earlier diagnosis and treatment of these neoplasms, ultimately resulting in a survival advantage and in cost savings, remains to be determined through carefully-designed studies.

Key words: Capsule endoscopy; Enteroscopy; Obscure gastrointestinal bleeding; Small-bowel tumors; Polyposis syndromes

Peer reviewer: Klaus R Herrlinger, Hepatology and Endocrinology, Robert-Bosch-Hospital, Auerbachstrasse. 110, D-70376 Stuttgart, Germany

Pennazio M, Rondonotti E, de Franchis R. Capsule endoscopy in neoplastic diseases. *World J Gastroenterol* 2008; 14(34): 5245-5253 Available from: URL: http://www.wjgnet.com/1007-9327/14/5245.asp DOI: http://dx.doi.org/10.3748/wjg.14.5245

INTRODUCTION

Tumors of the small intestine present a unique challenge to the clinicians across medical specialties. Although the small bowel represents 75% of the length and 90% of the overall mucosal surface of the alimentary tract and despite its anatomic location between two regions of high cancer risk, the small bowel is generally considered as a rare location for the development of neoplasms, accounting for only 1%-3% of all primary gastrointestinal (GI) tumors[1-3].

The overall age-adjusted incidence of small-bowel cancers estimated in population based studies in Western countries ranges between 0.9 and 1.4 (Table 1)[1,4-9]; malignant tumors account for about one half of all new cases of small-bowel tumors reported[10]. The incidence rate of small-bowel cancer varies among populations: cancer rates are high among the Maori of New Zealand (about 4 cases per 100 000 per year) and among ethnic Hawaiians, and low in India, Romania, and other parts of Eastern Europe[1]. Some recently published studies reported an increasing incidence of these neoplasms over the last 20 years (Figure 1)[1,9].

Because small-bowel tumors are relatively rare compared with other neoplasms of the gastrointestinal tract, several factors have been proposed to explain or understand this disparity: (1) a quick transit allowing only short contact of possible carcinogens from food

Table 1 Incidence of small-bowel tumors (modified from Neugut *et al*[11])

Population/area	Ref.	Time interval	Cases of SB tumor	Incidence per million
Los Angeles County	4	1972-1985	264	-
Nine SEER Registers	5	1973-1982	366	9.6
Cancer register of British Columbia, Alberta, Saskatchewan, Manitoba	6	1975-1989	263	11
Utah Cancer registry	7	1966-1999	442	14
Nine SEER registers	8	1973-1991	892	13
Connecticut Tumor registry	9	1980-2000	1260	8.8

SEER: Surveillance epidemiology and end result.

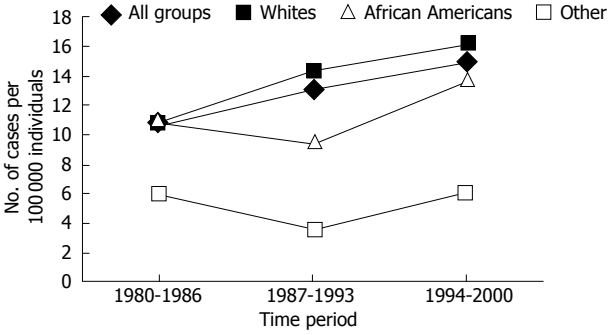


Figure 1 Incidence of small-bowel tumors per race in the Connecticut tumor registry per periods of 7 years since 1980 to 2000[5].

with the intestinal mucosa; (2) the intestinal content is mixed together with a great volume of intestinal juices decreasing the concentration of irritating agents; (3) a decrease in mechanical and/or chemical inflammation of the mucosa because of the liquidity and alkaline pH of the small-bowel contents; (4) the high concentration of lymphatic tissue and of immunoglobulin exerts an effective immune surveillance; (5) the low bacteria concentration in the small intestine processing the intestinal content produces a low amount of carcinogens; (6) the rapid turnover of epithelial cells should decrease the potential growth and development of neoplastic cells[1,10,11].

Genetics could also play a role in some particular subgroups of patients; subjects affected by familiar adenomatous polyposis, hereditary non-polypoid colorectal cancer, Crohn's disease, celiac disease, Peutz-Jeghers syndrome, and several other diseases must be surveyed for the risk of small intestine tumor[9,12]. A relevant role of genetics has also been described in patients with sporadic gastrointestinal stromal tumors (GISTs) in which four different regions (exon 9, exon 11, exon 13, and exon 17) of the *KIT* gene have found to be mutated[13].

Approximately 40 different histological types of small intestinal tumors have been identified[14]. Among malignant tumors, about 30%-50% are adenocarcinomas, 25%-30% are carcinoids, and 15%-20% are lymphomas. A recently published study, including 1260 cases of small-bowel tumor, showed that they seem to be frequently located in the ileum (about 30% of cases) or in the duodenum (about 25% of cases)[9]; the sites at highest risk for malignant neoplasms have been reported to be the duodenum for adenocarcinomas and the ileum for carcinoids and lymphomas[1]. One reason why adenocarcinomas tend to arise in the duodenum may implicate bile or its metabolites in the etiology of the neoplasm at this site[15]. However, among patients with Crohn's disease, which generally affects the ileum rather than the more proximal small bowel, adenocarcinomas tend to occur in the terminal ileum[1].

Secondary neoplastic involvement of the small intestine has been reported to be more frequent than primary small intestinal neoplasms. Primary tumors of the colon, ovary, uterus, and stomach can involve the small bowel (by direct invasion or by intraperitoneal spread) whereas primaries from breast, lung, and melanoma metastasize to the small bowel by the hematogenous route[16]. SB metastases from melanoma have been described in 1.5%-4.4% of patients[17,18] with previously removed skin melanoma and in 58% of post-mortem specimens[17].

In the majority of cases, the diagnosis of small-bowel tumors is delayed. This could be due to several factors: (Ⅰ) Small-bowel tumors grow slowly, extraluminally, remaining asymptomatic for years or presenting insidiously with non-specific complaints such as abdominal pain, diarrhea, iron deficiency anemia, bleeding, extra intestinal symptoms (flushing, paraneoplastic syndromes)[19]. Obstruction is also a common presentation; indeed, small-bowel tumors are the third most common cause of small-bowel obstruction in the United States[20]. (Ⅱ) The rare incidence of small-bowel tumors may contribute to the relatively low index of clinical suspicion for their presence.(Ⅲ) Routine laboratory tests and other diagnostic tests may frequently be inconclusive; as a consequence, diagnostic laparoscopy or exploratory laparotomy may be indicated not only to deliver an effective treatment but also to reach a definitive diagnosis.

Since the introduction in clinical practice of capsule endoscopy, several case reports describing primary and secondary tumors affecting the small bowel have been published. More recently, a few retrospective studies collecting series of patients in which this technology was able to show the presence of a small-bowel tumor have also been published.

SMALL-BOWEL TUMORS: DIAGNOSTIC TOOLS

Historically the small bowel has been considered a difficult organ to evaluate. For many years, visualization of the small-bowel mucosa and the diagnosis of small-bowel tumors were feasible only in a surgical setting and this organ has been considered a sort of "black box". This situation derived both from the anatomical characteristics of the small bowel and the limitations of available techniques. The length of the small intestine,

the distance between the organ and external orifices (mouth and anus), its sinuousness, its ability to produce huge amounts of fluids and the continuous contractions long hampered accurate inspection of the small-bowel mucosa.

Traditional radiological techniques, including small-bowel follow-through and small-bowel enteroclysis, allow an indirect evaluation of the entire small bowel, however the difficulty to place a specific catheter in the right position (enteroclysis), the low pressure and the dilution of the contrast medium (small-bowel follow-through) contribute to a high miss rate for small and/or flat lesions. Conventional cross-sectional imaging techniques, such as computed tomography (CT) and magnetic resonance imaging (MRI), can be helpful in identifying large small-bowel masses or extraintestinal disease, but are unable to provide precise data about the intestinal wall. Endoscopy has the advantage of visualizing intestinal mucosa directly and, above all, of carrying out targeted biopsies. Upper GI endoscopy, when performed to the ligament of Treitz, is suited for identifying duodenal tumors; however, lesions located distally pose a unique diagnostic challenge. Push enteroscopy (PE) is an effective diagnostic and therapeutic procedure which entails the oral insertion of a dedicated enteroscope; however, it only allows thorough examination of the distal duodenum and proximal jejunum to approximately 50-100 cm beyond the ligament of Treitz. Because of its ability to examine the entire small intestine, sonde enteroscopy has been utilized to diagnose small-bowel tumors[21]. However, this technically-challenging procedure has been today completely abandoned. Intraoperative enteroscopy (IOE) is the most complete, but also the most invasive means of examining the small bowel. It is a difficult, time-consuming technique, often traumatic to the bowel, with a substantial risk of complications and even mortality.

The development and the introduction in the clinical practice of the capsule endoscopy (CE) has revolutionized the field of small-bowel imaging, not only opening up this sort of "Pandora's box", but also stimulating the development of other imaging techniques aimed at studying the small bowel.

Magnetic resonance enteroclysis (MRI-enteroclysis) combines the advantages of cross-sectional resonance with those of volume challenge of conventional enteroclysis in the detection and characterization of small-bowel wall abnormalities, such as initial neoplasms. Small-bowel tumors usually exhibit moderate signal intensity on true-FISP images, as opposed to the high signal intensity of the distended lumen and the mesenteric fat. Post-gadolinium 3D FLASH with fat saturation may be the most important sequence for the identification and characterization of small-bowel tumors by their enhancement pattern. The degree of prestenotic dilatation, the peritoneal extension of the neoplasm and associated lymphadenopathy is well visualized in all MRI-enteroclysis sequences[22,23]. The multidetector row computed tomography (MRCT) has the potential to provide high-resolution images and a precise delineation of pathology. The multiplanar reformatted images obtained using MRCT have spatial resolution similar to that of the axial plane without any loss of information. These advantages of MDCT imaging lead to a more accurate demonstration of the site of the tumor and possible complications of underlying small-bowel tumors including small-bowel obstruction, intussusception, perforation and bleeding[24]. The administration of methyl-cellulose as a neutral luminal contrast material in a 4%-15% water-soluble solution or a diluted (1%) barium solution as positive luminal contrast in patients undergoing MRCT results in a computed tomography enteroclysis (CT-enteroclysis). As previously described for MRI-enteroclysis, this technique combines the advantages of enteral volume challenge and the ability of cross-sectional imaging to depict extra intestinal manifestations of the disease[25]. Both MRI enteroclysis and CT-enteroclysis require the placement of a specific catheter into the third part of the duodenum (fluoroscopic monitored), administration of medications (anti-motility agents and sedative medications) and small-bowel preparation with laxatives (PEG-based solutions, 2 to 4 L)[24]. Up to now, there are only few, but promising, publications about the role of these three techniques in the diagnostic algorithm of small-bowel tumors.

In the attempt to design a new scope that would allow a large part of the small-bowel mucosa to be visualized, overcoming the limits of PE and IOE, Yamamoto *et al*[25] developed a new method of push-and-pull enteroscopy (PPE) using a double-balloon technique. PPE affords inspection of the entire small bowel, combining the oral and anal approaches, with the advantage of enabling biopsies and endoscopic interventions to be performed in all parts of the small bowel without laparotomy. It is, however, invasive, time-consuming, and requires conscious sedation[26].

CAPSULE ENDOSCOPY IN THE DIAGNOSIS OF SMALL-BOWEL TUMORS

In a recently published paper, the hypothesis of an increased incidence of small-bowel tumors in recent years was put forward, based on the increasing number of cases diagnosed by means a non-invasive methods such as CE and small-bowel ultrasound[11]. In fact, compared with previously mentioned diagnostic techniques for the study of the small bowel, CE seems to be an ideal tool to recognize the presence of neoplastic lesions along the small bowel. The potential of CE for the diagnosis of small-bowel tumors, as well as for the surveillance of subjects at increased risk of developing them, depends largely on the technical characteristics of this diagnostic device. CE is a non-invasive tool, well accepted by patients, who can allow the visualization of the entire small bowel; high-quality images of the small-bowel mucosa may be captured and small and flat lesions recognized, without exposure to radiation.

In fact, since the introduction of CE in clinical

Table 2 Summary of CE studies for small-bowel tumors

Study[ref]	Population	Tumor cases (%)	Mean age of patients with tumors (yr)	Malignant tumors (%)	Tumors leading to capsule retention (%)
Cobrin *et al*[28]	562	50 (8.9)	63	48	0
Bailey *et al*[29]	416	27 (6.3)	61	63	3/26 (11.5)
Urbain *et al*[31]	443	11 (2.5)	63	100	0
Estevez *et al*[30]	320	23 (7.8)	63	NA	NA
Schwartz *et al*[32]	NA	87 (NA)	60	60	NA
Pasha *et al*[33]	1000	16 (1.6)	67	86	4/16 (25)
Rondonotti *et al*[34]	5129	124 (2.4)	59	NA	12/124 (9.7)

NA: Not applicable (these data are not reported in the paper).

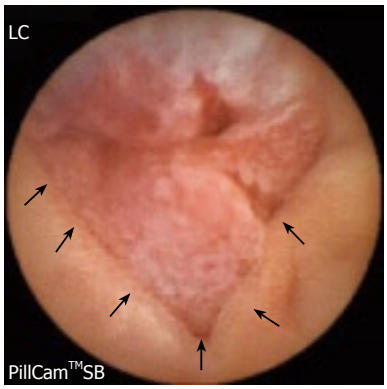


Figure 2 Infiltrating, stenotic, mass (arrows) in the ileum in a patient with hereditary non-polyposis colorectal cancer syndrome. Histology revealed an adenocarcinoma.

practice, some studies have been published[27-32] reporting a frequency of small-bowel tumors higher than previously expected, ranging between 3.6% and 9%. All these studies were retrospective; each of them collected about 350-500 patients undergoing CE, described the frequency of small-bowel tumors in a highly selected group of patients with symptoms (obscure GI bleeding in the majority of cases) and sometimes the diagnosis was based only on the endoscopic images (one study[30] reported 35% of lesions described as tumor without histological confirmation). Two recent studies, coming from the USA and Europe, only published in abstract form[33,34], examined a large population of patients undergoing CE (respectively, 1000[33] and more than 5000[34]) in whom the definitive diagnosis was confirmed by means of tissue sampling (Table 2). They reported a small-bowel tumor frequency only slightly above that reported in previous surgical series, ranging from 1.6% to 2.4%, and also confirmed that the main clinical indication to CE in patients with small-bowel tumors is obscure GI bleeding (in about 90% of cases). Other indications for CE in these two studies were: chronic diarrhea, abdominal pain, para-neoplastic syndromes or, in a small group of patients, presence of conditions increasing the risk to develop a small-bowel tumor (such as refractory celiac disease, familial adenomatous polyposis or Peutz-Jeghers syndrome). In some rare cases CE was also used to confirm the presence of a tumor previously suspected by other imaging modalities. Although Cobrin *et al*[28] underlined that in their study the percentage of patients with tumor was greater among patients younger than 50 years, the median age of patients enrolled the above mentioned large studies ranged between 59[34] and 67 years[33] (Table 2).

Confirming data previously reported in surgical series[9,10] the majority of tumors identified by CE (from 63%[29] to 86%[33]) are malignant neoplasms and the most frequent histological types are adenocarcinomas and carcinoids (in about 20% of cases each[28,29,32]), while GISTs represent the most frequently identified benign neoplasm. Of note, this tumor accounted for more than one third of all collected cases in the large multi-center European study[34]. As far as small-bowel metastases are concerned, these lesions mainly (about 1/3 of cases[34]) derived from previously removed skin melanomas[35], but there are also some papers reporting lesions derived from colorectal cancers[29], from hepatocellular carcinoma or from rare tumors such as seminomas[34].

Small-bowel tumors appear at CE as masses or polyps in about 70%-80% of cases[28-34] and as ulcers (sometimes actively bleeding) or stenoses in 20%-30% of cases (Figure 2). Unfortunately, it is very difficult to identify pathology and tumor type based on the capsule endoscopic appearance of lesions[36]. These tumors are mostly located in the jejunum, 40%-60% of cases, in the ileum, 25%-40% of cases, and less frequently in the duodenum, in 15%-20% of cases[28-34]. The location of the majority of lesions in the mid-small bowel could be a partial explanation of the extensive (and mainly negative) diagnostic work-up performed in patients enrolled in all these studies. Each patient underwent a mean of 2%-4.6%[29,32] examinations before CE while, focusing only on exams addressed to evaluate the small bowel (particularly small-bowel series and/or small-bowel follow-through and/or PE and/or CT-enteroclysis), the mean number of examinations performed per patient ranged between 1 and 2[28,29,32]. Despite the extensive number of examinations performed before CE, this technique was found to have a positive impact on diagnosis (defined as the capability to identify a neoplasm not shown by other diagnostic techniques or as the ability to provide crucial information leading to change the subsequent patient management) in about 65%-80% of cases[31,34]. Urbain *et al*[31], trying to evaluate the impact of CE on the therapeutic choices of malignant small-bowel tumors, found that CE may influence directly the therapeutic work-up in about 55% of cases by providing

information about size, location and appearance of the lesion.

Because the early diagnosis and treatment of cancer usually affects outcome, some authors[28,29] suggest that the capability of CE to discover small-bowel tumors at an early stage may have an impact on prognosis for patients with these lesions. All the papers previously mentioned reported that in patients with small-bowel neoplasm identified by CE, surgery alone or surgery plus chemotherapy is the treatment of choice in about 85%-90% of the cases[28-30,33,34] but, to date, there is only one published paper describing the follow-up of these patients. Bailey *et al*[29] reported that surgical treatment was performed in 88% of patients with small-bowel tumor, in half of the cases with curative aim. None of the patients who underwent a curative resection developed tumor recurrence at follow-up (range, 26-51 mo). These authors also reported that none of the patients with benign tumors discovered by CE and treated according to CE findings had recurrence of either overt or occult obscure GI bleeding at follow-up (3-51 mo).

CAPSULE ENDOSCOPY FOR SPECIFIC SMALL-BOWEL TUMORS

Thanks to its capability to identify a small-bowel lesion in most patients with a prior negative diagnostic work-up, several case reports, but also some small series, aimed at evaluating the possible role of the CE in the diagnosis of specific tumors in particular clinical conditions, have been published over the last few years.

Van Tuyl *et al*[37], in a prospective descriptive study, evaluated 20 patients with liver metastases, mesenteric metastases or both, originated from a neuroendocrine tumor (NET) with unknown primary location. All these patients had undergone several examinations including small-bowel enteroclysis, abdominal CT, pentetreotide scintigraphy and laboratory tests. In this particular subset of patients, CE showed a diagnostic yield (60%) significantly higher than enteroclysis and CT scan. Pentetreotide scintigraphy had an even higher diagnostic yield than CE, but without differentiation between intestinal and mesenteric localization. In this study, the absence of findings at CE in patients with abnormalities at nuclear imaging was interpreted to be related to the presence of NET restricted to the mesentery or to a false-negative CE. On the ground of these data, the authors suggested that patients with a metastatic NET and an unknown primary tumor should undergo CE. Conversely, in a small retrospective study of 8 patients[38], CE detected NETs of the small bowel with high specificity, but slightly lower sensitivity than did CT enteroclysis. It was concluded that CE should not be used as a routine method for diagnosing NET in the small bowel.

As far as small-bowel metastases are concerned, Prakoso and Selby[35] performed a retrospective analysis of a prospective database identifying 13 patients with previous or recurrent malignant melanoma referred for CE. The indication for CE were overt GI bleeding in three patients, anemia in six, abnormal imaging in two, abdominal pain in one, and one patient had positive fecal occult blood test. In these patients, CE was able not only to show small-bowel metastases (in 5 patients), but also to provide a different possible explanation of symptoms in three other patients (NSAID-related ulcers, artero-venous malformation or aphtoid lesions). The authors concluded that since the optimal investigation for the detection of small-bowel metastases in patients with melanoma has still to be determined, CE can be considered an ideal method to do so because it appears to be more sensitive than small-bowel follow-through and CT scan.

Flieger *et al*[39] explored the potential contribution of CE to the diagnosis and staging of gastrointestinal lymphomas describing capsule endoscopic features of these tumors. They studied with CE a total of 27 consecutive patients with newly diagnosed gastrointestinal lymphoma: 20 patients with histologically confirmed gastric lymphoma and seven patients with intestinal lymphoma. All seven patients with primary intestinal lymphomas were found to have pathological findings at CE (ulcerations, nodules or villous atrophy), while 5 of the 20 patients with gastric lymphoma had pathological findings in the small bowel (including abnormal villi, white nodules or villous atrophy). In this study, the authors found that CE is able to identify pathological intestinal findings in patients with gastrointestinal lymphoma more frequently than previously thought and suggest that knowledge of small-bowel involvement can lead to changes in the therapeutic strategy in individual cases.

Lymphomatous polyposis (LP), first described by Cornes in 1961[40,41], is a rare condition; however, since the introduction of CE and PPE in clinical practice, a few reports[42,43] have been published on this topic. LP is defined as polypoid mucosal involvement of long segments of the GI tract by neoplastic lymphoid cells[40]. For many years LP has been considered the macroscopic appearance of the mantle cell lymphoma, but it has recently been suggested that it can be also the macroscopic manifestation of mucosa-associated lymphoid tissue (MALT) lymphoma and follicular B cell lymphoma[44]. In patients with LP, CE is a valuable tool because it may recognize the presence of nodules, evaluate the extent of the small-bowel involvement and drive further investigations (i.e. the decision about the PPE approach).

Another peculiar clinical condition is represented by patients with refractory celiac disease. It is known that these patients have an increased risk to develop small-bowel neoplasms, mainly enteropathy associated T-cell lymphoma (EATL). However, in this particular subgroup of patients CE is aimed at identifying not only a malignant neoplasm, but also some other possible complications such as ulcerative jejunitis. To date, two papers have been published on this topic[45,46] showing that CE is a useful tool in the assessment of complicated

celiac disease, especially in patients with refractory celiac disease type Ⅱ[45].

CAPSULE ENDOSCOPY: RISKS AND LIMITATIONS IN PATIENTS WITH SMALL-BOWEL TUMORS

Several papers[47-49] described risks and limitations related to the use of CE in everyday clinical practice. Some limitations can be present in any procedure performed regardless of the clinical indication ("general limitations"); these limitations are mainly related to the technical characteristics of the device or to the anatomical structure of the small bowel, for example, due to the duration of battery life (about 8 h), the capsule allows an evaluation of the entire small bowel only in 75%-85%[47,49] of cases. In addition, sometimes the presence of fecal debris, particularly in the distal small bowel, can hamper the accurate visualization of the small-bowel mucosa.

Among general limitations, capsule retention is certainly the more feared one because it can significantly modify the subsequent management of the patient. It is generally recognized that the frequency of capsule retention is mostly dependent on the clinical indication to CE (Table 3), ranging between 0% in healthy subjects to 21% in patients with intestinal obstruction[50,51]. Patients with small-bowel tumors, which frequently appear as lesions protruding into the small-bowel lumen or as stenoses, in both cases capable of narrowing the lumen of the small bowel, have a high probability to develop capsule retention. However, although capsule retention at the site of the lesion has been described in 10%[29,34] to 25%[33] of these patients (Table 2), most authors consider this situation as a minor complication. In fact, although some case reports describing possible acute obstructions due to capsule retained at the site of the tumor[52,53] exist, none of the 15 patients with capsule retention described in large published series[29,33,34] developed acute small-bowel obstruction. In these patients the subsequent surgical intervention, allowing capsule retrieval, was planned basically to treat the tumor (or because of symptoms persistence) rather than to retrieve the capsule. We must also keep in mind that surgical intervention aimed to retrieve the capsule can be done in a laparoscopic way[54] and that PPE can also allow capsule retrieval when surgical intervention is contraindicated or not feasible[55]. In addition, the recently developed Patency capsule[56] (given imaging, Yoqneam, Israel) can be used in selected patients as a screening method to prevent capsule retention.

The capsule can also have some problems in sizing lesions because of the shape of its dome, its magnification capability, the lack of air insufflation and of remote orientation. This issue has recently been highlighted in papers addressed to study patients with small-bowel inherited polyposis syndromes[57,58] in which the authors found that MRI seems to be more accurate and reliable than CE in the estimation of location and size of polyps[58]. The ingestion of "reference granules" of mesalazine 15-20 min before CE has recently been proposed to increase the accuracy of the procedure[59].

Table 3 Frequency of capsule retention in patients undergoing capsule endoscopy (modified from Pennazio[50])

Clinical indication	Frequency of capsule retention (%)
Healthy volunteers	0
Obscure GI bleeding	1.5
Suspected Crohn's disease	1.4
Known Crohn's disease	4-13
Small-bowel tumor	10-25
Suspected small-bowel obstruction	21

Another general limitation, that can be critical in the field of small-bowel tumors, is the accurate localization of the lesion along the small bowel. To estimate the location of a lesion we can correlate the time when the lesion appears to the small-bowel transit time divided in three equal thirds[60], or we can refer to the localization system[61]; both these systems are time-consuming, depend on some reference points established by the reader, are not suitable when the capsule does not reach the ileo-cecal valve during examination time and the localization software is reliable only considering a two dimension plan. Despite all these obvious limitations, in one large study[33] the capsule was able to correctly estimate the location of the lesion in a surprisingly high percentage of patients (about 85%).

Unfortunately, in the field of small-bowel neoplasms, in addition to these general limitations there are some other related to the intrinsic characteristics of these lesions ("tumor-related limitations").

Several studies[30,62-64] reported patients with negative CE in whom further examinations showed small-bowel tumors (false negative capsule endoscopy). Lewis *et al*[63], analyzing data from an industry-maintained trial database, found that in about 1.5% of patients with small-bowel tumors CE was completely negative. These authors estimated that the miss rate of CE in neoplastic diseases can reach 18.9%. Although this percentage is substantially lower than that reported in the same paper for other diagnostic techniques (63.2%) it remains still alarming, especially if one keeps in mind the clinical relevance of these missing findings. Obviously, there are several reasons contributing to that miss rate, but probably the crucial one is related, in this particular subset of patients, to the fact that sometime it is arduous, on the ground of CE findings, to discriminate masses from bulges. A bulge is defined as a round smooth, large base protrusion in the lumen having an ill defined edge on the surrounding mucosa; it can be a prominent normal fold or the luminal expression of intestinal loop angulation and stiffness, and sometimes it can be virtually indistinguishable from a small submucosal tumor. Some visual clues may help distinguishing masses from bulges (i.e. changes in mucosal characteristics, presence of bridging folds, of transit abnormalities, of repetitive images, and of synchronous lesions), but unfortunately all these are indirect indicators and often

are completely lacking.

Pasha *et al*[33] described 51 patients with polypoid lesions revealed at CE that were not confirmed at further examinations (false positive capsule endoscopy). This problem, highlighted also in other studies[30], can significantly influence the subsequent management; in fact a positive CE requires further invasive examinations (PPE or surgical interventions). For this reason, the final interpretation of a finding identified by CE must be done taking into account not only the endoscopic images, but also the patient's clinical history and other diagnostic examinations performed.

CAPSULE ENDOSCOPY IN INHERITED POLYPOSIS SYNDROMES

On the ground of its own technical characteristics (i.e. high-quality endoscopic images of the whole small bowel, no need for radiations) and of the patients' acceptance, CE has also been proposed in patients with inherited polyposis syndromes for both surveillance over time and in case of symptomatic disease.

In Peutz-Jeghers syndrome (PJS) the polyps are chiefly located in the small bowel (Figure 3) and may give rise to complications in the form of intussusception, bleeding and obstruction of the intestine, depending on the number and size of the polyps present, as well as to small-bowel malignancy. Several studies have explored the possible diagnostic role of CE in these patients[57,58,65,66] showing that this tool seems to be superior to small-bowel follow-through[57]. Unfortunately, the same studies also underlined that CE (as discussed above) is not reliable for accurate sizing of polyps. At the present time, it is suggested that CE should be performed at diagnosis in all patients with PJS, as the primary surveillance modality every 2-3 years from the age of 10, and as part of the investigation of patients with symptoms[50]. Additional information to evaluate the size and location of polyps, which is useful for planning the appropriate therapeutic strategy, can be provided by CT/MRI[57,58]. The coupling of CE with PPE and polypectomy may offer an ideal follow-up and treatment method for these patients, possibly avoiding surgery[67].

The role of CE is less clear in familial adenomatous polyposis (FAP). CE may miss duodenal/periampullary polyps due to a quick passage of the device in the descending duodenum. In a recently published prospective study, Wong *et al*[68] compared CE with push enteroscopy and with lower GI endoscopy in 32 patients with FAP. They showed that, in a defined segment of the small bowel, CE diagnosed significantly fewer small-bowel polyps than standard endoscopy, showed only fair agreement with PE in determining polyp counts, and was fairly inaccurate in determining the size of the largest polyp and also in detecting large polyps. For these reasons, CE is not presently recommended when the diagnosis of FAP is well established, but it may be considered as a part of surveillance for patients with severe duodenal polyposis (Spigelman stage Ⅲ-Ⅳ; Figure 4)[65,66]. Moreover, in FAP patients with known mesenteric desmoids, caution is recommended before performing CE for the possible risk of capsule retention.

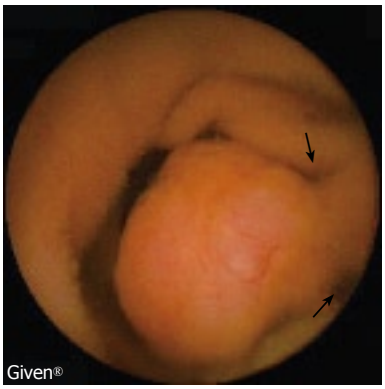


Figure 3 Small sessile, plaque-like, polyp (arrows) in the distal duodenum in a patient with familial adenomatous polyposis.

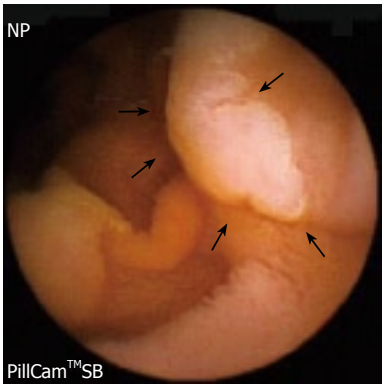


Figure 4 Pedunculated jejunal polyp (arrows denote stalk) in a patient with Peutz-Jeghers syndrome.

CONCLUSION

Small-bowel tumors are a small, but significant proportion of GI neoplasms. Using new diagnostic modalities, their frequency has been shown to be slightly superior than previously thought. Until recently, diagnosis and management of these tumors were delayed by the difficulty of access to the small bowel and the poor diagnostic capabilities of the available diagnostic techniques. An array of new methods has recently been developed, increasing the possibility of detecting these tumors at an earlier stage. Despite its limitations, CE plays a pivotal role in this setting. Whether the use of CE in combination with other new diagnostic (MRI or multidetector CT enterography) and therapeutic (PPE) techniques will lead to earlier diagnosis and treatment of these neoplasms, ultimately resulting in a survival advantage and in cost savings, remains to be determined through carefully-designed studies.

REFERENCES

1 **Neugut AI**, Jacobson JS, Suh S, Mukherjee R, Arber N. The epidemiology of cancer of the small bowel. *Cancer Epidemiol Biomarkers Prev* 1998; **7**: 243-251
2 **Rossini FP**, Risio M, Pennazio M. Small bowel tumors and polyposis syndromes. *Gastrointest Endosc Clin N Am* 1999; **9**: 93-114
3 **DiSario JA**, Burt RW, Vargas H, McWhorter WP. Small bowel cancer: epidemiological and clinical characteristics from a population-based registry. *Am J Gastroenterol* 1994; **89**: 699-701
4 **Ross RK**, Hartnett NM, Bernstein L, Henderson BE. Epidemiology of adenocarcinomas of the small intestine: is bile a small bowel carcinogen? *Br J Cancer* 1991; **63**: 143-145
5 **Weiss NS**, Yang CP. Incidence of histologic types of cancer of the small intestine. *J Natl Cancer Inst* 1987; **78**: 653-656
6 **Gabos S**, Berkel J, Band P, Robson D, Whittaker H. Small bowel cancer in western Canada. *Int J Epidemiol* 1993; **22**: 198-206
7 **Chow JS**, Chen CC, Ahsan H, Neugut AI. A population-based study of the incidence of malignant small bowel tumours: SEER, 1973-1990. *Int J Epidemiol* 1996; **25**: 722-728
8 **Severson RK**, Schenk M, Gurney JG, Weiss LK, Demers RY. Increasing incidence of adenocarcinomas and carcinoid tumors of the small intestine in adults. *Cancer Epidemiol Biomarkers Prev* 1996; **5**: 81-84
9 **Hatzaras I**, Palesty JA, Abir F, Sullivan P, Kozol RA, Dudrick SJ, Longo WE. Small-bowel tumors: epidemiologic and clinical characteristics of 1260 cases from the connecticut tumor registry. *Arch Surg* 2007; **142**: 229-235
10 **Blanchard DK**, Budde JM, Hatch GF 3rd, Wertheimer-Hatch L, Hatch KF, Davis GB, Foster RS Jr, Skandalakis JE. Tumors of the small intestine. *World J Surg* 2000; **24**: 421-429
11 **Kala Z**, Valek V, Kysela P, Svoboda T. A shift in the diagnostics of the small intestine tumors. *Eur J Radiol* 2007; **62**: 160-165
12 **Ashley SW**, Wells SA Jr. Tumors of the small intestine. *Semin Oncol* 1988; **15**: 116-128
13 **Miettinen M**, Lasota J. Gastrointestinal stromal tumors: review on morphology, molecular pathology, prognosis, and differential diagnosis. *Arch Pathol Lab Med* 2006; **130**: 1466-1478
14 **O'Riordan BG**, Vilor M, Herrera L. Small bowel tumors: an overview. *Dig Dis* 1996; **14**: 245-257
15 **Lowenfels AB**. Does bile promote extra-colonic cancer? *Lancet* 1978; **2**: 239-241
16 **Gill SS**, Heuman DM, Mihas AA. Small intestinal neoplasms. *J Clin Gastroenterol* 2001; **33**: 267-282
17 **Das Gupta TK**, Brasfield RD. Metastatic melanoma of the gastrointestinal tract. *Arch Surg* 1964; **88**: 969-973
18 **Reintgen DS**, Thompson W, Garbutt J, Seigler HF. Radiologic, endoscopic, and surgical considerations of melanoma metastatic to the gastrointestinal tract. *Surgery* 1984; **95**: 635-639
19 **Talamonti MS**, Goetz LH, Rao S, Joehl RJ. Primary cancers of the small bowel: analysis of prognostic factors and results of surgical management. *Arch Surg* 2002; **137**: 564-570; discussion 570-571
20 **Fitzgibbons RJ**, Filippi CJ, Quinn TH. Inguinal hernias. In: Brunicardi FC, Andersen DK, Billiar TR, Dunn DL, Hunter JG, Pollock RE. Schwartz's Principles of Surgery. 8th ed. New York: McGraw-Hill, 2005: 230-246
21 **Lewis BS**, Kornbluth A, Waye JD. Small bowel tumours: yield of enteroscopy. *Gut* 1991; **32**: 763-765
22 **Semelka RC**, John G, Kelekis NL, Burdeny DA, Ascher SM. Small bowel neoplastic disease: demonstration by MRI. *J Magn Reson Imaging* 1996; **6**: 855-860
23 **Gourtsoyiannis N**, Papanikolaou N, Grammatikakis J, Prassopoulos P. MR enteroclysis: technical considerations and clinical applications. *Eur Radiol* 2002; **12**: 2651-2658
24 **Ramachandran I**, Sinha R, Rajesh A, Verma R, Maglinte DD. Multidetector row CT of small bowel tumours. *Clin Radiol* 2007; **62**: 607-614
25 **Yamamoto H**, Kita H, Sunada K, Hayashi Y, Sato H, Yano T, Iwamoto M, Sekine Y, Miyata T, Kuno A, Ajibe H, Ido K, Sugano K. Clinical outcomes of double-balloon endoscopy for the diagnosis and treatment of small-intestinal diseases. *Clin Gastroenterol Hepatol* 2004; **2**: 1010-1016
26 **May A**, Nachbar L, Schneider M, Ell C. Prospective comparison of push enteroscopy and push-and-pull enteroscopy in patients with suspected small-bowel bleeding. *Am J Gastroenterol* 2006; **101**: 2016-2024
27 **de Franchis R**, Rondonotti E, Abbiati C, Beccari G, Signorelli C. Small bowel malignancy. *Gastrointest Endosc Clin N Am* 2004; **14**: 139-148
28 **Cobrin GM**, Pittman RH, Lewis BS. Increased diagnostic yield of small bowel tumors with capsule endoscopy. *Cancer* 2006; **107**: 22-27
29 **Bailey AA**, Debinski HS, Appleyard MN, Remedios ML, Hooper JE, Walsh AJ, Selby WS. Diagnosis and outcome of small bowel tumors found by capsule endoscopy: a three-center Australian experience. *Am J Gastroenterol* 2006; **101**: 2237-2243
30 **Estevez E**, Gonzalez-Conde B, Vazquez-Iglesias JL, Alonso PA, Vazquez-Millan Mde L, Pardeiro R. Incidence of tumoral pathology according to study using capsule endoscopy for patients with obscure gastrointestinal bleeding. *Surg Endosc* 2007; **21**: 1776-1780
31 **Urbain D**, De Looze D, Demedts I, Louis E, Dewit O, Macken E, Van Gossum A. Video capsule endoscopy in small-bowel malignancy: a multicenter Belgian study. *Endoscopy* 2006; **38**: 408-411
32 **Schwartz GD**, Barkin JS. Small-bowel tumors detected by wireless capsule endoscopy. *Dig Dis Sci* 2007; **52**: 1026-1030
33 **Pasha SF**, Sharma VK, Carey EJ, Shiff AD, Heigh RI, Gurudu SR, Erickson PJ, Post JK, Hara AK, Fleischer DE, Leighton JA. Utility of video capsule endoscopy in the detection of small bowel tumors. A single center experience of 1000 consecutive patients. Proceedings of the 6th International Conference on Capsule Endoscopy, 2007 June 8-10; Madrid, Spain, 2007: 45
34 **Rondonotti E**, Pennazio M. Small bowel tumors detected by capsule endoscopy (VCE): preliminary data from the ECEG (European Capsule Endoscopy Group) database. *Gastroint Endosc* 2007: **65**: AB90-AB371
35 **Prakoso E**, Selby WS. Capsule endoscopy in patients with malignant melanoma. *Am J Gastroenterol* 2007; **102**: 1204-1208
36 **Pennazio M**. Small-intestinal pathology on capsule endoscopy: tumors. *Endoscopy* 2005; **37**: 1008-1017
37 **van Tuyl SA**, van Noorden JT, Timmer R, Stolk MF, Kuipers EJ, Taal BG. Detection of small-bowel neuroendocrine tumors by video capsule endoscopy. *Gastrointest Endosc* 2006; **64**: 66-72
38 **Johanssen S**, Boivin M, Lochs H, Voderholzer W. The yield of wireless capsule endoscopy in the detection of neuroendocrine tumors in comparison with CT enteroclysis. *Gastrointest Endosc* 2006; **63**: 660-665
39 **Flieger D**, Keller R, May A, Ell C, Fischbach W. Capsule endoscopy in gastrointestinal lymphomas. *Endoscopy* 2005; **37**: 1174-1180
40 **Cornes JS**. Multiple lymphomatous polyposis of the gastrointestinal tract. *Cancer* 1961; **14**: 249-257
41 **Yatabe Y**, Nakamura S, Nakamura T, Seto M, Ogura M, Kimura M, Kuhara H, Kobayashi T, Taniwaki M, Morishima Y, Koshikawa T, Suchi T. Multiple polypoid lesions of primary mucosa-associated lymphoid-tissue lymphoma of colon. *Histopathology* 1998; **32**: 116-125
42 **Higuchi K**, Komatsu K, Wakamatsu H, Kawasaki H, Murata M, Miyazaki K, Oikawa K, Ohwada M, Nanjo H, Otaka M, Watanabe S, Komatsu K. Small intestinal follicular lymphoma with multiple tumor formations diagnosed by double-balloon enteroscopy. *Intern Med* 2007; **46**: 705-709

43 **Esaki M**, Matsumoto T, Nakamura S, Suekane H, Ohji Y, Yao T, Iida M. Capsule endoscopy findings in intestinal follicular lymphoma. *Endoscopy* 2007; **39** Suppl 1: E86-E87

44 **Kodama T**, Ohshima K, Nomura K, Taniwaki M, Nakamura N, Nakamura S, Kohno S, Yamamoto J, Karube K, Yamasita Y, Shirakusa T, Kikuchi M. Lymphomatous polyposis of the gastrointestinal tract, including mantle cell lymphoma, follicular lymphoma and mucosa-associated lymphoid tissue lymphoma. *Histopathology* 2005; **47**: 467-478

45 **Daum S**, Wahnschaffe U, Glasenapp R, Borchert M, Ullrich R, Zeitz M, Faiss S. Capsule endoscopy in refractory celiac disease. *Endoscopy* 2007; **39**: 455-458

46 **Culliford A**, Daly J, Diamond B, Rubin M, Green PH. The value of wireless capsule endoscopy in patients with complicated celiac disease. *Gastrointest Endosc* 2005; **62**: 55-61

47 **Ho KK**, Joyce AM. Complications of capsule endoscopy. *Gastrointest Endosc Clin N Am* 2007; **17**: 169-178, viii-ix

48 **Cheifetz AS**, Lewis BS. Capsule endoscopy retention: is it a complication? *J Clin Gastroenterol* 2006; **40**: 688-691

49 **Rondonotti E**, Herrerias JM, Pennazio M, Caunedo A, Mascarenhas-Saraiva M, de Franchis R. Complications, limitations, and failures of capsule endoscopy: a review of 733 cases. *Gastrointest Endosc* 2005; **62**: 712-716; quiz 752, 754

50 **Pennazio M**. Capsule endoscopy: where are we after 6 years of clinical use? *Dig Liver Dis* 2006; **38**: 867-878

51 **Cave D**, Legnani P, de Franchis R, Lewis BS. ICCE consensus for capsule retention. *Endoscopy* 2005; **37**: 1065-1067

52 **Strosberg JR**, Shibata D, Kvols LK. Intermittent bowel obstruction due to a retained wireless capsule endoscope in a patient with a small bowel carcinoid tumour. *Can J Gastroenterol* 2007; **21**: 113-115

53 **Lin OS**, Brandabur JJ, Schembre DB, Soon MS, Kozarek RA. Acute symptomatic small bowel obstruction due to capsule impaction. *Gastrointest Endosc* 2007; **65**: 725-728

54 **Dominguez EP**, Choi Y, Raijman IL, Sweeney JF. Laparoscopic approach for the retrieval of retained video capsule endoscopy. *JSLS* 2006; **10**: 496-498

55 **Tanaka S**, Mitsui K, Shirakawa K, Tatsuguchi A, Nakamura T, Hayashi Y, Sakamoto C, Terano A. Successful retrieval of video capsule endoscopy retained at ileal stenosis of Crohn's disease using double-balloon endoscopy. *J Gastroenterol Hepatol* 2006; **21**: 922-923

56 **Spada C**, Shah SK, Riccioni ME, Spera G, Marchese M, Iacopini F, Familiari P, Costamagna G. Video capsule endoscopy in patients with known or suspected small bowel stricture previously tested with the dissolving patency capsule. *J Clin Gastroenterol* 2007; **41**: 576-582

57 **Brown G**, Fraser C, Schofield G, Taylor S, Bartram C, Phillips R, Saunders B. Video capsule endoscopy in peutz-jeghers syndrome: a blinded comparison with barium follow-through for detection of small-bowel polyps. *Endoscopy* 2006; **38**: 385-390

58 **Caspari R**, von Falkenhausen M, Krautmacher C, Schild H, Heller J, Sauerbruch T. Comparison of capsule endoscopy and magnetic resonance imaging for the detection of polyps of the small intestine in patients with familial adenomatous polyposis or with Peutz-Jeghers' syndrome. *Endoscopy* 2004; **36**: 1054-1059

59 **Racz I**, Janoki M, Kovacs V. Measurement of small-bowel polyp size in patients with Peutz-Jeghers syndrome by using reference granules during video capsule endoscopy. *Endoscopy* 2007; **39** Suppl 1: E41

60 **Biagi F**, Rondonotti E, Campanella J, Villa F, Bianchi PI, Klersy C, De Franchis R, Corazza GR. Video capsule endoscopy and histology for small-bowel mucosa evaluation: a comparison performed by blinded observers. *Clin Gastroenterol Hepatol* 2006; **4**: 998-1003

61 **Fischer D**, Schreiber R, Levi D, Eliakim R. Capsule endoscopy: the localization system. *Gastrointest Endosc Clin N Am* 2004; **14**: 25-31

62 **Mehdizadeh S**, Ross A, Gerson L, Leighton J, Chen A, Schembre D, Chen G, Semrad C, Kamal A, Harrison EM, Binmoeller K, Waxman I, Kozarek R, Lo SK. What is the learning curve associated with double-balloon enteroscopy? Technical details and early experience in 6 U.S. tertiary care centers. *Gastrointest Endosc* 2006; **64**: 740-750

63 **Lewis BS**, Eisen GM, Friedman S. A pooled analysis to evaluate results of capsule endoscopy trials. *Endoscopy* 2005; **37**: 960-965

64 **Chong AK**, Chin BW, Meredith CG. Clinically significant small-bowel pathology identified by double-balloon enteroscopy but missed by capsule endoscopy. *Gastrointest Endosc* 2006; **64**: 445-449

65 **Schulmann K**, Hollerbach S, Kraus K, Willert J, Vogel T, Moslein G, Pox C, Reiser M, Reinacher-Schick A, Schmiegel W. Feasibility and diagnostic utility of video capsule endoscopy for the detection of small bowel polyps in patients with hereditary polyposis syndromes. *Am J Gastroenterol* 2005; **100**: 27-37

66 **Burke CA**, Santisi J, Church J, Levinthal G. The utility of capsule endoscopy small bowel surveillance in patients with polyposis. *Am J Gastroenterol* 2005; **100**: 1498-1502

67 **Ohmiya N**, Taguchi A, Shirai K, Mabuchi N, Arakawa D, Kanazawa H, Ozeki M, Yamada M, Nakamura M, Itoh A, Hirooka Y, Niwa Y, Nagasaka T, Ito M, Ohashi S, Okamura S, Goto H. Endoscopic resection of Peutz-Jeghers polyps throughout the small intestine at double-balloon enteroscopy without laparotomy. *Gastrointest Endosc* 2005; **61**: 140-147

68 **Wong RF**, Tuteja AK, Haslem DS, Pappas L, Szabo A, Ogara MM, DiSario JA. Video capsule endoscopy compared with standard endoscopy for the evaluation of small-bowel polyps in persons with familial adenomatous polyposis (with video). *Gastrointest Endosc* 2006; **64**: 530-537

S- Editor Zhong XY **L- Editor** Rippe RA **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5254

World J Gastroenterol 2008 September 14; 14(34): 5254-5260
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Miguel Angel Muñoz-Navas, Profesor, *Series Editors*

Esophageal capsule endoscopy

Ignacio Fernandez-Urien, Cristina Carretero, Raul Armendariz, Miguel Muñoz-Navas

Ignacio Fernandez-Urien, Cristina Carretero, Raul Armendariz, Miguel Muñoz-Navas, Department of Gastroenterology, University of Navarra, Pamplona 31080, Spain
Author contributions: Fernandez-Urien I and Carretero C wrote the paper; Armendariz R reviewed bibliography and selected pictures; Muñoz-Navas M reviewed the paper.
Correspondence to: Ignacio Fernandez-Urien, MD, Department of Gastroenterology, University of Navarra, Av. Pio XII, 36, Pamplona 31080, Spain. ifurien@unav.es
Telephone: +34-948-255400 Fax: +34-948-296500
Received: February 26, 2008 Revised: August 23, 2008
Accepted: August 30, 2008
Published online: September 14, 2008

Abstract

Capsule endoscopy is now considered as the first imaging tool for small bowel examination. Recently, new capsule endoscopy applications have been developed, such as esophageal capsule endoscopy and colon capsule endoscopy. Esophageal capsule endoscopy in patients with suspected esophageal disorders is feasible and safe, and could be also an alternative procedure in those patients refusing upper endoscopy. Although large-scale studies are needed to confirm its utility in GERD and cirrhotic patients, current results are encouraging and open a new era in esophageal examination.

Key words: Capsule endoscopy; Esophagus; Gastroesophageal reflux disease; Varices; Esophagoscopy; PillCam ESO

Peer reviewer: Giovanni D De Palma, Professor, Department of Surgery and Advanced Technologies, University of Naples Federico II, School of Medicine, Naples 80131, Italy

Fernandez-Urien I, Carretero C, Armendariz R, Muñoz-Navas M. Esophageal capsule endoscopy. *World J Gastroenterol* 2008; 14(34): 5254-5260 Available from: URL: http://www.wjgnet.com/1007-9327/14/5254.asp DOI: http://dx.doi.org/10.3748/wjg.14.5254

INTRODUCTION

Since its introduction by Iddan *et al*[1], capsule endoscopy (CE) has acquired a well-established role in the investigation of suspected small bowel (SB) diseases. In fact, over 500 000 CE-procedures have been performed worldwide. Demands for CE are hoped to increase because of its proven superiority to conventional techniques for small bowel examination[2-11] and emerging indications, such as esophageal capsule endoscopy (ECE) and colon capsule endoscopy. Currently, upper endoscopy (EGD) is considered by most authors as the best method to explore the esophagus. However, because of the discomfort of intubation, conscious sedation is usually required resulting in increased costs, risks and patients acceptability[12-14]. In fact, because of these concerns, some patients are reluctant to undergo EGD even when it is indicated. So, it seems that there is a need for an alternative, simple and less invasive diagnostic tool for the evaluation of patients with known or suspected esophageal disorders. The esophageal capsule (PillCam™ ESO), which was approved by the FDA in November 2004, allows direct visualization of the esophagus without the need of sedation. Advantages include also its invasiveness and painless nature, the ability to pursue normal daily activities after the procedure and patients acceptability. Clinical data on its use and current indications, although quite limited, have opened a promising era for esophageal endoscopic examination.

PILLCAM™ ESO

The PillCam™ ESO (Given Imaging Ltd. Yoqneam, Israel) is an ingestible and disposable capsule measuring 11 mm × 26 mm (similar to PillCam™ SB) that acquires video images from both ends at a combined rate of 14 frames per second (7 from each side of the capsule) during its natural passage through the esophagus (Figure 1). The battery expires in 20-30 min approximately, resulting in more than 15 000 images captured per procedure, which are usually enough to explore the entire esophagus and sometimes, part of the stomach. The images, transmitted via digital radio frequency communication channel to the data recorder unit located outside the body, are captured by an antenna array located on the upper chest and the abdominal wall of the patient. Upon completion of the procedure, the images are transmitted to the Rapid® Workstation for processing and interpretation, which takes only a few minutes. Recently, a new complementary tool has been developed by Given Imaging: the RAPID® Access

Figure 1 PillCam™ ESO.

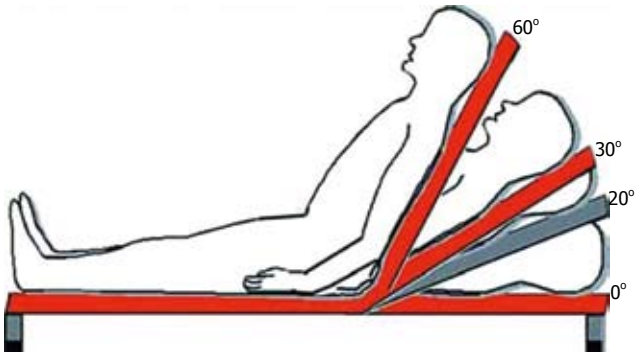


Figure 2 Ingestion protocol.

RT. During the ECE-procedure, the RAPID® Access RT allows real time visualization of capsule images. This is extremely useful in certain circumstances as the physician can intervene to optimize the procedure by changing patient position or administering medications such as laxatives depending on the images obtained in real time.

PROCEDURE

ECE is a quite simplex procedure, which can take only 4-5 min to physicians. This procedure requires implementation of a specific ingestion procedure to assure effective coverage of the esophagus (Figure 2). After a 6 h fast and before capsule ingestion, the patient is asked to drink swiftly a small amount of water (100 mL) in a standing position to clear saliva from the esophagus. Then, the capsule is swallowed in the supine position helped by a small sip of water (10 mL) if required. The patient must remain in this position for 2 min and then must be rose to an inclination of 30 degrees. The patient must remain in this position for 2 min and then the inclination must be increased to 60 degrees. One minute later, the patient is asked to drink a small sip of water (10 mL) and then, allowed to sit upright and to drink again 10 mL of water. In this moment, the patient is allowed to get up and walk in the waiting room for 15-20 min. During procedure in bed, the patient is instructed not to talk. Once the batteries have expired, the procedure is over and downloading

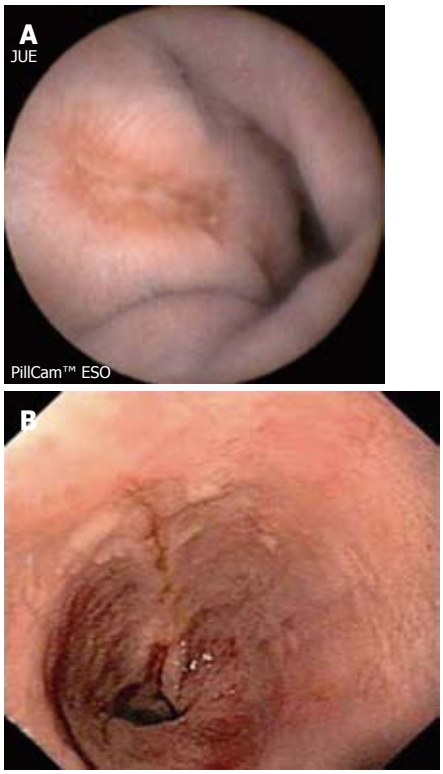


Figure 3 **A**: PillCam™ ESO image of erosive esophagitis; **B**: Upper endoscopy image of distal esophagus in the same patient.

process begins (4-5 min). Recently, an article published by Gralnek *et al*[15] has evaluated a new simplified ingestion procedure (SIP) in healthy volunteers who swallowed the capsule in the right supine position. Although esophageal transit time was shorter in comparison with the original ingestion procedure (mean: 38 s *vs* 225 s, respectively; $P < 0.001$), results showed that the SIP provides significantly improved visualization of the Z-line (visualization of $\geqslant$ 2 quadrants of the Z-line in 100% *vs* 75% of the cases; $P = 0.025$). Therefore, the authors recommend testing the proposed SIP in patients undergoing ECE.

ECE IN GERD PATIENTS (TABLE 1, FIGURES 3 AND 4)

Persistent heartburn is one of the most frequent gastrointestinal symptoms in western countries. Symptomatic GERD affects at least 5%-7% of the global population and in western countries, up to 30% of the population is affected by this disorder[16-18]. Complications of GERD include erosive esophagitis, ulcers, strictures or Barrett's (BE) esophagus. Up to 30% of subjects with GERD are found to have esophagitis while ulcers or strictures occur in 5% of patients[19]. Barrett's esophagus, which carries a risk of 0.5% per patient-years of esophageal adenocarcinoma, may occur in up to 10% of patients with chronic GERD[20]. Therefore, international guidelines recommend screening EGD in all GERD patients. However, as demonstrated, its cost and invasiveness limits its utilization in many patients[21].

Table 1 ECE in GERD patients: results in published studies

Author	Yr	*n*	Indication	Capsule	Ingestion	S (%)	E (%)	PPV (%)	NPV (%)
Neu[22]	2003	8	Esophagitis	SB	Supine	37.5	(-)	(-)	(-)
Ramirez[23]	2005	50	BE	String SB	Standing	100	100	100	100
Eliakim[24]	2004	17	Esophagitis BE	ESO 4-fps	Supine	100	80	92	100
Eliakim[25]	2005	93	Esophagitis	ESO 4-fps	Supine	89	99	97	94
Eliakim[25]	2005	13	BE	ESO 4-fps	Supine	97	99	97	99
Koslowsky[26]	2006	25	Esophagitis BE	ESO 4-fps	Supine	81	61	74	79
Koslowsky[26]	2006	25	Esophagitis BE	ESO 14-fps	Supine	100	74	100	77
Sharma[27]	2007	53	BE (suspected-known)	ESO 14-fps	Supine	67-79	87-78	60-94	90-44
Sharma[27]	2007	41	Esophagitis	ESO 14-fps	Supine	50	90	56	88
Lin[28]	2007	90	BE	ESO 14-fps	Supine	67	84	22	98

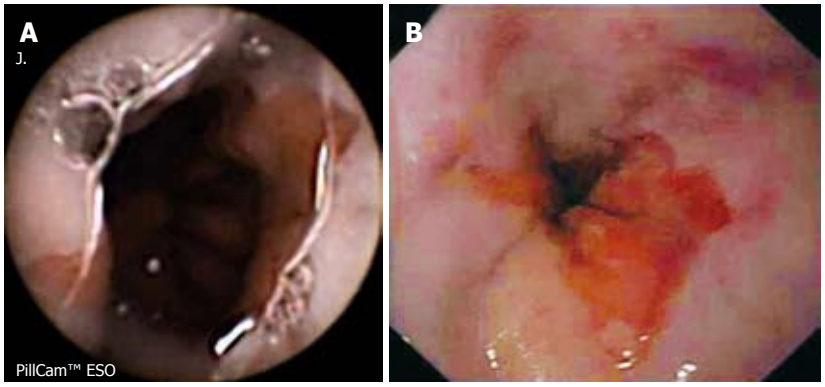


Figure 4 **A**: PillCam™ ESO image of short segment Barrett´s esophagus; **B**: Subsequent upper endoscopy image which confirms capsule findings.

In 2003, Neu *et al*[22] published the first article regarding ECE. Using SB capsules (single camera at a frame rate of 2/s), they evaluated the accuracy of capsule endoscopy in 8 patients with known esophagitis. All patients swallowed the capsule in the supine position. The capsule detected only 3 of 8 (37.5%) patients with esophagitis and an adequate visualization of 50% and 100% of the Z-line circumference was achieved in 12.5% and 37.5% of the patients, respectively. They also evaluated the quality of images obtained by the capsule in 58 patients examined for suspected small bowel pathology with poor results (0% of 100% of the Z-line circumference visualization), due to the short esophageal transit time (these patients swallowed the capsule in standing position). They concluded that distal esophageal assessment by SB capsules was not feasible.

Few months later, Ramirez *et al*[23] used SB capsules attached to strings allowing capsule control up and down the esophagus. Fifty patients with Barrett's esophagus were enrolled in this study. The mean recording time in this study was much longer than in the previous study by Neu *et al* (7.9 min *vs* 3 s). All 50 patients with BE were detected by the capsule. The majority of patients (92%) preferred string-capsule endoscopy to EGD because usually, none or minimal discomfort was associated with capsule ingestion. They concluded that string esophageal capsule endoscopy is feasible, safe and highly acceptable by patients with esophageal disorders.

In 2004, Eliakim *et al*[24] published a pilot study of ECE using specifically-designed capsules for esophageal examination (double camera at a frame rate of 4 per second). They compared the diagnostic yield of ECE to EGD (used as gold standard) in 17 patients with suspected esophageal disorders. All patients swallowed the capsule in supine position to avoid rapid esophageal transit of the capsule. All patients with positive findings at EGD (12/17) were detected by the capsule. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were 100%, 80%, 92% and 100%, respectively. Of 15 patients asked, 12 (80%) preferred the capsule experience to EGD which was performed under sedation with Midazolam 2.5-5 mg. They concluded that ECE with the new device proposed is an accurate, convenient, safe and well-tolerated method for patients with esophageal disorders. These results were encouraging but the small sample size of this pilot study was an obstacle to elaborate solid conclusions. Then, largescale studies seemed to be necessary.

A similar study conducted at 7 sites in 2005[25] evaluated the new developed PillCam™ ESO (double camera at a frame rate of 4 per second) compared to EGD in 106 patients (93 with GERD and 13 with BE). All patients swallowed the capsule in the supine position. Sensitivity, specificity, PPV and NPV for esophagitis were 89%, 99%, 97% and 94%, respectively, and 97%, 99%, 97% and 99%, respectively, for BE. ECE was preferred over EGD by all patients. These results were consistent with those obtained by Eliakim *et al*[24] in 2004. They concluded that ECE was a convenient and sensitive method for visualization of esophageal mucosal pathology and may provide an effective method to evaluate patients for esophageal disease.

However, some experiences have shown that the speed of the capsule in the proximal esophagus can reach up to 20 cm per second. It means that using the

Table 2 ECE in portal hypertension: Results in published studies

Author	Yr	*n*	Indication	Capsule	Ingestion	S	E	PPV	NPV
Ramirez[40]	2005	30	Varices	String SB	Standing	96%	100%	100%	83.3%
Eisen[41]	2006	32	Varices	ESO 14-fps	Supine	100%	89%	(-)	(-)
Eisen[41]	2006	32	PHG	ESO 14-fps	Supine	100%	77%	(-)	(-)
Lapalus[42]	2006	21	Varices	ESO 14-fps	Supine	81.2%	100%	100%	57%

4-frame per second capsule, only one image could be taken per 10 cm length in some instances. Koslowsky *et al*[26] speculated in 2006 that the diagnostic yield of the ECE might be improved by using a 14-frame per second capsule. Fifty patients (42 suffering from GERD symptoms and 8 with confirmed BE) were included in this study and all of them swallowed the capsule in the supine position: 25 underwent ECE with the 4-frame per second capsule and 25 underwent ECE with the 14-frame per second capsule. Using EGD as gold standard, the 4-frame per second capsule sensitivity, specificity, PPV and NPV were 81%, 61%, 74% and 79%, respectively, and the 14-frame per second capsule sensitivity, specificity, PPV and NPV were 100% ($P < 0.02$), 74%, 100% and 77%, respectively. The upper esophageal sphincter and the entire esophagus were assessed by the 4-fps capsule in 25% and 12% of the cases, respectively, and in 81% ($P < 0.01$) and 76% ($P < 0.01$) of the cases by the 14-fps capsule, respectively. They concluded that ECE using the 14-fps capsule has a greater sensitivity and allows better visualization of the entire esophagus than the 4-fps capsule.

Recently, two prospective, blinded and well-designed studies have compared the diagnostic accuracy of ECE using the 14-fps capsule *vs* EGD in both GERD and BE. Sharma *et al*[27] included 100 patients with GERD and BE. Ninety-four of these patients swallowed the capsule in the supine position. Results reported showed a higher diagnostic accuracy for BE than for erosive esophagitis. The sensitivity, specificity, PPV and NPV for BE in GERD patients were 67%, 87%, 60% and 90%, respectively, and for known Barrett's esophagus in patients undergoing surveillance were 79%, 78%, 94% and 44%, respectively. The diagnostic accuracy of ECE for long Barrett's segment esophagus (LSBE) was greater than for short Barrett's segments esophagus (SSBE). For erosive esophagitis, the sensitivity was 50%, the specificity 90%, the PPV 56% and the NPV 88%. These results were quite different than those obtained in previous studies. These differences might be attributed in part, to the diagnostic skills of examiners and to the ingestion protocol in the supine position. Anyway, the authors require for the future an improvement in technology and learning curve assessments. The other study, published at the same time by Lin *et al*[28], included 96 patients with chronic gastroesophageal reflux and BE undergoing surveillance. Again, the selected ingestion protocol was in the supine position. ECE sensitivity, specificity, PPV and NPV for BE were 67%, 84%, 22% and 98%, respectively. There were no differences between SSDE and LSBE detection. These results were similar to the study by Sharma *et al*[27] but again, quite different to those showed in previous studies with the 4-fps capsule. The authors attributed these differences to the patient adjudication process in previous studies (unblinded investigators). They conclude that ECE is not, at present, suitable as a primary screening tool for BE but may be used in patients unwilling to undergo EGD. Precisely, one study by Sanchez-Yague *et al*[29] published in 2006, reviewed 30 cases of ECE in patients refusing conventional endoscopy. They demonstrated that ECE is an adequate alternative diagnostic method for the study of patients with suspected esophageal diseases.

ECE IN PATIENTS WITH PORTAL HYPERTENSION (TABLE 2, FIGURES 5 AND 6)

The presence of esophageal varices is one of the most common complications of portal hypertension in cirrhotic patients. Although they are present in about 50% of the patients when cirrhosis is diagnosed, most of these patients develop varices during their lifetime[30,31]. Severe upper gastrointestinal bleeding as a complication of portal hypertension occurs in about 30%-40% of cirrhotic patients and in most cases because of the presence of esophageal varices[32,33]. Despite recent improvements in the diagnosis and treatment of esophagogastric variceal haemorrhage, the mortality rate of first variceal haemorrhage remains high (20%-35%)[33-36]. The risk of bleeding is related to the hepatic venous pressure, the Child- Pugh class and the endoscopic appearance of the varices[37]. Therefore, one of the challenges is to identify those cirrhotic patients who have esophageal varices and are also at risk of bleeding. Recently, the Baveno Ⅲ Consensus Conference on portal hypertension recommended that all cirrhotic patients should be screened by means of upper endoscopy for esophageal varices when liver cirrhosis is diagnosed, at 2-3 years intervals in compensated patients without varices and at 1-2 years intervals in compensated patients with previous small varices[38]. However, sedation during EGD in cirrhotic patients carries increased risks of cardiopulmonary complications because they are more susceptible to oversedation than those with normal liver function[12,39].

At the moment, three published studies have evaluated the role of ECE in portal hypertension. The first study was published in 2005 by Ramirez *et al*[40] who used the string-capsule endoscopy to evaluate portal

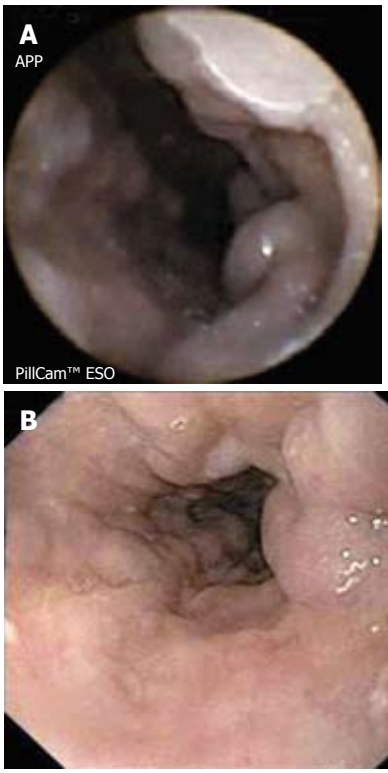


Figure 5 **A**: PillCam ESO™ image showing esophageal varices; **B**: Upper endoscopy image of distal esophagus in the same patient.

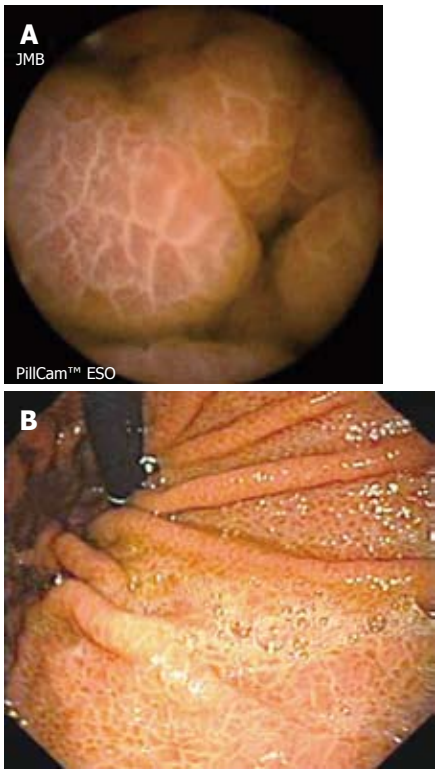


Figure 6 **A**: PillCam ESO™ image of the gastric wall showing portal hypertension gastropathy; **B**: Subsequent upper endoscopy image which confirms capsule findings.

hypertension in 30 cirrhotic patients. They reported an overall accuracy of 96.7% for varices (sensitivity of 96%, specificity of 100%, PPV of 100% and NPV of 83.3%, respectively) and all patients preferred string-capsule endoscopy to EGD.

On the other hand, two comparative studies have been recently published. Both of them used the new 14-fps capsule, which were swallowed in the supine position, and compared ECE *vs* EGD for portal hypertension in cirrhotic patients. Eisen *et al*[41] included 32 cirrhotic patients who were undergoing EGD for varices screening or surveillance. They reported a sensitivity of 100%, specificity of 89%, positive likelihood ratio of 9.1 and negative likelihood ratio of 0.0 for esophageal varices detection in comparison with EGD. There was complete agreement in the grading of varices in 65% of the cases and in 95% of the cases within one grade. They also evaluated the accuracy of the capsule to detect portal hypertension gastropathy (PHG). Sensitivity, specificity, positive likelihood ratio and negative likelihood ratio for PHG were 100%, 77%, 4.3 and 0.0, respectively. This pilot study led to a multicenter study with more than 300 patients included which is now finished but not yet published. The other comparative study by Lapalus *et al*[42], simultaneously published, included 21 cirrhotic patients who were undergoing unsedated EGD for varices screening. Results showed that ECE accurately assessed the presence of esophageal varices in 85% of the cases and correctly indicated a need for primary prophylaxis in 100% of the cases. All patients preferred ECE to unsedated EGD which was performed with a small-diameter upper gastrointestinal endoscope.

CONCLUSION

Capsule endoscopy has opened a new era in small bowel examination. Its indications are now well-defined and currently, wireless capsule endoscopy is considered as the first-line imaging tool for the diagnosis of small bowel diseases. ECE has been shown to be feasible, safe (no ECE-related complications have been reported with the PillCam™ ESO) and a good alternative technique in patients refusing conventional endoscopy. Although results reported in both GERD and cirrhotic patients are encouraging, great differences in terms of accuracy (particularly in GERD patients) have been found in published studies. These differences have been attributed to study designs, the lack of adequate experience and inconvenience of ingestion protocols. In summary, more large-scale studies evaluating the new 14-fps capsule, adequate ECE-experience and new modified ingestion protocols are still needed.

REFERENCES

1 **Iddan G**, Meron G, Glukhovsky A, Swain P. Wireless capsule endoscopy. *Nature* 2000; **405**: 417
2 **Ell C**, Remke S, May A, Helou L, Henrich R, Mayer G. The first prospective controlled trial comparing wireless capsule endoscopy with push enteroscopy in chronic gastrointestinal bleeding. *Endoscopy* 2002; **34**: 685-689
3 **Lewis BS**, Swain P. Capsule endoscopy in the evaluation of patients with suspected small intestinal bleeding: Results of a pilot study. *Gastrointest Endosc* 2002; **56**: 349-353
4 **Mylonaki M**, Fritscher-Ravens A, Swain P. Wireless capsule endoscopy: a comparison with push enteroscopy in patients with gastroscopy and colonoscopy negative gastrointestinal bleeding. *Gut* 2003; **52**: 1122-1126

5 **Saurin JC**, Delvaux M, Gaudin JL, Fassler I, Villarejo J, Vahedi K, Bitoun A, Canard JM, Souquet JC, Ponchon T, Florent C, Gay G. Diagnostic value of endoscopic capsule in patients with obscure digestive bleeding: blinded comparison with video push-enteroscopy. *Endoscopy* 2003; **35**: 576-584

6 **Hartmann D**, Schilling D, Bolz G, Hahne M, Jakobs R, Siegel E, Weickert U, Adamek HE, Riemann JF. Capsule endoscopy versus push enteroscopy in patients with occult gastrointestinal bleeding. *Z Gastroenterol* 2003; **41**: 377-382

7 **Eliakim R**, Fischer D, Suissa A, Yassin K, Katz D, Guttman N, Migdal M. Wireless capsule video endoscopy is a superior diagnostic tool in comparison to barium follow-through and computerized tomography in patients with suspected Crohn's disease. *Eur J Gastroenterol Hepatol* 2003; **15**: 363-367

8 **de Franchis R**, Rondonotti E, Abbiati C, Beccari G, Merighi A, Pinna A, Villa E. Capsule enteroscopy in small bowel transplantation. *Dig Liver Dis* 2003; **35**: 728-731

9 **Appleyard M**, Fireman Z, Glukhovsky A, Jacob H, Shreiver R, Kadirkamanathan S, Lavy A, Lewkowicz S, Scapa E, Shofti R, Swain P, Zaretsky A. A randomized trial comparing wireless capsule endoscopy with push enteroscopy for the detection of small-bowel lesions. *Gastroenterology* 2000; **119**: 1431-1438

10 **Costamagna G**, Shah SK, Riccioni ME, Foschia F, Mutignani M, Perri V, Vecchioli A, Brizi MG, Picciocchi A, Marano P. A prospective trial comparing small bowel radiographs and video capsule endoscopy for suspected small bowel disease. *Gastroenterology* 2002; **123**: 999-1005

11 **Liangpunsakul S**, Chadalawada V, Rex DK, Maglinte D, Lappas J. Wireless capsule endoscopy detects small bowel ulcers in patients with normal results from state of the art enteroclysis. *Am J Gastroenterol* 2003; **98**: 1295-1298

12 **Lapalus MG**, Saurin JC. [Complications of gastrointestinal endoscopy: gastroscopy and colonoscopy] *Gastroenterol Clin Biol* 2003; **27**: 909-921

13 **Daneshmend TK**, Bell GD, Logan RF. Sedation for upper gastrointestinal endoscopy: results of a nationwide survey. *Gut* 1991; **32**: 12-15

14 **Froehlich F**, Gonvers JJ, Fried M. Conscious sedation, clinically relevant complications and monitoring of endoscopy: results of a nationwide survey in Switzerland. *Endoscopy* 1994; **26**: 231-234

15 **Gralnek IM**, Rabinovitz R, Afik D, Eliakim R. A simplified ingestion procedure for esophageal capsule endoscopy: initial evaluation in healthy volunteers. *Endoscopy* 2006; **38**: 913-918

16 **Johanson JF**. Epidemiology of esophageal and supraesophageal reflux injuries. *Am J Med* 2000; **108** Suppl 4a: 99S-103S

17 **Locke GR 3rd**, Talley NJ, Fett SL, Zinsmeister AR, Melton LJ 3rd. Prevalence and clinical spectrum of gastroesophageal reflux: a population-based study in Olmsted County, Minnesota. *Gastroenterology* 1997; **112**: 1448-1456

18 **Farup C**, Kleinman L, Sloan S, Ganoczy D, Chee E, Lee C, Revicki D. The impact of nocturnal symptoms associated with gastroesophageal reflux disease on health-related quality of life. *Arch Intern Med* 2001; **161**: 45-52

19 **Frazzoni M**, De Micheli E, Savarino V. Different patterns of oesophageal acid exposure distinguish complicated reflux disease from either erosive reflux oesophagitis or non-erosive reflux disease. *Aliment Pharmacol Ther* 2003; **18**: 1091-1098

20 **Sharma P**, Falk GW, Weston AP, Reker D, Johnston M, Sampliner RE. Dysplasia and cancer in a large multicenter cohort of patients with Barrett's esophagus. *Clin Gastroenterol Hepatol* 2006; **4**: 566-572

21 **Inadomi JM**, Sampliner R, Lagergren J, Lieberman D, Fendrick AM, Vakil N. Screening and surveillance for Barrett esophagus in high-risk groups: a cost-utility analysis. *Ann Intern Med* 2003; **138**: 176-186

22 **Neu B**, Wettschureck E, Rosch T. Is esophageal capsule endoscopy feasible? Results of a pilot. *Endoscopy* 2003; **35**: 957-961

23 **Ramirez FC**, Shaukat MS, Young MA, Johnson DA, Akins R. Feasibility and safety of string, wireless capsule endoscopy in the diagnosis of Barrett's esophagus. *Gastrointest Endosc* 2005; **61**: 741-746

24 **Eliakim R**, Yassin K, Shlomi I, Suissa A, Eisen GM. A novel diagnostic tool for detecting oesophageal pathology: the PillCam oesophageal video capsule. *Aliment Pharmacol Ther* 2004; **20**: 1083-1089

25 **Eliakim R**, Sharma VK, Yassin K, Adler SN, Jacob H, Cave DR, Sachdev R, Mitty RD, Hartmann D, Schilling D, Riemann JF, Bar-Meir S, Bardan E, Fennerty B, Eisen G, Faigel D, Lewis BS, Fleischer DE. A prospective study of the diagnostic accuracy of PillCam ESO esophageal capsule endoscopy versus conventional upper endoscopy in patients with chronic gastroesophageal reflux diseases. *J Clin Gastroenterol* 2005; **39**: 572-578

26 **Koslowsky B**, Jacob H, Eliakim R, Adler SN. PillCam ESO in esophageal studies: improved diagnostic yield of 14 frames per second (fps) compared with 4 fps. *Endoscopy* 2006; **38**: 27-30

27 **Sharma P**, Wani S, Rastogi A, Bansal A, Higbee A, Mathur S, Esquivel R, Camargo L, Sampliner RE. The diagnostic accuracy of esophageal capsule endoscopy in patients with gastroesophageal reflux disease and Barrett's esophagus: a blinded, prospective study. *Am J Gastroenterol* 2008; **103**: 525-532

28 **Lin OS**, Schembre DB, Mergener K, Spaulding W, Lomah N, Ayub K, Brandabur JJ, Bredfeldt J, Drennan F, Gluck M, Jiranek GC, McCormick SE, Patterson D, Kozarek RA. Blinded comparison of esophageal capsule endoscopy versus conventional endoscopy for a diagnosis of Barrett's esophagus in patients with chronic gastroesophageal reflux. *Gastrointest Endosc* 2007; **65**: 577-583

29 **Sanchez-Yague A**, Caunedo-Alvarez A, Garcia-Montes JM, Romero-Vazquez J, Pellicer-Bautista FJ, Herrerias-Gutierrez JM. Esophageal capsule endoscopy in patients refusing conventional endoscopy for the study of suspected esophageal pathology. *Eur J Gastroenterol Hepatol* 2006; **18**: 977-983

30 **Lay CS**, Tsai YT, Teg CY, Shyu WS, Guo WS, Wu KL, Lo KJ. Endoscopic variceal ligation in prophylaxis of first variceal bleeding in cirrhotic patients with high-risk esophageal varices. *Hepatology* 1997; **25**: 1346-1350

31 **D'Amico G**, Pagliaro L, Bosch J. Pharmacological treatment of portal hypertension: an evidence-based approach. *Semin Liver Dis* 1999; **19**: 475-505

32 **Conn HO**, Lindenmuth WW, May CJ, Ramsby GR. Prophylactic portacaval anastomosis. *Medicine* (Baltimore) 1972; **51**: 27-40

33 **Pagliaro L**, D'Amico G, Sorensen TI, Lebrec D, Burroughs AK, Morabito A, Tine F, Politi F, Traina M. Prevention of first bleeding in cirrhosis. A meta-analysis of randomized trials of nonsurgical treatment. *Ann Intern Med* 1992; **117**: 59-70

34 **Kovacs TOG**, Jensen DM. Initial management of UGI hemorrhage in patients with portal hypertension. In: Rutherford RB, ed. Vascular surgery. 5th ed. Philadelphia: Saunders, 1999: 1554–1566

35 **Sarin SK**, Lamba GS, Kumar M, Misra A, Murthy NS. Comparison of endoscopic ligation and propranolol for the primary prevention of variceal bleeding. *N Engl J Med* 1999; **340**: 988-993

36 **D'Amico G**, Pagliaro L, Bosch J. The treatment of portal hypertension: a meta-analytic review. *Hepatology* 1995; **22**: 332-354

37 **Jensen DM**. Endoscopic screening for varices in cirrhosis: findings, implications, and outcomes. *Gastroenterology* 2002; **122**: 1620-1630

38 **D'Amico G**, Garcia-Tsao G, Cale's P, Escorsell A, Nevens F, Cestari R, Caletti G. Diagnosis of portal hypertension: how and when. In: De Franchis R, ed. Proceedings of the Third

Baveno International Consensus Workshop on Definitions, Methology and Therapeutic Strategies. Oxford: Blackwell Science, 2001: 36-63

39 **Madhotra R**, Mokhashi M, Willner I, Hawes RH, Reuben A. Prospective evaluation of a 3.1-mm battery-powered esophagoscope in screening for esophageal varices in cirrhotic patients. *Am J Gastroenterol* 2003; **98**: 807-812

40 **Ramirez FC**, Hakim S, Tharalson EM, Shaukat MS, Akins R. Feasibility and safety of string wireless capsule endoscopy in the diagnosis of esophageal varices. *Am J Gastroenterol* 2005; **100**: 1065-1071

41 **Eisen GM**, Eliakim R, Zaman A, Schwartz J, Faigel D, Rondonotti E, Villa F, Weizman E, Yassin K, deFranchis R. The accuracy of PillCam ESO capsule endoscopy versus conventional upper endoscopy for the diagnosis of esophageal varices: a prospective three-center pilot study. *Endoscopy* 2006; **38**: 31-35

42 **Lapalus MG**, Dumortier J, Fumex F, Roman S, Lot M, Prost B, Mion F, Ponchon T. Esophageal capsule endoscopy versus esophagogastroduodenoscopy for evaluating portal hypertension: a prospective comparative study of performance and tolerance. *Endoscopy* 2006; **38**: 36-41

S- Editor Zhong XY **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5261

World J Gastroenterol 2008 September 14; 14(34): 5261-5264
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Miguel Angel Muñoz-Navas, Profesor, *Series Editors*

Role of videocapsule endoscopy for gastrointestinal bleeding

Cristina Carretero, Ignacio Fernandez-Urien, Maite Betes, Miguel Muñoz-Navas

Cristina Carretero, Ignacio Fernandez-Urien, Maite Betes, Miguel Muñoz-Navas, Department of Gastroenterology, University of Navarra, Pamplona 31008, Spain
Author contributions: Carretero C and Fernandez-Urien I contributed equally to this paper, both performing research and writing the paper; Betes M and Muñoz-Navas M reviewed the paper.
Correspondence to: Cristina Carretero, MD, Department of Gastroenterology, University of Navarra, Av. Pio XII, 36, Pamplona 31008, Spain. ccarretero@unav.es
Telephone: +34-948-255400 Fax: +34-948-296500
Received: March 31, 2008 Revised: July 26, 2008
Accepted: August 2, 2008
Published online: September 14, 2008

Abstract

Obscure gastrointestinal bleeding (OGIB) is defined as bleeding of an unknown origin that persists or recurs after negative initial upper and lower endoscopies. Several techniques, such as endoscopy, arteriography, scintigraphy and barium radiology are helpful for recognizing the bleeding source; nevertheless, in about 5%-10% of cases the bleeding lesion cannot be determined. The development of videocapsule endoscopy (VCE) has permitted a direct visualization of the small intestine mucosa. We will analyze those techniques in more detail. The diagnostic yield of CE for OGIB varies from 38% to 93%, being in the higher range in those cases with obscure-overt bleeding.

Key words: Capsule endoscopy; Bleeding; Small bowel; Obscure; Hemorrhage

Peer reviewer: Ronan A Cahill, Department of General Surgery, Waterford Regional Hospital, Waterford, Cork, Ireland

Carretero C, Fernandez-Urien I, Betes M, Muñoz-Navas M. Role of videocapsule endoscopy for gastrointestinal bleeding. *World J Gastroenterol* 2008; 14(34): 5261-5264 Available from: URL: http://www.wjgnet.com/1007-9327/14/5261.asp DOI: http://dx.doi.org/10.3748/wjg.14.5261

INTRODUCTION

Obscure gastrointestinal bleeding (OGIB) is defined as bleeding of an unknown origin that persists or recurs after negative initial upper and lower endoscopies[1]. It can be subclassified as either obscure-occult, detected only by positive fecal occult blood tests (FOBT) and/or iron deficiency anemia (IDA), or obscure-overt with recurrent or persistent visible episodes of bleeding[2].

Approximately 5% of all gastrointestinal bleeding[3] are of obscure origin, and the most frequent causes are found among these: esophagitis, Cameron ulcers, Dieulafoy lesions, angiodysplasias, GAVE, portal hypertensive gastropathy, small bowel neoplasms, hemobilia, Meckel diverticulum, Crohn's disease, medication-induced mucosal lesions and a few others[2].

Several techniques, such as endoscopy, arteriography, scintigraphy and barium radiology, are helpful for recognizing the bleeding source; nevertheless, in about 5%-10% of cases the bleeding lesion cannot be determined[4]. Before 2001, the study of OGIB was deficient as the small bowel could not be reviewed reliably in its whole, but the development of videocapsule endoscopy (VCE) has permitted a direct visualization of the small intestine mucosa.

DIAGNOSTIC TECHNIQUES

Capsule endoscopy (CE)

Capsule endoscopy (CE) is a disposable 26 mm × 11 mm plastic capsule (Figure 1) consisting of an optical dome, 4 light-emitting electrodes, a sensor, 2 batteries and a micro transmitter. It acquires and transmits 2 frames per second (until the battery expires after 7 h ± 1 h[5]) to a sensor array attached to the patient[2]. Image features include a 140-degree field of view, 1:8 magnification, 1 to 30 mm depth of field and a minimum size for detection of about 0.1 mm[5].

It is passively propelled by peristalsis and it captures images of the entire length of the small intestine. The main limitations are the lack of air insufflation, the unavailability of rinsing, taking biopsies or/and treating lesions[2].

The incidence of capsule retention accounts of less than 1%[2] and is generally related to the presence of endoluminal narrowing. For that reason, and intending to predict the risk of retention, patients with high suspicion of bowel narrowing (history of NSAID's intake, Crohn's Disease, occlusive symptoms or ischemic bowel disease) should undergo capsule endoscopy exploration after performing other techniques such as CT scan, small

Figure 1 Hand holding a small bowel PillCam.

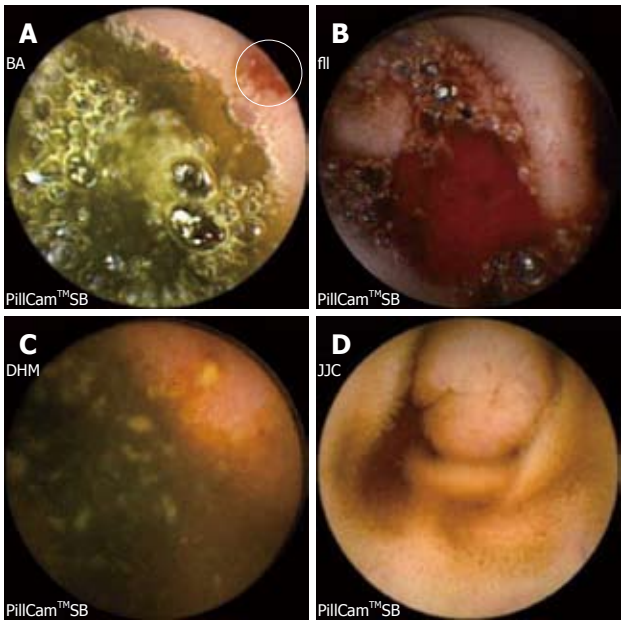


Figure 2 Lesions in the small bowel suspicious of being responsible for bleeding that can be found on CE: Angioectasia (**A**); Active bleeding (**B**); Ileal ulceration (**C**); Polypoid lesion (**D**).

bowel series or M2A patency capsule.

The diagnostic yield of CE for OGIB varies from 38% to 93%[5], being in the higher range in those cases with obscure-overt bleeding[3]. The ability to exclude bleeding lesions is between 82.6% and 100%[2]; nevertheless, in up to 35% of cases the capsule doesn't reach the cecum, probably due to slow gastric transit[2]. The most commonly detected lesions in the small bowel suspicious of being responsible for bleeding, that can be found on CE, are: angioectasia (Figure 2A), fresh blood (Figure 2B), ulceration (Figure 2C), polypoid or tumoral lesions (Figure 2D) and varices[5], and it seems that there is no significant difference in the diagnostic yield of CE in obscure-overt and obscure-occult bleeding[5,6]. One of the difficulties while reading the CE is to determine what has to be considered a positive finding with clinical significance, and a consensus still has to be reached[3]. In general, nonspecific mucosal changes (red spots in Figure 3, white spots, *etc*) are not considered positive

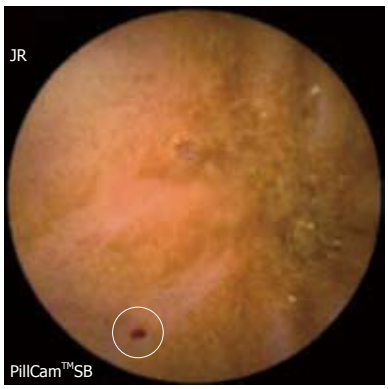


Figure 3 Red spot, with no relevant clinical significance.

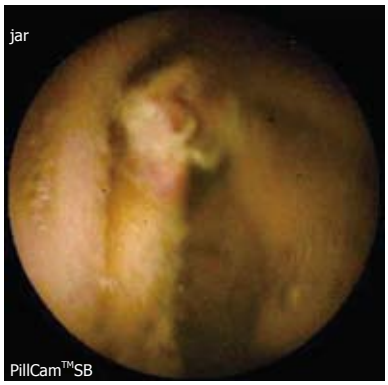


Figure 4 Ulcerated polyp.

findings; while angioectasias, tumors (Figure 4), masses or mucosal breaks should be included as positive findings.

This wide range of diagnostic yield can be related to several reasons, and one that has been suggested is the performance of CE within 15 days which can improve it (91% *vs* 34%, $P < 0.001$)[4]. Despite this improvement, some bleedings are still from an unknown origin. It has been suggested that a repeated CE can come across new findings in about 75% of cases, leading to changes in patient management in 62.5%[7] of cases. One of the main reasons to repeat a CE is the limited visualization that happens in about 44% of cases[7]. Recurrent bleeding is another reason for repeating CE. It can be helpful if the lesion responsible for bleeding is present intermittently or there was no bleeding source recognized in the first CE[7].

However, not only is it important to have a high diagnostic yield, but also to know if patient outcomes are improved after performing a CE. Carey *et al* considered measures of patient outcome the number of hospitalizations, units of blood transfused and the number of tests or procedures related to GI bleeding. Considering these measures, patient outcome appeared to improve after CE[3].

Lai *et al*[8] reported, in 2006, the results of a long-term follow-up of patients with obscure gastrointestinal bleeding. In 63.3% of cases, CE was able to determine the bleeding source and 32.7% of patients presented re-bleeding within the follow-up (median follow-up period: 19 mo)[8]. Patients with angiodysplasia were more susceptible to re-bleed (58.3% of cases were due to this condition), followed by patients with active

bleeding during the CE procedure with no identified bleeding source (53.8%)[8]. They found that the probability of re-bleeding was significantly higher in patients with positive CE than in those with negative CE ($P = 0.003$)[8].

Repeat upper endoscopy and colonoscopy

Between 35% to 75% of patients can be under-diagnosed at the initial endoscopic study[2]. Missed lesions occur due to their size, location, presence of clots or the absence of active bleeding while the endoscopy is being performed. Other possible causes are anemia, volume contraction, the effect of sedatives, as they can result in paleness of vascular lesions, and the timing of the endoscopy, as it is more probably to identify the bleeding source if the endoscopy is performed within 48 hours of the acute event[2].

It has been recommended to repeat upper and lower endoscopy ("second-look endoscopy")[2], nevertheless, about 5% to 10% of patients will remain undiagnosed.

Push enteroscopy

Enteroscopy consists of a peroral insertion into the jejunum of a long endoscope, using either a pediatric colonoscope or an enteroscope, and with or without the use of an overtube in order to avoid gastric looping[2]. This technique allows the examination of 15 to 160 cm beyond the ligament of Treitz[5] and it is generally considered the next diagnostic step after upper and lower negative endoscopic studies.

In 38%-75% of cases, the lesion causing OGIB can be identified with this technique[2,5]. However, in 28% to 75% of cases, it is reachable with a gastroscope[5]. Common findings are angiodysplasias (20%-46%), peptic ulcer disease, benign and malignant jejunal tumors, diverticulum, esophagitis and varices[2]. A meta-analysis has been published showing that the yield of CE for all findings is 63% *vs* 28% for Push Enteroscopy, with an incremental yield of 35%, $P < 0.00001$[9]. Yield of clinically significant findings is 56% for CE *vs* 26% for Push Enteroscopy[9].

Small bowel series and enteroclysis

Before the development of CE, a radiologic study of the small bowel was mandatory for the study of OGIB. The diagnostic yield is about 6%[2,5], and the most common missing lesions include angioectasia, ulcers and erosions[5]. The diagnostic yield can be improved (diagnostic yield of 10%-20%)[5] by performing enteroclysis. This technique consists in the introduction of a catheter into the small intestine followed by the injection of barium and methylcellulose. The barium coats the intestine and the methylcellulose distends the lumen to give a double contrast exam that allows for fluoroscopic visualization of the entire small bowel. The sensitivity of enteroclysis for small bowel tumors is higher than small bowel series[5].

A recent meta-analysis has shown that the diagnostic yield for all findings for CE is 67%, compared with an 8% for small bowel series. Diagnostic yield for significant findings is 42% for CE *vs* 6% for small bowel radiography[9].

Bleeding scanning with technetium-99-labeled RBC and angiography

This test can be useful if active bleeding is present as it may detect the source of hemorrhage if the bleeding rate ranges from 0.1 to 0.4 mL/min. Angiography can also be performed for diagnosis, as it is able to detect bleeding rates over 0.5 mL/min[2]. If the bleeding scanning is positive, an angiography should be performed to detect bleeding lesions and treat them if possible[2]. The diagnostic yield of these techniques varies from 44% to 68%[2].

Computed tomographic angiography (CTA)

Angiographic images can be obtained, not only by routine angiographic techniques, but also by CT scan. CTA is noninvasive and potentially useful for the diagnosis of GI bleeding, as it avoids the risks of standard angiography[10]. The CTA is able to identify the bleeding source in 24% of patients[10]. As has been recently published, CE is able to identify the bleeding source in a higher proportion of patients than CTA (72% *vs* 24%)[10].

Intraoperative enteroscopy (IE)

IE is the final diagnostic procedure for OGIB. It consists of performing an enteroscopy with the help of the surgeon, who helps by pushing the bowel over the enteroscope as the endoscopist examines it. The insertion can be done transoral or directly through a small enterotomy, but the last is more likely to achieve a complete examination of the small bowel. One of the drawbacks of this technique is that the manipulation of the bowel can create artifacts, which can be considered as bleeding sources although they are really not[2]. The direct effect of this handicap is that the examination of the mucosa has to be done while the intubation and not while the withdrawal of the endoscope. IE can achieve a diagnostic yield of a 70 to 93 which is comparable to CE[9].

Double balloon enteroscopy (DBE)

DBE is a novel endoscopic technique that allows for visualization of the entire small bowel, tissue sampling and therapeutic interventions. It consists of an enteroscope with 2 latex balloons, one attached to the endoscope and the other attached to an overtube. The procedure is performed by inflating and deflating the balloons, allowing the deep intubation through the small bowel. The route of insertion (perorally or transanally) selected depends on the patients' symptoms (hematochezia, melena *etc*).

A potential bleeding cause can be found in 75.7% of patients[11] and approximately a quarter of patients might need both anterograde and retrograde approaches[11]. DBE can change patient management in about 83.5% of cases, with an average therapeutic endoscopic intervention of 15.7%[11]. Hadithi *et al*[12] have published that CE is able to detect the presence of a possible

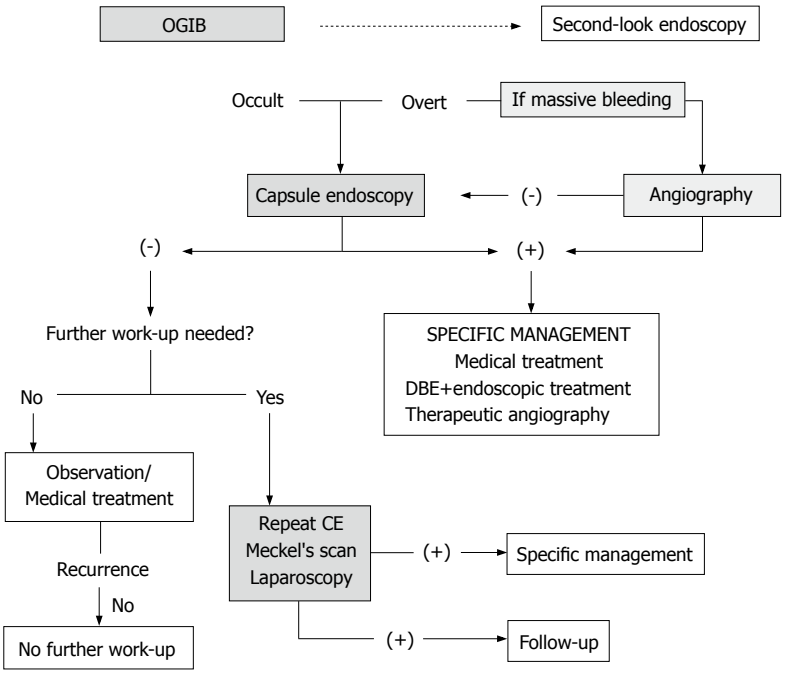


Figure 5 Suggested algorithm for OGIB.

bleeding source in a higher proportion of patients than DBE (80% *vs* 60%).

CONCLUSION

As explained before, there are some diagnostic techniques that can be used for the study of OGIB. With so many different tools, it is important to establish which should be performed first, not only taking into account the diagnostic yield but also the cost effectiveness of each one. Finally the suggested algorithm for OGIB by the International Consensus on Capsule Endoscopy is shown in Figure 5.

REFERENCES

1 **Zuckerman GR**, Prakash C, Askin MP, Lewis BS. AGA technical review on the evaluation and management of occult and obscure gastrointestinal bleeding. *Gastroenterology* 2000; **118**: 201-221
2 **Concha R**, Amaro R, Barkin JS. Obscure gastrointestinal bleeding: diagnostic and therapeutic approach. *J Clin Gastroenterol* 2007; **41**: 242-251
3 **Carey EJ**, Leighton JA, Heigh RI, Shiff AD, Sharma VK, Post JK, Fleischer DE. A single-center experience of 260 consecutive patients undergoing capsule endoscopy for obscure gastrointestinal bleeding. *Am J Gastroenterol* 2007; **102**: 89-95
4 **Bresci G**, Parisi G, Bertoni M, Tumino E, Capria A. The role of video capsule endoscopy for evaluating obscure gastrointestinal bleeding: usefulness of early use. *J Gastroenterol* 2005; **40**: 256-259
5 **Tang SJ**, Haber GB. Capsule endoscopy in obscure gastrointestinal bleeding. *Gastrointest Endosc Clin N Am* 2004; **14**: 87-100
6 **Ben Soussan E**, Antonietti M, Herve S, Savoye G, Ramirez S, Lecleire S, Ducrotte P, Lerebours E. Diagnostic yield and therapeutic implications of capsule endoscopy in obscure gastrointestinal bleeding. *Gastroenterol Clin Biol* 2004; **28**: 1068-1073
7 **Jones BH**, Fleischer DE, Sharma VK, Heigh RI, Shiff AD, Hernandez JL, Leighton JA. Yield of repeat wireless video capsule endoscopy in patients with obscure gastrointestinal bleeding. *Am J Gastroenterol* 2005; **100**: 1058-1064
8 **Lai LH**, Wong GL, Chow DK, Lau JY, Sung JJ, Leung WK. Long-term follow-up of patients with obscure gastrointestinal bleeding after negative capsule endoscopy. *Am J Gastroenterol* 2006; **101**: 1224-1228
9 **Triester SL**, Leighton JA, Leontiadis GI, Fleischer DE, Hara AK, Heigh RI, Shiff AD, Sharma VK. A meta-analysis of the yield of capsule endoscopy compared to other diagnostic modalities in patients with obscure gastrointestinal bleeding. *Am J Gastroenterol* 2005; **100**: 2407-2418
10 **Saperas E**, Dot J, Videla S, Alvarez-Castells A, Perez-Lafuente M, Armengol JR, Malagelada JR. Capsule endoscopy versus computed tomographic or standard angiography for the diagnosis of obscure gastrointestinal bleeding. *Am J Gastroenterol* 2007; **102**: 731-737
11 **Sun B**, Rajan E, Cheng S, Shen R, Zhang C, Zhang S, Wu Y, Zhong J. Diagnostic yield and therapeutic impact of double-balloon enteroscopy in a large cohort of patients with obscure gastrointestinal bleeding. *Am J Gastroenterol* 2006; **101**: 2011-2015
12 **Hadithi M**, Heine GD, Jacobs MA, van Bodegraven AA, Mulder CJ. A prospective study comparing video capsule endoscopy with double-balloon enteroscopy in patients with obscure gastrointestinal bleeding. *Am J Gastroenterol* 2006; **101**: 52-57

S- Editor Zhong XY **L- Editor** Rippe RA **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5265

World J Gastroenterol 2008 September 14; 14(34): 5265-5268
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Miguel Angel Muñoz-Navas, Profesor, *Series Editors*

Colon capsule endoscopy

Ignacio Fernandez-Urien, Cristina Carretero, Ana Borda, Miguel Muñoz-Navas

Ignacio Fernandez-Urien, Cristina Carretero, Ana Borda, Miguel Muñoz-Navas, Department of Gastroenterology, University of Navarra, Pamplona 31080, Spain
Author contributions: Fernandez-Urien I and Carretero C wrote the paper; Borda A performed picture selection; Muñoz-Navas M reviewed the paper.
Correspondence to: Ignacio Fernandez-Urien, MD, Department of Gastroenterology, University of Navarra, Av. Pio XII, 36, Pamplona 31080, Spain. ifurien@unav.es
Telephone: +34-948-255400 Fax: +34-948-296500
Received: August 2, 2008 Revised: August 18, 2008
Accepted: August 25, 2008
Published online: September 14, 2008

Abstract

Wireless capsule endoscopy has become the first imaging tool for small bowel examination. Recently, new capsule endoscopy applications have been developed, such as esophageal capsule endoscopy and colon capsule endoscopy. Clinical trials results have shown that colon capsule endoscopy is feasible, accurate and safe in patients suffering from colonic diseases. It could be a good alternative in patients refusing conventional colonoscopy or when it is contraindicated. Upcoming studies are needed to demonstrate its utility for colon cancer screening and other indications such us ulcerative colitis. Comparative studies including both conventional and virtual colonoscopy are also required.

Key words: Capsule endoscopy; Colon; Polyp; Colon cancer; Screening; Colonoscopy

Peer reviewers: Burton I Korelitz, MD, Department of Gastroenterology, Lenox Hill Hospital, 100 East 77th Street, 3 Achelis, New York, N.Y 10021, United States; Francesco Costa, Dr, Dipartimento di Medicina Interna-U.O. di Gastroenterologia Università di Pisa-Via Roma, 67-56122-Pisa, Italy

Fernandez-Urien I, Carretero C, Borda A, Muñoz-Navas M. Colon capsule endoscopy. *World J Gastroenterol* 2008; 14(34): 5265-5268 Available from: URL: http://www.wjgnet.com/1007-9327/14/5265.asp DOI: http://dx.doi.org/10.3748/wjg.14.5265

INTRODUCTION

Colorectal cancer (CRC) is the second most frequent cause of cancer-related death in western countries -skin tumors excluded-, after lung cancer in men and breast cancer in women. One out of three patients suffering from CRC will not survive[1]. Nevertheless, it can be considered as a preventable and curable condition. Firstly a preventable condition because in most cases, it develops from colonic adenomas. In fact, colonic adenomas are found in 11% to 40% of average risk population[2-4]. And secondly, a curable condition, because the 5-year survival rate in early stages can reach 90%[1]. For these reasons, conventional colonoscopy is suggested to be the optimal technique to be used for CRC screening programs in high-risk population, allowing a 90% decrease in CRC incidence[5]. However, it has to be considered that no more than 25% of compliance has been achieved in screening programs[5]. This low compliance can be explained by the drawbacks of conventional colonoscopy, such as being painful, patient's embarrassment or the need of sedation. Non-invasive techniques for colonoscopy, such as CT colonography[6-8] and Colon Capsule Endoscopy[9-11] are currently being evaluated as alternatives to conventional colonoscopy in order to improve the compliance to screening programs.

PILLCAM™ COLON CAPSULE

A large number of clinical trials have been performed testing different capsule designs in healthy volunteers. Finally, Given Imaging Ltd. has developed the final prototype for colon examination, which is called PillCam™ Colon. The PillCam™ Colon capsule has some differences from those used to study the small bowel and the esophagus. It measures 31 mm in length (4 mm longer than the PillCam™ ESO and SB) and 11 mm in diameter (the same as PillCam™ ESO and SB).

Figure 1 shows some morphologic differences between the three capsules commercially available. The PillCam™ Colon capsule has also some technical improvements, such as being equipped with cameras on both ends taking 4 images per second (2 images per camera). Each camera contains an automatic lighting control and has improved optics, which capture more

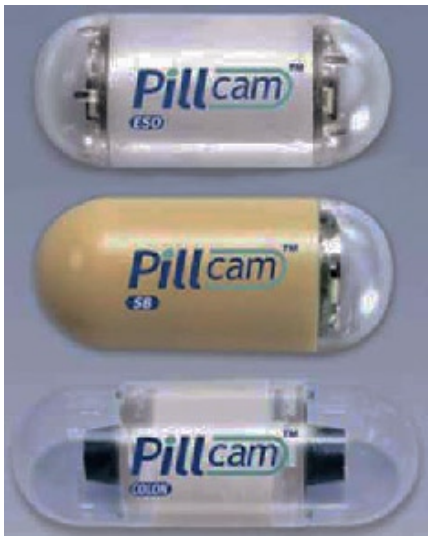


Figure 1 PillCam™ SB, ESO and Colon.

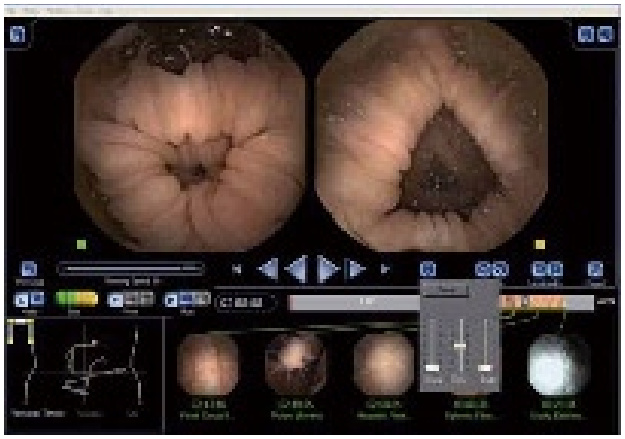


Figure 2 New Software for Colon Capsule reading: RAPID® 5 Scientific Edition.

than twice the coverage area and depth of field of PillCam™ SB resulting in a superior observation field. Other specific features are the presence of a longer battery (lasts 9-10 h on average), which can also "hibernate" minutes to hours after ingestion in order to conserve power before the capsule enters into the colon.

The accessory devices (sensor arrays and Data Recorder) are similar to those ones used by the PillCam™ ESO and SB. The RAPID® software used for images visualization during first clinical trials is a scientific version very similar to RAPID® 4, which includes (Ⅰ) a larger image display (round-rectangular shape), (Ⅱ) a complementary capsule localization system and an (Ⅲ) image enhancement (IE) features (Figure 2). The localization display is similar to the one already in use for the small bowel, but it also includes a schematic diagram of the colon that helps the physician to identify the location of findings, i.e., right, transverse and left colon segments once the main anatomic landmarks (first cecal image, the hepatic flexure, the splenic flexure and the body exit) have been selected. Moreover, this software allows the physician to enhance the appearance of the image by changing their color, brightness and sharpness.

Table 1 Procedure protocols

	Eliakim[10]	Schoofs[9]	Lewis[11]
Day-2	Low fiber diet	(-)	(-)
Day-1	19:00-20:00 PEG 2 L	18:00-21:00 PEG 3 L	18:00-21:00 PEG 3 L
Day 0[1]	07:00-08:00	06:00-07:00	07:00-08:00
	PEG 1 L	PEG 1 L	PEG 1 L
	+	+	+
	08:15	07:45	08:15
	Tegaserod 6 mg	Motilium 20 mg	Tegaserod 6 mg
	+	+	+
	08:30	08:00	08:30
	Capsule ingestion	Capsule ingestion	Capsule ingestion
	+	+	+
	10:30	10:00	10:30
	NaP 30 mL[2]	NaP 45 mL[2]	NaP 30 mL[2]
	+	+	+
	13:00	14:00	13:00
	Tegaserod 6 mg	NaP 30 mL	Tegaserod 6 mg
	+	+	+
	14:00	16:30	14:00
	NaP 15 mL	Bisacodyl	NaP 15 mL
	+	Suppository 10 mg	+
	16:30		16:30
	Bisacodyl		Bisacodyl
	suppository 10 mg		suppository 10 mg

[1]If the capsule was excreted, the regimen was discontinued; [2]Only if the capsule has exit the stomach.

RAPID® Access RT by Given Imaging allows real time visualization of capsule images. This is extremely useful in certain circumstances as the physician can intervene to optimize the procedure by changing patient position or administering medications such as laxatives depending on the images obtained in real time. In the PillCam™ Colon procedure, the importance of the real time viewer is that -as we will see later in more detail- 2 h post PillCam™ Colon Capsule ingestion, the patient has to drink a small amount of Sodium Phosphate. It is well known that Sodium Phosphate can delay gastric emptying time; therefore before giving it to the patient, it is recommended to check if the capsule has left the stomach, which can be easily done with the real time viewer.

PROCEDURE AND CLEANLINESS

The procedure of bowel cleansing until capsule ingestion is similar to that used for traditional colonoscopy. It usually begins one day before capsule ingestion, with the administration of laxatives to the patient. Patients are usually asked to maintain a low fiber diet 2 d before capsule ingestion. After the capsule has been ingested additional laxative and prokinetic agents are provided to the patient in order to (Ⅰ) maintain the cleanliness of the colon throughout the transit of the capsule and (Ⅱ) enhance capsule propulsion and excretion within 9-10 h post ingestion. The laxative and prokinetic agents are commercially available, and are provided within their permitted dose. Detailed information of the prep and procedure regimen used in recent trials[9-11] is shown in Table 1.

First results using the same prep as conventional colonoscopy showed low capsule excretion rates (about

Table 2 Results of PillCam™ Colon trials

	Yr	*n*	S	E	PPV	NPV
Results for polyps (any size)						
Eliakim[9]	2006	91	69%	81%	74%	78%
Schoofs[10]	2006	41	76%	64%	83%	54%
Results for significant polyps (> 6 mm or > 3 polyps > 3 mm)						
Eliakim[9]	2006	91	63%	94%	67%	91%
Schoofs[10]	2006	41	60%	73%	46%	83%
Results for other lesions						
Eliakim[9]	2006	91	78%	76%	47%	93%
Schoofs[10]	2006	41	76%	63%	82%	52%

S: Sensitivity; E: Specificity; PPV: Positive predictive value; NPV: Negative predictive value.

20%) which meant low rates of complete colonoscopies. Changes in prep regimens were then introduced (see Table 1) and higher excretion rates were reported by Eliakim *et al*[9], Schoofs *et al*[10] and Lewis *et al*[11] (78%, 84% and 90%, respectively). Moreover, the colon cleansing level reported by Eliakim *et al*[9] and Schoofs *et al*[10] was good to excellent in 84.4% and 88% of the patients, respectively. Recently, an undergoing European multicenter study published in abstract form[12] has reported a capsule excretion rate of 93% and good to excellent colon cleansing level in 71% of the patients. All these results are consistent with those obtained by conventional colonoscopy. As the goal of colon capsule endoscopy is to improve patient compliance to CRC screening, other simplified ingestion regimens including Moviprep® as the main laxative product or capsule procedures during the night are currently under evaluation.

LESIONS DETECTION

The long term primary objective of the PillCam™ Colon capsule is the average risk population undergoing CRC screening. In order to evaluate the accuracy of the new capsule device, it is being tested in those patients with known or suspected lesions (i.e. polyps or tumors). At the moment, encouraging results has been reported. Two European feasibility studies[9,10] including a total of 132 patients and one American study[11] published in abstract form including 25 patients, have recently evaluated the role of the PillCam™ Colon capsule in detecting colonic lesions. In all of these studies, conventional colonoscopy was considered the gold standard and the American[11] study included also the virtual colonoscopy as an additional comparative procedure. Preliminary results from these studies are resumed in Table 2. The European studies showed a capsule sensitivity (S) for polyps of any size of 69% and 76%, specificity (E) of 81% and 64%, positive predictive value (PPV) of 74% and 83% and negative predictive value (NPV) of 78% and 54%, respectively. Those polyps greater than 6 mm or 3 polyps of 3 mm were considered significant lesions. The accuracy of the colon capsule for significant lesions was very similar as well as for inflammatory lesions (i.e. diverticula, ulcerative colitis, *etc*). These results are consistent with those obtained in the American study

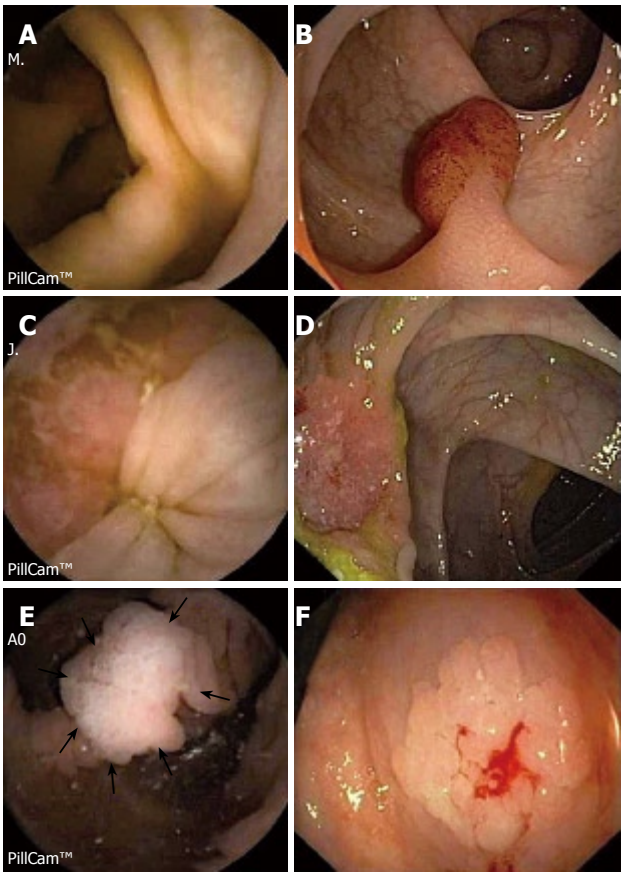


Figure 3 Images captured by the Pillcam™ Colon and conventional colonoscopy. **A** and **B**: Pedunculated polyp in the sigmoid colon; **C** and **D**: Ulcerated tumor in the transverse colon; **E** and **F**: Flat adenoma in the ascending colon.

which also showed that conventional colonoscopy was more accurate than colon capsule endoscopy and virtual colonoscopy (81%, 63% and 54%, respectively). In the European multicenter study[12], S, E, PPV and NPV for significant lesions were 66%, 82%, 72% and 77%, respectively; S, E, PPV and NPV for polyps > 6 mm were 64%, 84%, 60% and 86%, respectively and S, E, PPV and NPV for polyps > 10 mm were 60%, 98%, 83% and 93%, respectively. These results are very similar to those obtained by previous studies. On the other hand, the Z line is clearly visualized in 60% of cases by the capsule, even if the capsule is ingested in the standing position[10]. It means that patients undergoing CRC screening by PillCam™ Colon capsule endoscopy could be also screened for Barrett´s esophagus. Figure 3 shows some images from PillCam™ Colon capsule endoscopy.

SAFETY

The capsule colonoscopy seems to be a safe procedure. Capsule or laxatives-related complications during procedures has nor been reported by first feasibility studies[9-11]. On the other hand, 2 of 126 patients (1.6%) were unable to swallow the capsule in the study by Eliakim *et al*[9]. However, in these patients, the capsule can be easily introduced into the stomach or duodenum by means of the capsule deliver system (US Endoscopy).

COST-EFFECTIVENESS

As demonstrated by several studies, patients' compliance for CRC screening is still much lower that for other common neoplastic diseases such as breast and prostate cancer. Therefore, alternative procedures such as colon capsule endoscopy or CT colonography, which may increase patients´ compliance, are welcome. In fact, colon capsule endoscopy is an attractive non-invasive method for CRC screening, especially for those patients who are non-compliant to current screening procedures. Whether colon capsule endoscopy will be cost-effective has not been widely evaluated. However, a recent paper by Hassan *et al*[13] based on a mathematical Markov model concludes that colon capsule endoscopy may be cost-effective compared with colonoscopy if a 30% patients´ compliance increase is achieved. Moreover, as polyp detection by capsule endoscopy is expected to be more accurate in the future, it may be cost-effective even if compliance rates achieved remains lower than 30%.

CONCLUSION

Based on current available studies, PillCam™ Colon capsule colonoscopy is a feasible, effective and safe procedure that allows the visualization of the entire colon in most of the cases. It may be complementary to conventional colonoscopy and could be an appropriate exam for those patients who have received incomplete colonoscopy, contraindicated or are unwilling to undergo conventional colonoscopy. Further studies are needed to confirm these results and the possibilities of this new modality for endoscopic examination of the colon and for CRC screening. As colon capsule endoscopy has still some limitations (cannot insufflate air, clean or take biopsies), future capsule prototypes seem to be necessary. Moreover, it is anticipated that future procedures with modified regimens that may be performed at home, possibly over the weekend, can offer a unique method and further enhance patient compliance.

REFERENCES

1 **Ibanez MB**, Ribon CC, de la Torre FT, Munoz-Navas M. Evidencia científica en cribado del cáncer colorrectal: manual de actuacion. Madrid: International Marketing & Communication, 2006

2 **Betes M**, Munoz-Navas MA, Duque JM, Angos R, Macias E, Subtil JC, Herraiz M, De La Riva S, Delgado-Rodriguez M, Martinez-Gonzalez MA. Use of colonoscopy as a primary screening test for colorectal cancer in average risk people. *Am J Gastroenterol* 2003; **98**: 2648-2654

3 **Betes Ibanez M**, Munoz-Navas MA, Duque JM, Angos R, Macias E, Subtil JC, Herraiz M, de la Riva S, Delgado-Rodriguez M, Martinez-Gonzalez MA. Diagnostic value of distal colonic polyps for prediction of advanced proximal neoplasia in an average-risk population undergoing screening colonoscopy. *Gastrointest Endosc* 2004; **59**: 634-641

4 **Levine JS**, Ahnen DJ. Clinical practice. Adenomatous polyps of the colon. *N Engl J Med* 2006; **355**: 2551-2557

5 **Winawer SJ**, Zauber AG, Ho MN, O'Brien MJ, Gottlieb LS, Sternberg SS, Waye JD, Schapiro M, Bond JH, Panish JF. Prevention of colorectal cancer by colonoscopic polypectomy. The National Polyp Study Workgroup. *N Engl J Med* 1993; **329**: 1977-1981

6 **Silva AC**, Wellnitz CV, Hara AK. Three-dimensional virtual dissection at CT colonography: unraveling the colon to search for lesions. *Radiographics* 2006; **26**: 1669-1686

7 **Johnson KT**, Carston MJ, Wentz RJ, Manduca A, Anderson SM, Johnson CD. Development of a cathartic-free colorectal cancer screening test using virtual colonoscopy: a feasibility study. *AJR Am J Roentgenol* 2007; **188**: W29-W36

8 **Chaoui AS**, Barish MA. Virtual colonoscopy: a new tool for colorectal cancer screening. *Curr Opin Gastroenterol* 2001; **17**: 78-85

9 **Eliakim R**, Fireman Z, Gralnek IM, Yassin K, Waterman M, Kopelman Y, Lachter J, Koslowsky B, Adler SN. Evaluation of the PillCam Colon capsule in the detection of colonic pathology: results of the first multicenter, prospective, comparative study. *Endoscopy* 2006; **38**: 963-970

10 **Schoofs N**, Deviere J, Van Gossum A. PillCam colon capsule endoscopy compared with colonoscopy for colorectal tumor diagnosis: a prospective pilot study. *Endoscopy* 2006; **38**: 971-977

11 **Lewis B**, Rex D, Lieberman D. Capsule Colonoscopy: An Interim Report of a Pilot 3 Arm, Blinded Trial of Capsule Colonoscopy, Virtual Colonoscopy and Colonoscopy. *Am J Gastroenterol* 2006; **101**: S545-S561 (A1470)

12 **Deviere J**, Munoz-Navas M, Fernandez-Urien I, Carretero C, Gay G, Delvaux M, Lapalus MG, Ponchon T, Costamagna G, Riccioni ME, Spada C, Neuhaus H, Philipper M, Frazer DM, Postgate A, Fitzpatrick A, Hagenmuller F, Keuchel M, Schoofs N, Van Gossum AM. PillCam® Colon Capsule Endoscopy Compared to Colonoscopy in Detection of Colon Polyps and Cancers. *Gastroenterology* 2008; **134**: 282 (A38)

13 **Hassan C**, Zullo A, Winn S, Morini S. Cost-effectiveness of capsule endoscopy in screening for colorectal cancer. *Endoscopy* 2008; **40**: 414-421

S- Editor Zhong XY **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5269

World J Gastroenterol 2008 September 14; 14(34): 5269-5273
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Miguel Angel Muñoz-Navas, Profesor, *Series Editors*

Patency© and agile© capsules

Ángel Caunedo-Álvarez, Javier Romero-Vazquez, Juan M Herrerias-Gutierrez

Ángel Caunedo-Álvarez, Javier Romero-Vazquez, Juan M Herrerias-Gutierrez, Gastroenterology Service, "Virgen Macarena" University Hospital, Av Dr. Fedriani, Seville 41071, Spain
Correspondence to: Ángel Caunedo Álvarez, MD, Gastroenterology Service, "Virgen Macarena" University Hospital, Avda. Dr. Fedriani, Seville 41071, Spain. acaunedoa@meditex.es
Telephone: +34-955-008801 Fax: +34-955-008805
Received: February 15, 2008 Revised: July 26, 2008
Accepted: August 2, 2008
Published online: September 14, 2008

Abstract

Small bowel strictures can be missed by current diagnostic methods. The Patency capsule is a new non-endoscopic dissolvable capsule which has as an objective of checking the patency of digestive tract, in a non-invasive manner. The available clinical trials have demonstrated that the Patency© capsule is a good tool for assessment of the functional patency of the small bowel, and it allows identification of those patients who can safely undergo a capsule endoscopy, despite clinical and radiographic evidence of small-bowel obstruction. Some cases of intestinal occlusion have been reported with the Patency© capsule, four of them needed surgery. So, a new capsule with two timer plugs (Agile© capsule) has been recently developed in order to minimize the risk of occlusion. This new device stars its dissolution process earlier (30 h after ingestion) and its two timer plugs have been designed to begin the disintegration even when the device is blocked in a tight stricture.

Key words: Capsule endoscopy; Patency capsule; Agile capsule; Small bowel strictures

Peer reviewer: Amado S Peña, Professor, Department of Pathology, Immunogenetics, VU University Medical Centre, De Boelelaan 1117, PO Box 7057, Amsterdam 1007 MB, The Netherlands

Caunedo-Álvarez Á, Romero-Vazquez J, Herrerias-Gutierrez JM. Patency© and agile© capsules. *World J Gastroenterol* 2008; 14(34): 5269-5273 Available from: URL: http://www.wjgnet.com/1007-9327/14/5269.asp DOI: http://dx.doi.org/10.3748/wjg.14.5269

INTRODUCTION AND DEVICE DESCRIPTION

Since Iddan *et al*[1] reported the new wireless endoscopy system the capsule endoscopy (CE) has become one of the most significant technical innovations of Gastroenterology in recent years. Regarding the complications of the techniques, some incidental cases of impaction in a Meckel´s diverticulum[2], a Zenker´s diverticulum[3], or in the cricopharyngeal muscle[4], as well as aspiration of the capsule[5-7], solved without complications, have been reported. However, the most frequent side effect is undoubtedly the non-natural excretion (NNE) of the capsule due to a stricture or a tumor in the small bowel. The retrospective analysis of some series indicates that the incidence of NNE depends on the indication for the capsule exam: 0% in healthy controls[8], 1.4% in obscure gastrointestinal bleeding[9-13], 1.48% in suspected Crohn´s disease[14-17], 5%-13% in known Crohn´s disease[17-19] and 21% in suspected small bowel obstruction[20].

In the vast majority of cases, capsule retention, regardless of its cause, is asymptomatic, being evidenced by the absence of excretion and confirmed by means of radiology. On the other hand, capsule retention frequently allows for the identification of the bowel pathology which caused the symptoms in the patient and which could not have been diagnosed by means of standard methods, furthermore facilitating the location of the stricture or the tumor for the surgeon after "milking" the capsule through the bowel. For these reasons, many authors have described NNE as a "therapeutic complication" of the CE[21-23]. However, although this consideration is valid for patients with tumors or those with stricture due to NSAIDs, radiotherapy or previous abdominal surgery, it may not be valid in patients with stenoses secondary to Crohn's disease. Unlike the previously mentioned groups, in patients with Crohn's disease, surgery is not curative and may present more complications, thus in this case, NNE of the capsule can become more an "undesirable circumstance" than a "therapeutic complication". The same can be said of those patients showing high surgical risk or who would not be willing to undergo surgery in the event that NNE occurs.

Current imaging techniques can show long or medium stenoses, with great reduction of the lumen size; however, short stenoses usually cannot be detected by standard methods. This fact explains that in most

Table 1 Summary of the main seria on Patency Capsule (%)

Author	Inclusion criteria	*n*	Diagnosis known or suspected before Pillcam	Capsule integrity at egestion	Pts with uneventful PillCam after PC excreted intact
Spada *et al*[24] 2005	Suspected or confirmed small bowel stricture based on radiological exams	34	Crohn's disease: 30/34 (88.23) Adhesional syndrome: 3/34 (8.82) Ischemic enteritis: 1/34 (2.94)	30/34 (88.23)	10/10 (100)
Boivin *et al*[25] 2005	Obstructive small bowel symptoms, and/or radiographic evidence of structuring small bowel disease	22	Crohn's disease: 15/22 (68.18) Adhesional syndrome: 4/22 (18.18) Others: 3/22 (13.64)	16/22 (72.73)	13/13 (100)
Delvaux *et al*[26] 2005	Suspected or confirmed small bowel stricture based on either clinical background or radiological exams	22	Crohn's disease: 12/22 (54.54) NSAIDs stricture: 3/22 (13.64) Tumors: 3/22 (13.64) Others: 4/22 (18.18)	16/22 (72.73)	5/5 (100)
Signorelli *et al*[27] 2006	Risk of capsule retention because of clinical background or radiological exams	32	Crohn's disease: 18/32 (56.25) Intestinal surgery: 7/32 (21.87) Others: 7/32 (21.87)	26/32 (81.25)	25/25 (100)
Caunedo *et al*[28] 2003	Suspected or confirmed small bowel stricture based on radiological exams	29	Crohn's disease: 15/29 (51.72) Adhesional syndrome: 6/29 (20.69) Tumors: 3/29 (10.34) Others: 5/29 (17.24)	13/29 (44.83)	12/12 (100)
Spada *et al*[31] 2007	Radiologic findings suggesting small bowel stricture without clinical evidence of obstruction	27	Crohn's disease: 24/27 (88.89) Adhesional syndrome: 2/27 (7.41) Ischemic enteritis: 1/27 (3.70)	15/27 (55.55)	15/15 (100)
Total		166	Crohn's disease: 114/166 (68.67) Adhesional syndrome: 15/166 (9.04) Tumors: 6/166 (3.61) Intestinal surgery: 7/166 (4.22) Others: 24/166 (14.46)	116/166 (69.88)	80/80 (100)

of the reported cases of non-natural excretion (NNE) of the capsule, the previous performance of the usual radiological studies was not capable of diagnosing the intestinal strictures which the capsule clearly showed[9,11-13,19-21]. It is, therefore, proven that the lack of findings in radiological techniques does not rule out the existence of a bowel stenosis.

The manufacturing company of the PillCamSB has recently developed a new system (Given® M2A Patency System) which has as an objective of checking the patency of digestive tract, in a non-invasive manner. The Patency Capsule (PC) consists of a small identification tag (RFID), detectable by radiofrequency, which is surrounded by an absorbable material with a small amount of barium, all this covered by an external cover (Figure 1). PC has the same dimensions (11.4 mm × 26.4 mm) and the same shape as the standard capsule. PC is designed to remain intact in the gastrointestinal tract for about 80 h. After this period, if still within the body, it spontaneously disintegrates, except for the identification tag, whose small size (3 mm × 13 mm) allows it to pass through a stenosis of a very reduced lumen size. The persistence of the PC inside the organism can be verified by means of radiology, or with a radiofrequency emitting external detector device locating the identification tag.

CLINICAL TRIALS WITH PATENCY CAPSULE

Clinical experience with PC is still limited. A prospective, multi-center trial was designed to assess the clinical usefulness and safety of the PC capsule in patients with intestinal strictures suspected from clinical and/or radiological data. The global data of this trial have not been published, but the results of four of these centers have been reported (Table 1)[24-27]. In the series reported by Spada *et al*[24], 30 out of 34 (88.2%) patients with known or suspected small bowel stricture retrieved the capsule in the stool. After the excretion, the PC was intact in 20 cases (median transit time 22 h), and disintegrated in 10 patients (median transit time 53 h). Ten patients underwent video capsule endoscopy following the patency capsule examination. In all of these the video capsule passed through the small-bowel stricture without complication. The rate of patients with the PC excreted intact was similar in the seria of Boivin *et al*[25] (16/22, 73%), Delvaux *et al*[26] (16/22, 73%), Signorelli *et al*[27] (26/32, 81%). In our center, the percentage of cases with PC excreted intact was significant lower (13/29, 45%), probably because of patient selection[28]. In all the seria, the patency system scanner showed a good agreement (94%-100%) with fluoroscopy findings in identifying the presence of the tag in the body and may be used to detect the presence of the patency capsule without the need for radiology.

All these authors conclude that PC was unable to detect the presence of a small bowel stricture as previously defined by radiological techniques, but it added crucial information on the functional patency of the stenoses, and this information could allow a distinction between rigid fibrotic strictures and flexible ones. Boivin *et al*[25] found that passage of an intact capsule that is

Table 2 Complications reported in the main seria on Patency Capsule (%)

Author	Pts with abdominal pain during procedure	Severity of adverse event (abdominal pain)	Action taken
Spada *et al*[24] 2005	6/34 (17.64)	Mild: 5/34 (14.71) Moderate: 0/34 (0) Severe: 1/34 (2.94)	Nothing: 5/34 (14.71) Medical therapy: 1/34 (2.94) Surgery: 0/34 (0)
Boivin *et al*[25] 2005	6/22 (27.27)	Mild: 1/22 (4.54) Moderate: 1/22 (4.54) Severe: 4/22 (18.18)	Nothing or medical therapy: 5/22 (22.73) Surgery: 1/22 (4.54)
Delvaux *et al*[26] 2005	3/22 (13.64)	Mild: 1/22 (4.54) Moderate: 0/22 (0) Severe: 2/22 (9.09)	Nothing: 1/22 (4.54) Medical therapy: 0/22 (0) Surgery: 2/22 (9.09)
Signorelli *et al*[27] 2006	2/32 (6.25)	Mild: 2/32 (1.44) Moderate: 0/32 (0) Severe: 0/32 (0)	Nothing: 2/32 (1.44) Medical therapy: 0/32 (0) Surgery: 0/32 (0)
Caunedo *et al*[28] 2003	10/29 (34.48)	Mild: 4/29 (13.79) Moderate: 4/29 (13.79) Severe: 2/29 (6.90)	Nothing: 3/29 (10.35) Medical therapy: 7/29 (24.14) Surgery: 0/29 (0)
Spada *et al*[31] 2007	6/27 (22.22)	Mild: 5/27 (18.52) Moderate: 0/27 (0) Severe: 1/27 (3.70)	Nothing or medical therapy: 5/27 (18.52) Surgery: 1/27 (3.70)
Total	33/166 (19.88)	Mild: 18/166 (10.84) Moderate: 5/166 (3.01) Severe: 10/166 (6.02)	Nothing or medical therapy: 29/166 (17.47) Surgery: 4/166 (2.41)

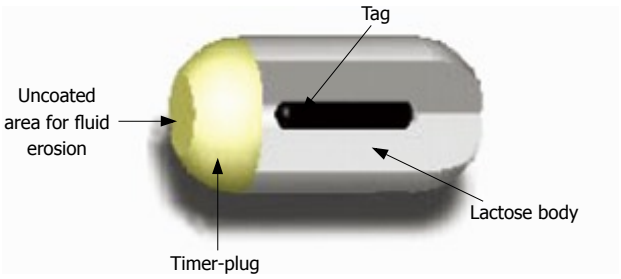


Figure 1 Schematic drawing of M2A® patency Capsule.

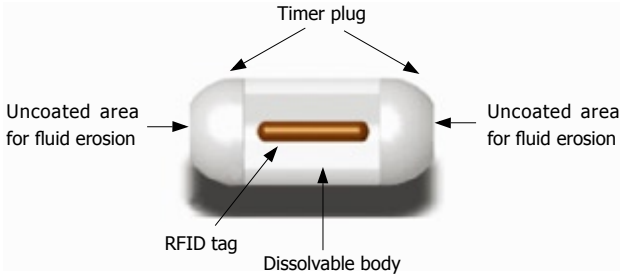


Figure 2 Schematic drawing of AGILE® patency Capsule.

accompanied by severe pain, similar to disintegration of the capsule with or without pain, seems indicative of a clinically relevant small-bowel stricture and is associated with a high probability of surgery. This observation seems to be confirmed by a recent retrospective analysis of 42 patients who underwent PC with known or suspected small bowel stricture[29]. In this study, the rate of patients who need to be operated in a period of three months was significantly higher in those with a delayed excretion, with the capsule excreted deformed, or with pain during the procedure. Moreover, in patients where painless natural expulsion of the intact PC occurred, CE could be applied without problems despite radiographic evidence of small-bowel strictures. This is an important finding since it might open the path to CE for about 60% of patients where video capsule investigation would otherwise be denied on grounds of history and radiological findings.

Abdominal pain during the procedure seems to be the main complication of PC (Table 2), observed in almost 20% of the cases reported to date. Probably, this pain is secondary to symptomatic intestinal occlusion, and it resolves spontaneously when the disintegration process concludes. However, at least four cases of occlusion did not respond adequately to conservative treatment and needed surgery. According to Gay *et al*[30], the problem could be that when the PC entrapped in a very tight stenosis it might not have been in contact with fluids and, therefore, only started to dissolve 48 h later, after moving back to the enlarged intestinal loop where it encountered fluids. Another possible factor is proposed by Gay *et al*[30]; PC is mainly made of lactose and the presence of lactase, an enzyme produced by intestinal mucosal cells, may be of importance in initiating the dissolution process. As lactase is mainly produced in the jejunum, one may also assume the enzyme is massively destroyed before the intestinal content reaches the ileum and that in the cases of occlusion, the enzyme could not have interacted with the capsule material. These authors[26,31] conclude that the start of dissolution at 40 h after ingestion is too slow to prevent episodes of intestinal occlusion, and so, it should be used cautiously under clinical surveillance in patients with Crohn´s disease.

THE AGILE© PATENCY CAPSULE

In order to reduce the risk of obstruction, a new dissolvable capsule with two timer plugs, one at each end, has been recently developed (Figure 2). With these

two timer plugs, the dissolution process starts earlier (30 h), and the contact with intestinal fluids is ensured even if the device is blocked in a tight stricture[32]. The new capsule, named Agile© Patency Capsule, has been evaluated in a multicenter clinical trial[33] designed to assess its safety in patients with known strictures and its ability to help physicians identify which patients may safely undergo CE. In this study, the intestinal tract was considered to be sufficiently patent if the capsule was excreted intact, or if the capsule was not detected by the scanner at 30 h after ingestion. If patency was established, then the patient underwent CE. Fifty-nine out (56%) of the 106 included patients excreted the Agile© capsule intact and subsequently underwent CE. There were no cases of retention of the video capsule and no Agile© capsules were found to have dissolved before 30 h after ingestion. Significant findings on CE were found in 24 patients (41%). A total of 17 (17/106) subjects had an adverse event, of these, 11 (11/106) consisted of abdominal pain. The pain resolved with conservative management within 48 h in all except for a patient with Crohn´s disease who need surgery (1/106). This patient developed obstruction after ingestion of the AGILE capsule and underwent surgery with resection of the terminal ileum and proximal colon, no remnants of the capsule were found at surgery. The physicians involved felt that the capsule did not lead to the obstruction. All of the other adverse events resolved within 48 h with conservative management.

In summary, the AGILE Patency capsule seems to be a useful, non-invasive tool to identify which patients with suspected strictures could safely ingest the standard video capsule. It has been designed to minimize the risk of intestinal occlusion.

REFERENCES

1 **Iddan G**, Meron G, Glukhovsky A, Swain P. Wireless capsule endoscopy. *Nature* 2000; **405**: 417
2 **Gortzak Y**, Lantsberg L, Odes HS. Video Capsule entrapped in a Meckel's diverticulum. *J Clin Gastroenterol* 2003; **37**: 270-271
3 **Feitoza AB**, Gostout CJ, Knipschield MA, Rajan E. Video capsule endoscopy: Is the recording time ideal? *Am J Gastroenterology* 2002; **97**: S307
4 **Fleischer DE**, Heigh RI, Nguyen CC, Leighton JA, Sharma VK, Musil D. Videocapsule impaction at the cricopharyngeus: a first report of this complication and its successful resolution. *Gastrointest Endosc* 2003; **57**: 427-428
5 **Schneider AR**, Hoepffner N, Rosch W, Caspary WF. Aspiration of an M2A capsule. *Endoscopy* 2003; **35**: 713
6 **Sinn I**, Neef B, Andus T. Aspiration of a capsule endoscope. *Gastrointest Endosc* 2004; **59**: 926-927
7 **Buchkremer F**, Herrmann T, Stremmel W. Mild respiratory distress after wireless capsule endoscopy. *Gut* 2004; **53**: 472
8 **Goldstein JL**, Eisen GM, Lewis B, Gralnek IM, Zlotnick S, Fort JG. Video capsule endoscopy to prospectively assess small bowel injury with celecoxib, naproxen plus omeprazole, and placebo. *Clin Gastroenterol Hepatol* 2005; **3**: 133-141
9 **Barkin JS**, Friedman S. Wireless capsule endoscopy requiring surgical intervention: The world's experience. *Am J Gastroenterol* 2002; **97**: A83
10 **Pennazio M**, Santucci R, Rondonotti E, Abbiati C, Beccari G, Rossini FP, De Franchis R. Outcome of patients with obscure gastrointestinal bleeding after capsule endoscopy: report of 100 consecutive cases. *Gastroenterology* 2004; **126**: 643-653
11 **Sears DM**, Avots-Avotins A, Culp K, Gavin MW. Frequency and clinical outcome of capsule retention during capsule endoscopy for GI bleeding of obscure origin. *Gastrointest Endosc* 2004; **60**: 822-827
12 **Rondonotti E**, Herrerias JM, Pennazio M, Caunedo A, Mascarenhas-Saraiva M, de Franchis R. Complications, limitations, and failures of capsule endoscopy: a review of 733 cases. *Gastrointest Endosc* 2005; **62**: 712-716; quiz 752, 754
13 **Caunedo A**, Rodriguez-Tellez M, Garcia-Montes JM, Gomez-Rodriguez BJ, Guerrero J, Herrerias JM Jr, Pellicer F, Herrerias JM. Usefulness of capsule endoscopy in patients with suspected small bowel disease. *Rev Esp Enferm Dig* 2004; **96**: 10-21
14 **Fireman Z**, Mahajna E, Broide E, Shapiro M, Fich L, Sternberg A, Kopelman Y, Scapa E. Diagnosing small bowel Crohn's disease with wireless capsule endoscopy. *Gut* 2003; **52**: 390-392
15 **Eliakim R**, Fischer D, Suissa A, Yassin K, Katz D, Guttman N, Migdal M. Wireless capsule video endoscopy is a superior diagnostic tool in comparison to barium follow-through and computerized tomography in patients with suspected Crohn's disease. *Eur J Gastroenterol Hepatol* 2003; **15**: 363-367
16 **Herrerias JM**, Caunedo A, Rodriguez-Tellez M, Pellicer F, Herrerias JM Jr. Capsule endoscopy in patients with suspected Crohn's disease and negative endoscopy. *Endoscopy* 2003; **35**: 564-568
17 **Cheifetz AS**, Kornbluth AA, Legnani P, Schmelkin I, Brown A, Lichtiger S, Lewis BS. The risk of retention of the capsule endoscope in patients with known or suspected Crohn's disease. *Am J Gastroenterol* 2006; **101**: 2218-2222
18 **Mow WS**, Lo SK, Targan SR, Dubinsky MC, Treyzon L, Abreu-Martin MT, Papadakis KA, Vasiliauskas EA. Initial experience with wireless capsule enteroscopy in the diagnosis and management of inflammatory bowel disease. *Clin Gastroenterol Hepatol* 2004; **2**: 31-40
19 **Buchman AL**, Miller FH, Wallin A, Chowdhry AA, Ahn C. Videocapsule endoscopy versus barium contrast studies for the diagnosis of Crohn's disease recurrence involving the small intestine. *Am J Gastroenterol* 2004; **99**: 2171-2177
20 **Cheifetz AS**, Lewis BS. Capsule endoscopy retention: is it a complication? *J Clin Gastroenterol* 2006; **40**: 688-691
21 **Cave D**, Legnani P, de Franchis R, Lewis BS. ICCE consensus for capsule retention. *Endoscopy* 2005; **37**: 1065-1067
22 **Brandt LJ**. Deformation of the anus: an alternative to rectal air suctioning for patient comfort after colonoscopy. *Gastrointest Endosc* 2004; **59**: 461
23 **Baichi MM**, Arifuddin RM, Mantry PS. What we have learned from 5 cases of permanent capsule retention. *Gastrointest Endosc* 2006; **64**: 283-287
24 **Spada C**, Spera G, Riccioni M, Biancone L, Petruzziello L, Tringali A, Familiari P, Marchese M, Onder G, Mutignani M, Perri V, Petruzziello C, Pallone F, Costamagna G. A novel diagnostic tool for detecting functional patency of the small bowel: the Given patency capsule. *Endoscopy* 2005; **37**: 793-800
25 **Boivin ML**, Lochs H, Voderholzer WA. Does passage of a patency capsule indicate small-bowel patency? A prospective clinical trial? *Endoscopy* 2005; **37**: 808-815
26 **Delvaux M**, Ben Soussan E, Laurent V, Lerebours E, Gay G. Clinical evaluation of the use of the M2A patency capsule system before a capsule endoscopy procedure, in patients with known or suspected intestinal stenosis. *Endoscopy* 2005; **37**: 801-807
27 **Signorelli C**, Rondonotti E, Villa F, Abbiati C, Beccari G, Avesani EC, Vecchi M, de Franchis R. Use of the Given Patency System for the screening of patients at high risk for capsule retention. *Dig Liver Dis* 2006; **38**: 326-330

28 **Caunedo A**, Rodriguez-Tellez M, Romero J, Hernandez-Duran M, Romero R, Pellicer FJ, Herrerias JM. Evaluation of the M2A Patency capsule in the gastrointestinal tract: one-centre preliminary data from a multicentre prospective trial. *Endoscopy* 2003; **35**: A182
29 **Caunedo-Alvarez A**, Romero-Vázquez J, Gomez-Rodriguez BJ, Sanchez-Yague A, Castro-Laria L, Herrerias-Gutierrez JM. Prognostic Factors of Short-Term Surgery in Patients with Known or Suspected Stricture Undergone to Patency Capsule. *Gastrointes Endoscopy* 2007; **65**: AB340
30 **Gay G**, Delvaux M, Laurent V, Reibel N, Regent D, Grosdidier G, Roche JF. Temporary intestinal occlusion induced by a "patency capsule" in a patient with Crohn's disease. *Endoscopy* 2005; **37**: 174-177
31 **Spada C**, Shah SK, Riccioni ME, Spera G, Marchese M, Iacopini F, Familiari P, Costamagna G. Video capsule endoscopy in patients with known or suspected small bowel stricture previously tested with the dissolving patency capsule. *J Clin Gastroenterol* 2007; **41**: 576-582
32 **Caunedo-Alvarez A**, Romero-Vazquez J, Gomez-Rodriguez BJ, Sanchez-Yague A, Castro-Laria L, Herrerias-Gutierrez JM. Evaluation of a new double-headed biodegradable device (AGILE Patency Capsule) for detecting functional patency of the small intestine: A prospective clinical trial. Proceedings of the 5th International Conference on Capsule. *Endoscopy* 2006
33 **Herrerias JM**, Leighton JA, Costamagna G, Infantolino A, Eliakim R, Fischer D, Rubin DT, Manten HD, Scapa E, Morgan DR, Bergwerk AJ, Koslowsky B, Adler SN. Agile patency system eliminates risk of capsule retention in patients with known intestinal strictures who undergo capsule endoscopy. *Gastrointest Endosc* 2008; **67**: 902-909

S- Editor Zhong XY **L- Editor** Rippe RA **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5274

World J Gastroenterol 2008 September 14; 14(34): 5274-5281
World Journal of Gastroenterology ISSN 1007-9327

COLORECTAL CANCER

Active chinese mistletoe lectin-55 enhances colon cancer surveillance through regulating innate and adaptive immune responses

Yan-Hui Ma, Wei-Zhi Cheng, Fang Gong, An-Lun Ma, Qi-Wen Yu, Ji-Ying Zhang, Chao-Ying Hu, Xue-Hua Chen, Dong-Qing Zhang

Yan-Hui Ma, Wei-Zhi Cheng, Fang Gong, An-Lun Ma, Qi-Wen Yu, Ji-Ying Zhang, Chao-Ying Hu, Shanghai Jiao Tong University School of Medicine, Shanghai Institute of Immunology, Shanghai 200025, China
Xue-Hua Chen, Shanghai Institute of Digestive Surgery, Ruijin Hospital, Shanghai 200025, China
An-Lun Ma, Centre de recherche du CHUM, Hôpital Notre-Dame, Université de Montréal, Montréal, Québec H2L 4M1, Canada
Author contributions: Ma YH and Cheng WZ contributed equally to this work; Zhang DQ, Ma YH and Cheng WZ designed research; Ma YH, Cheng WZ, Gong F, Ma AL, Yu QW, Zhang JY, Hu CY and Chen XH performed research; Chen XH contributed new reagents/analytic tools; Ma YH, Cheng WZ and Zhang DQ wrote the paper.
Supported by The National Natural Science Foundation of China, No. 30471593 and No. 30670939; the Shanghai Leading Academic Discipline Project, No. T0206; the Shanghai Commission of Science and Technology, No. 07JC14033; the Shanghai Institute of Immunology Project, No. 07-A02
Correspondence to: Dong-Qing Zhang, Shanghai Institute of Immunology,Shanghai Jiao Tong University School of Medicine, Shanghai 200025,
China. dqzhang1333@yahoo.com.cn
Telephone: +86-21-64453049 Fax: +86-21-64453049
Received: May 26, 2008 Revised: July 17, 2008
Accepted: July 24, 2008
Published online: September 14, 2008

Abstract

AIM: To investigate the potential role of Active Chinese mistletoe lectin-55 (ACML-55) in tumor immune surveillance.

METHODS: In this study, an experimental model was established by hypodermic inoculating the colon cancer cell line CT26 (5×10^5 cells) into BALB/c mice. The experimental treatment was orally administered with ACML-55 or PBS, followed by the inoculation of colon cancer cell line CT26. Intracellular cytokine staining was used to detect IFN-γ production by tumor antigen specific CD8+ T cells. FACS analysis was employed to profile composition and activation of CD4+, CD8+, γδ T and NK cells.

RESULTS: Our results showed, compared to PBS treated mice, ACML-55 treatment significantly delayed colon cancer development in colon cancer -bearing Balb/c mice *in vivo*. Treatment with ACML-55 enhanced both Ag specific activation and proliferation of CD4+ and CD8+ T cells, and increased the number of tumor Ag specific CD8+ T cells. It was more important to increase the frequency of tumor Ag specific IFN-γ producing-CD8+ T cells. Interestingly, ACML-55 treatment also showed increased cell number of NK, and γδT cells, indicating the role of ACML-55 in activation of innate lymphocytes.

CONCLUSION: Our results demonstrate that ACML-55 therapy can enhance function in immune surveillance in colon cancer-bearing mice through regulating both innate and adaptive immune responses.

Key words: Active chinese mistletoe lectin-55; Colon cancer; Immune surveillance; Tumor therapy; Ag-specific-CD8+ T cell

Peer reviewer: Yoshiharu Motoo, MD, PhD, FACP, FACG,Professor and Chairman, Department of Medical Oncology, Kanazawa Medical University,1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan

Ma YH, Cheng WZ, Gong F, Ma AL, Yu QW, Zhang JY, Hu CY, Chen XH, Zhang DQ. Active chinese mistletoe lectin-55 enhances colon cancer surveillance through regulating innate and adaptive immune responses. *World J Gastroenterol* 2008; 14(34): 5274-5281 Available from: URL: http://www.wjgnet.com/1007-9327/14/5274.asp DOI: http://dx.doi.org/10.3748/wjg.14.5274

INTRODUCTION

Mistletoe (Viscum album) is a semiparasitic plant with many unusual properties. It was used as a kind of herbal remedy in the ancient Chinese Pharmacopoeia and has been used in traditional Chinese medicine for diseases, such as gonorrhea, syphilis, hypertension and rheumatism for thousands of years. The aqueous extract of European Mistletoe (EM) has been used in conventional cancer therapy for decades[1]. Therapeutic efficacy is mostly attributed to the mistletoe lectins

(ML) ML-Ⅰ, ML-Ⅱ, ML-Ⅲ, that belong to the "toxic lectin family" and represent ribosome deactivating proteins class Ⅱ. They consist of one N-glycosidase (A chain) and one galactoside binding lectin (B chain) linked by a disulfide bridge. The lectins ML-Ⅰ and ML-Ⅲ preferentially bind to galactoside or N-acetylgalactosamine groups while ML-Ⅱ can bind to both carbohydrates[2].

EM has recently been found to act through several distinct bioactivities as a potent immune modulator. First, EM exerts its broad immunostimulatory activity by activating different types of cells[3-5] *in vivo* and *in vitro*. Incubation of lymphocytes with EM could result in anti-tumoral cytotoxic T lymphocytes bearing phosphorylated mistletoe ligands[6,7]. Second, EM favors bridging of natural killer tumor cell conjugates, enhancing its efficiency of killing[8-10]. Third, it has been found that EM could activate immune responses by modulating the complex network of cytokines that regulate leukocyte functions. EM caused increased secretion of tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 from isolated human mononuclear cells *in vitro*[11,12]. Finally, EM has also been described as an inducer of cell apoptosis. In the presence of EM, human mononuclear cells and many cell lines underwent apoptosis[1,13].

While the EM has been studied intensively, less is known about the Chinese mistletoe as an anti-cancer drug. In our prior study, a mistletoe lectin was purified from Chinese mistletoe and the effect of the active Chinese mistletoe mectin-55 (ACML-55) on human γδ T cell cytotoxicity, apoptosis and modulation of the cytokine network was reported[14,15]. Although these investigations suggest that ACML-55 may modulate the immune response against tumor development, the precise mechanism by which ACML-55 regulates the immune function has not been studied systematically. In this study, we demonstrate that ACML-55 enhances tumor immune surveillance against colon cancer formation by regulating both innate and adaptive immune responses. Our results suggest that ACML-55 may be a useful complementary therapy for treating colon cancer.

MATERIALS AND METHODS

Mice

BALB/c mice were purchased from Shanghai Experimental Animal Center, Chinese Academy of Science and were used at 6-8 wk of age in all experiments. All mice were maintained under specific pathogen-free conditions at Shanghai Jiao Tong University School of Medicine.

Administration of ACML-55

ACML-55 was dissolved in PBS at final concentration of 2 g/L. Mice were treated with ACML-55 or PBS orally (200 μL/mouse) once a day for 2 wk. Oral administration was achieved by gavage to ensure all mice received the entire dose.

Reagents

Recombinant murine IL-2 was purchased from R&D Systems (Minneapolis, MN, USA). Anti-mouse antibodies (CD3, NK1.1, CD4, CD8, CD62L, CD44, anti-αβ, anti-γδ and IFN-γ) used for phenotypic and cytokine analysis were purchased from BD Biosciences (San Jose, CA, USA).

Preparation of mistletoe lectins

Mistletoe lectins were isolated from extract of Chinese mistletoe, a subspecies of V. album according to previously described methods[14] with our own modifications. Briefly, the air-dried mistletoe (3 kg), collected from Sichuan province, China, was crushed and purified twice with 20 L methanol/water (1:1, V/V). The homogenate was filtered through a nylon cloth. After filtration, with its volume reduced to 2 liters, the aqueous phase was successively partitioned with cyclohexane, dichloromethane and ethyl acetate. Ethanol was added to the concentrated aqueous phase to a final concentration of 85% (V/V). A precipitate was obtained and separated from the supernatant by centrifugation (8000 *g*, 20 min). The supernatant was concentrated and ethanol was added to 85% (V/V). After centrifugation, the precipitate was collected and combined with the former precipitate. The final yield of ML extract was 100 g from 3 kg mistletoe. All the precipitate was dissolved in 100 mL phosphate buffer (10 mmol/L, pH 6.5) and the stock solution of mistletoe extract was stored at -80℃.

Purification of mistletoe lectins

To obtain the pure Chinese mistletoe protein, extract was further purified by CM-Sepharose column chromatography[14]. The aqueous layer (1 mL) was applied to a column of CM-Sepharose (1.5 cm × 20 cm) equilibrated with 10 mmol/L phosphate buffer (pH 6.5). After washing with 10 mmol/L phosphate buffer (pH 6.5) and 100 mmol/L NaCl in the same buffer at a rate of 0.5 mL/min, a peak eluted with 500 mmol/L NaCl in the same buffer was dialyzed with PBS (pH 7.4). The fractions containing hemagglutinating protein were collected and then applied to a column of Con A column (1.5 cm × 20 cm) equilibrated with 10 mmol/L PBS (pH 7.4). The column was washed with PBS (pH 7.4) and eluted with 300 mmol/L glucose in the same buffer. Fractions were subject to sodium dodecyl sulphate (SDS)-electrophoresis and fractions containing 55 kDa protein were pooled, dialyzed against water and freeze-dried.

SDS-polyacrylamide gel electrophoresis (PAGE)

The molecular mass and purity of ACML-55 was determined by SDS-PAGE. Twelve percent polyacrylamide gel was used as resolving gel and 5% was used as stacking gel. To further denature the proteins by reducing disulfide linkages, the samples were heated at 100ºC for 3 min in the presence of a reducing agent,

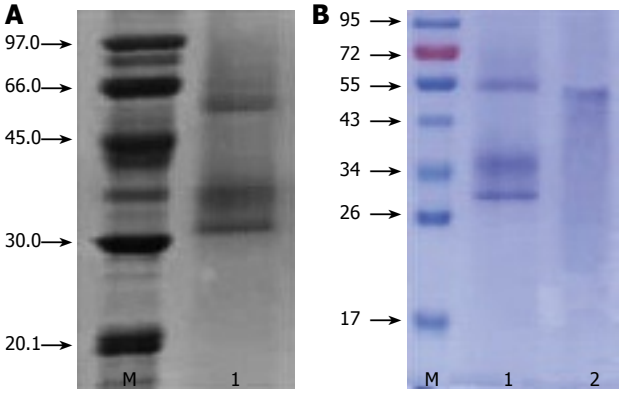


Figure 1 **A**: SDS-PAGE profiles of ACML-55. ACML-55 was determined by SDS-PAGE; ACML-55. **B**: ACML-55 was also determined by SDS-PAGE. ACML-55. ACML-55 in presence (lane 1) and absence (lane 2) of reducing agent.

then electrophoresed using electrophoresis system at 200 V for 75 min and lastly the gel was stained with Coomassie brilliant R-250 to show bands.

Tumor models

Colon cancer cell line CT26 and OVA-expressing EG7 cell line were purchased from ATCC (Manassas, VA, USA). For tumor induction, colon cancer cell line CT26 cells (5×10^5 cells/mouse) were injected subcutaneously, and tumor growth was monitored and recorded daily for over 3 wk as described in our previous studies[15]. For some experiments, EG7 tumor cells were also administered intraperitoneally (1.0×10^6 tumor cells/mouse).

IFN-γ production of tumor antigen specific CD8+ T cells

Mice were treated with ACML-55 or PBS for 2 wk as described above. These treated mice ($n = 5$ for each group) were immunized with 200 μg of CT26 tumor lysate emulsified in CFA in the hind footpad, as described in previous studies[16]. On day 7 post-immunization, draining lymph node cells were harvested, lymphocytes were cultured with comptumor air ratio RPMI-1640 containing 200 μg/mL CT26 tumor lysate for 24 h, with brefeldin A added for the last 3 h. Cells were then used for intracellular cytokine staining as described below.

Intracellular cytokine staining

Cultured draining lymph node cells were stained with FITC-anti-CD8 antibody followed by fixation with 2% formaldehyde and permeabilization with 0.5% saponin (w/v) for intracellular IFN-γ staining, using PE-anti-IFN-γ as described previously[17]. PE-conjugated rat IgG2a (BD Pharmingen) was used as an isotype control. Gating was performed on CD8+ T cells and the percentage of IFN-γ+ cells was reported.

Detecting tumor antigen specific CD8+ T cells

Both ACML-55 treated mice ($n = 4$) and control mice ($n = 4$) were inoculated intraperitoneally with 1.0×10^6 EG7 tumor cells. On day 10, splenocytes from these mice were isolated and stained with FITC-anti-CD8 and PE-tetramer antibodies for OVA. Percentage of tetramer positive CD8+ T cells was shown by FACS analysis.

Analysis of cell composition and activation

Both ACML-55 treated mice ($n = 4$) and control mice ($n = 4$) were inoculated intraperitoneally with 5×10^5 CT26 cells. On day 10, splenocytes from these mice were isolated and stained with one of the following antibody combinations: FITC-anit-CD3 and PE-anti-NK1.1; FITC-anti-γδ and PE-anti-αβ; PE-anti-CD62L, CyChrome- anti-CD44, FITC-anti-CD8a and APC-anti-CD4. Percentages of different cell subpopulations were shown by FACS analysis.

Statistical analysis

Statistical significance was evaluated by two tailed unpaired Student's test or non-parametric analysis if SDs were significantly different between two compared groups using InStat 2.03 for Macintosh software (Graph Pad Software). The incidence of tumor development was compared and analyzed using the log rank test, performed by Graph Pad Prism Version 3.0a for Macintosh (Graph Pad Software). $P < 0.05$ was used to denote statistical significance.

RESULTS

SDS-PAGE

Chinese ML extractions were analyzed by SDS-PAGE. In the presence of the reducing agent, it showed an estimated 55 kDa band consisting of two bands of a 30 kDa A chain and a 34 kDa B Chain (Figure 1).

Oral administration of ACML-55 enhances tumor immunesurveillance

Based on the findings that EM reduces the metastasis of rat mammary adenocarcinomas and its ability to modulate immune functions[18,12], we hypothesized that ACML-55 might enhance tumor immune surveillance. To assess the effect of ACML-55 on tumor development, ACML-55 or PBS was administered to sex- and age-matched BALB/c mice by gavage daily for 2 wk, followed by subcutaneous inoculation of CT26 cells (5×10^5 cells/mouse). Tumor growth was observed and recorded daily as previously described[16,19]. Compared to control group (PBS treated), ACML-55 treated mice showed delayed tumor development (Figure 2A) as well as reduced tumor size (Figrue 2B). ACML-55 treated mice were much more resistant to tumor cell growth upon subcutaneous tumor inoculation (Figrue 2C). The results indicate that ACML-55 significantly enhances tumor surveillance.

ACML-55 increases tumor specific activation of CD4+ and CD8+ T cells

To define the underlying molecular mechanisms of ACML-55 mediated anti-tumor immune response, we first tested the effect of ACML-55 on the adaptive immune response. Sex- and age- matched BALB/c mice

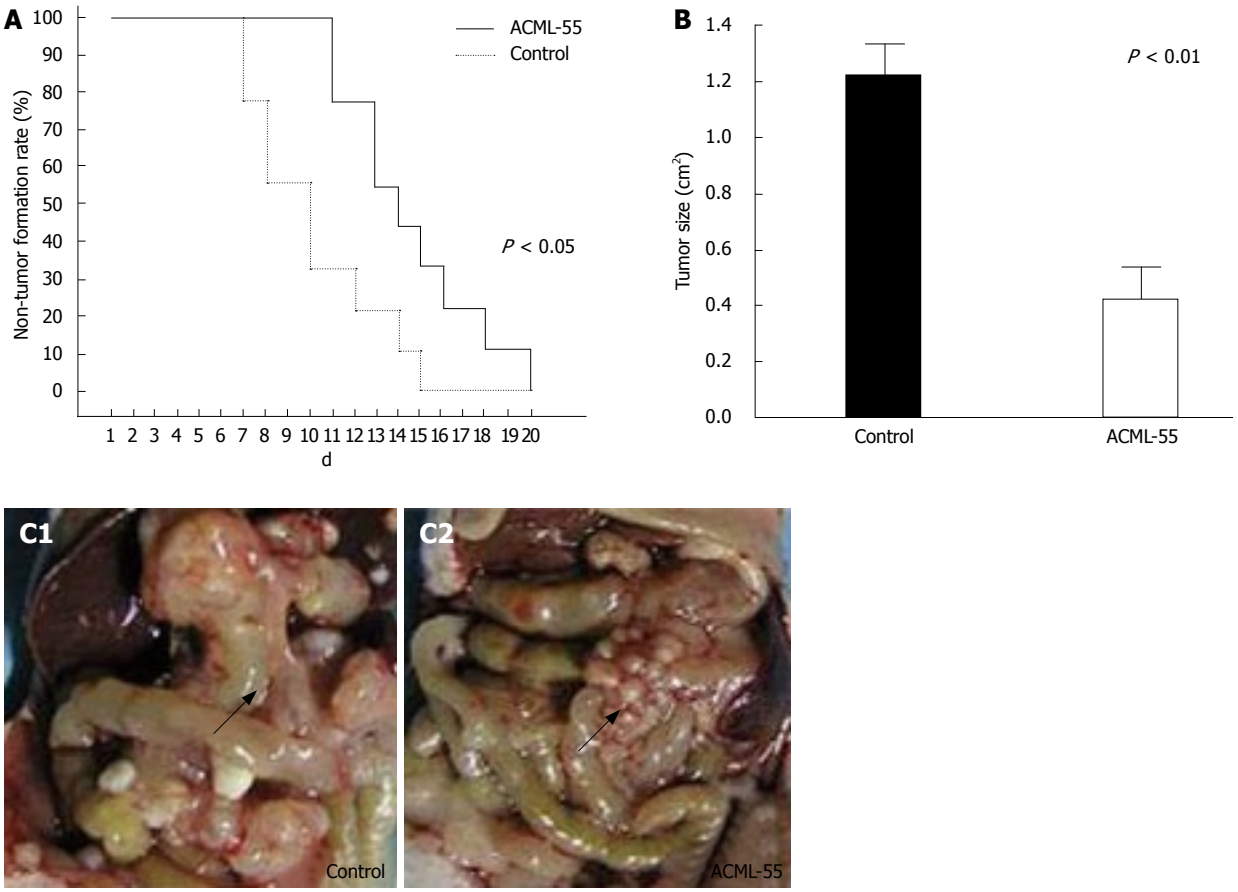


Figure 2 ACML-55 enhances tumor surveillance. **A**: ACML-55 treatment delays CT26 melanoma tumor formation. Sex- and age-matched BALB/c mice were administered orally either with 2 mg/mL (200 μL/mouse) ACML-55 or equivalent volume of PBS (control) daily for fifteen days (n = 20 for each group), followed by subcutaneous inoculation of CT26 melanoma tumor cells (5 × 10^5/mouse) on day 7 after the initiation of ACML-55 or PBS treatment. Tumor growth was recorded daily. Tumor size > 5 mm × 5 mm was considered positive. Data represents three independent experiments (P < 0.01). **B**: ACML-55 treatment inhibits tumor growth. The mean tumor size from ACML-55 and PBS-treated mice at day 20 is shown in this figure (P < 0.01). **C**: ACML-55 treatment significantly reduces intraperitoneal tumor formation. BALB/c mice were treated with ACML-55 or PBS followed by CT26 tumor cell inoculation intraperitoneally (n = 20 for each group) as described above, and tumor growth was monitored. A representative example of tumor formation is provided. Arrows point to intraperitoneal tumor.

were given ACML-55 or PBS daily for 2 wk, followed by intraperitoneal inoculation with EG7 tumor cells (EG7 tumor cells expressing OVA, 1.0 × 10^6 cell/mouse)[20]. On day 10 post-inoculation, harvested splenocytes were used for analysis of CD4+ and CD8+ T cell activation using specific activation markers. ACML-55 treatment significantly increased the number of activated CD4+ and CD8+ T cells. According to our findings, the percentage of $CD62L^{low}$ $CD44^{high}$ population in the spleen for each T cell subset was significantly higher in ACML-55 treated mice compared to those treated with PBS [14.29 ± 4.3 *vs* 7.63 ± 2.95 for CD4+ T cells, and 6.79 ± 1.41 *vs* 3.95 ± 1.97 for CD8+ T cells (Figure 3A), P = 0.0008 for CD4 and P = 0.0002 for CD8]. Representative data of the FACS profile for CD4+ and CD8+ T cells from ACML-55 or PBS treated mice are represented in Figure 3B.

ACML-55 promotes IFN-γ production in CD8+ T cells

IFN-γ has been shown previously to be a critical cytokine in tumor immunosurveillance[21]. To define the effect of ACML-55 on tumor antigen specific IFN-γ production, ACML-55 or PBS was administered to sex- and age- matched BALB/c mice (n = 6 for each group) as mentioned above for 2 wk, and then immunized in the hind footpad with CT26 tumor lysate in CFA. Eight days post-immunization, lymphocytes from the draining lymph nodes were isolated, cultured with 200 μg/mL tumor lysate for 24 h, with brefeldin A added during the last 3 h of culture. These cells were then fixed and permeabilized with 0.5% saponin for intracellular cytokine staining. The percentage of IFN-γ producing CD8+ T cells from ACML-55 treated mice (mean ± SD) was significantly higher than that of PBS treated mice (10.05 ± 2.3 and 2.30 ± 1.013, respectively, P = 0.0001; Figure 4A). An example of FACS analysis is represented in Figure 4B. In the same cultures, the percentage of IFN-γ producing CD4+ T cells from ACML-55 treated mice was also higher than those from PBS treated mice, although it did not reach significance (data not shown). ACML-55 treatment did not enhance the percentage of IFN-γ producing CD8+ T cells responding to control tumor lysate (different tumor cell line, data not shown). Our results demonstrate that ACML-55 not only enhances activation and proliferation of T cells, but also increases their capacity to produce

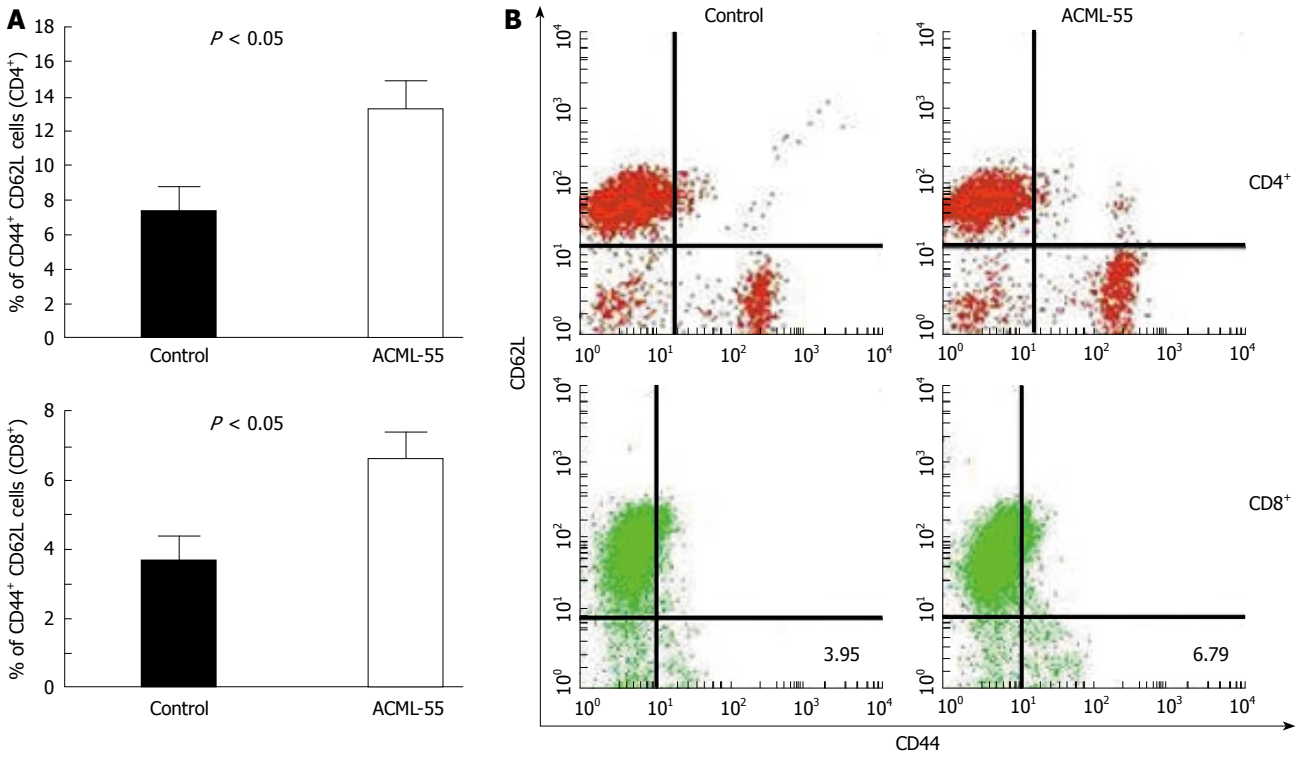


Figure 3 ACML-55 enhances CD4+ and CD8+ T cell activation. Sex- and age- matched BALB/c mice were administered orally with 2 mg/mL (200 μL/mouse) ACML-55 or equivalent volume of PBS daily for 15 days (n = 5 for each group), and on day 7, received an intraperitoneal inoculation with EG7 tumor cells (1×10^6 cells/mouse). Ten days postinoculation, splenoctyes were stained with antibodies against different surface molecules and analyzed by FACS. **A**: The percentage of activated CD4+ and CD8+ T cells (mean ± SD) is shown ($P < 0.05$); **B**: An example of the FACS profile for CD4+ and CD8+T cells is given.

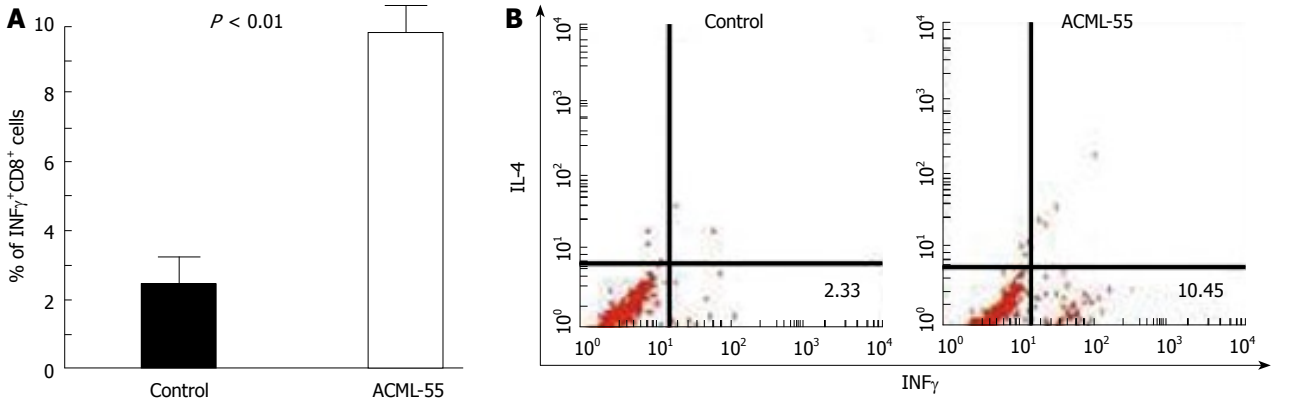


Figure 4 ACML-55 increases the number of IFN-γ+CD8+ T cells. Sex- and age- matched BALB/c mice were administered orally with 2 mg/mL (200 μL/mouse) ACML-55 or equivalent volume of PBS daily (n = 5 for each group) for fourteen days, followed by immunization with 200 μg of CT26 tumor lysate emulsified in CFA. After seven days, lymphocytes recovered from draining lymph nodes of immunized mice were cultured with 200 μg/mL of tumor lysate for 24 h, with the addition of brefeldin A to the culture for the remaining 3 h. Cells were then fixed with 2% formaldehyde and permeabilized with 0.5% saponin for intracellular IFN-γ staining. **A**: The percentage of IFN-γ producing cells (mean ± SD) from CD8+ T cells is shown; **B**: An example of intracellular cytokine staining upon gating on CD8+ T cells is shown.

IFN-γ cytokine.

ACML-55 increases the number of both NK and γδ T cells

Both NK cells and γδ T cells play a critical role in tumor immune surveillance. To test the effect of ACML-55 on these cell types, sex- and age- matched BALB/c mice were treated with ACML-55 or PBS (n = 6 for each group) as above for 2 wk, and the percentages of NK and γδ T cells in the spleen were analyzed by flow cytometry. Treatment with ACML-55 significantly increased the numbers of splenic NK cells and γδ T cells, with the percentage (mean ± SD) of NK1.1+ cells in ACML-55 treated mice *vs* control being 6.28 ± 0.90 *vs* 3.48 ± 0.77, and the percentage of CD3+ γδ+ cells for ACML-55 *vs* control being 6.51 ± 0.59 *vs* 3.85 ± 0.59, (Figure 5A, P = 0.0001). A representative result of FACS analysis for CD3 and NK1.1 as well as αβ/γδ T cell staining is presented in Figure 5B. Splenocytes from ACML-55 or PBS treated mice were cultured with anti-CD3 and anti-CD28 antibodies in the presence of brefeldin A for 6 h, and the percentage of IFN-γ + γδ+ T cells was analyzed by intracellular cytokine staining after gating TCR γδ positive cells. The results showed there is no significant difference in the percentage of

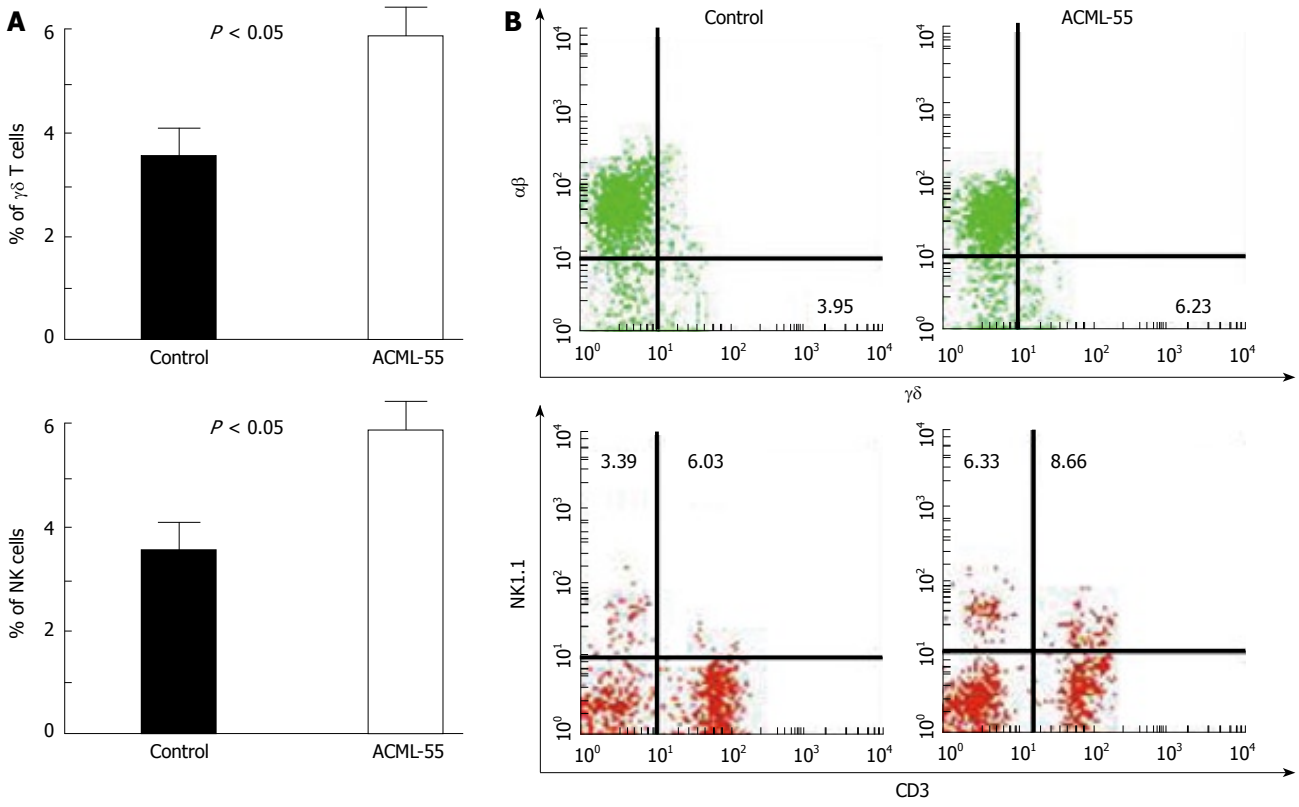


Figure 5 ACML-55 treatment increases the number of NK and γδ T cells. Sex- and age- matched BALB/c mice were administered orally with 2 mg/mL (200 μL/mouse) ACML-55 or equivalent volume of PBS daily (n = 5 for each group) for fourteen days, followed by inoculation with CT26 tumor cells intraperitoneally. Ten days post-inoculation, splenocytes were used for analysis of NK, NKT, αβ, and γδ T cells. **A**: The percentage of NK and γδ T cells (mean ± SD) is shown (P < 0.05); **B**: An example of the FACS analysis is given.

IFN-γ producing γδ T cells between two groups (data not shown). NKT cells (CD3+ NK1.1+) were also higher in ACML-55 treated mice compared to controls, although it did not reach significance. These results indicate that ACML-55 may enhance the antitumor immune response not only through modulating the adaptive immune response, but also working on innate immunity.

DISCUSSION

Extracts from European mistletoe are used widely in the treatment of cancer, but the mechanism of antitumor properties has not yet been clearly elucidated. Consumers often use EM as a complementary therapy for cancer treatment, and in some cases as an alternative to conventional cancer treatment[22,23]. Although Korean mistletoe, a subspecies of European mistletoe, has been used as a medicinal herb and shown to be cytotoxic against tumor cells as well[24], there are fewer systemic controlled studies to define the effect of EM in tumor immunity. In this study, we demonstrate that ACML-55 enhances tumor immune surveillance against both melanoma and lymphoma by regulating both innate and adaptive immune responses. We first illustrated that oral administration of ACML-55 prior to tumor inoculation could significantly delay the tumor growth and reduce tumor size (Figure 2A and B).The anti-tumor effect of ACML-55 is not limited to melanoma.

It has been shown that lymphocytes and IFN-γ both are essential components of tumor immune surveillance[25,26]. Different subsets of lymphocytes contribute to anti-tumor immune responses at different stages. CD4+ and CD8+ T cells are critical elements for the adaptive anti-tumor immune response. CD4+ T cells, especially Th1 subsets, produce IFN-γ to facilitate innate and adaptive immune responses[27]. These cells are also in favor of CD8+ T cells to develop memory response, whereas CD8+ T cells provide cytokines (IFN-γ and TNF-α) and cytotoxicity to exert their function to kill tumor cells directly. To explain the molecular mechanisms through which ACML-55 could mediate anti-tumor immune response, we sought to determine whether ACML-55 modulates the adaptive immune response. Our results indicate that ACML-55 treatment enhances activation and proliferation of both CD4+ and CD8+ T cells (Figure 3A and B). Moreover, administration of ACML-55 significantly increases the frequency of tumor antigen specific CD8+ T cells and their ability to produce higher level of IFN-γ (Figure 4A and 3B). Finally, ACML-55 treatment can make antigen specific CD8+ T cells expand more actively. The increasing number of CD8+ T cells partially contributes to the tumor resistance of ACML-55 treated mice. However, it is unclear how ACML-55 enhances the function of these T cells. It is possible that the mixture of polysaccharides in ACML-55 may activate the innate immune response

through undefined signaling pathways, such as Toll-like receptors and the downstream NF-κB pathway, which in turn helps to regulate the adaptive immune response. Consistently, it has been reported that ACML-55 enhances IL-12 production from macrophages[10] and increases nitric oxide concentration[12]. Further studies are needed to clarify the underlying mechanisms that mediate the effect of ACML-55 on the adaptive immune response.

A potential target of ACML-55 modulation within the innate immune system may be γδ T cells. These cells belong to a unique subset of T cells. They recognize protein or peptide independent of antigen presentation and function as innate like cells[28]. Our earlier studies have demonstrated that γδT cells predominantly produce IFN-γ upon activation[12,29] and play a critical role in tumor immune surveillance by providing an early source of IFN-γ[14]. Interestingly, ACML-55 treatment significantly increases the number of γδ T cells compared to those of PBS-treated mice (Figure 5). Since ACML-55 was given orally, it is possible that the effective components in ACML-55 might directly encounter γδ T cells lining the epithelial layer of the intestine resulting in their activation. In addition to γδ T cells, it has been well established that NK cells play an essential role in tumor immune surveillance. Interestingly, we found that treatment with ACML-55 also upregulates the number of NK cells upon tumor inoculation. Although the changes of NK T cells did not reach significance, the trend is clear. These results indicate that ACML-55 has multiple effects on the immune system.

Given the findings that ACML-55 could efficiently enhance several immune parameters (CD4+, CD8+ and γδ T cells), which were shown previously to be positive for tumor immune surveillance, it is likely that ACML-55 mediates its potential effects on tumor surveillance, at least in part, by upregulation these particular parameters. Future studies using different T cell subset from deficient mice will help to illuminate these questions.

In summary, we have presented clear picture that, as an active lectin from Chinese mistletoe, ACML-55 enhances tumor immune surveillance by regulating both the innate and the adaptive immune responses. Further studies are needed to define the molecular mechanisms mediating the effect of ACML-55 in tumor immunity.

COMMENTS

Background

Mistletoe is a semiparasitic plant with many unusual properties. In our prior study, a mistletoe lectin was purified from Chinese mistletoe and the effect of the active Chinese mistletoe lectin-55 (ACML-55) on human γδ T cell cytotoxicity, apoptosis and modulation of the cytokine network was reported. Although these investigations suggest that ACML-55 may modulate the immune response against tumor development, the precise mechanism by which ACML-55 regulates the immune function has not been studied systematically.

Research frontiers

In this study, an experimental model was established by hypodermically inoculating the colon cancer cell line CT26 into Balb/c mice. Intracellular cytokine staining used to detect tumor antigen specific CD8+ T cell IFN-γ production. The FACS profile for CD4+ and CD8+ T cells and NK or γδ T Cells composition and activation.

Innovations and breakthroughs

This study is investigates the potential effect of active Chinese mistletoe lectin-55 to enhance colon cancer immune surveillance through regulating both innate and adaptive immune responses.

Applications

The results demonstrate that ACML-55 can enhance colon cancer immune surveillance through regulating both innate and adaptive immune responses and also suggest that ACML-55 may be a useful complementary therapy for treating colon cancer.

Peer review

The study investigates the potential effect of ACML-55 to enhance colon cancer immune surveillance through regulating both innate and adaptive immune responses. Its scientific contents can reflect the advanced levels of basic research and the first report on Chinese mistletoe lectin-55.

REFERENCES

1 **Bussing A**, Suzart K, Bergmann J, Pfuller U, Schietzel M, Schweizer K. Induction of apoptosis in human lymphocytes treated with Viscum album L. is mediated by the mistletoe lectins. *Cancer Lett* 1996; **99**: 59-72

2 **Hajto T**. Immunomodulatory effects of iscador: a Viscum album preparation. *Oncology* 1986; **43** Suppl 1: 51-65

3 **Ribereau-Gayon G**, Dumont S, Muller C, Jung ML, Poindron P, Anton R. Mistletoe lectins I, II and III induce the production of cytokines by cultured human monocytes. *Cancer Lett* 1996; **109**: 33-38

4 **Beuth J**. Clinical relevance of immunoactive mistletoe lectin-I. *Anticancer Drugs* 1997; **8** Suppl 1: S53-S55

5 **Stein GM**, Berg PA. Mistletoe extract-induced effects on immunocompetent cells: in vitro studies. *Anticancer Drugs* 1997; **8** Suppl 1: S39-S42

6 **Fischer S**, Scheffler A, Kabelitz D. Activation of human gamma delta T-cells by heat-treated mistletoe plant extracts. *Immunol Lett* 1996; **52**: 69-72

7 **Fischer S**, Scheffler A, Kabelitz D. Stimulation of the specific immune system by mistletoe extracts. *Anticancer Drugs* 1997; **8** Suppl 1: S33-S37

8 **Hauer J**, Anderer FA. Mechanism of stimulation of human natural killer cytotoxicity by arabinogalactan from Larix occidentalis. *Cancer Immunol Immunother* 1993; **36**: 237-244

9 **Mueller EA**, Anderer FA. A Viscum album oligosaccharide activating human natural cytotoxicity is an interferon gamma inducer. *Cancer Immunol Immunother* 1990; **32**: 221-227

10 **Mueller EA**, Anderer FA. Synergistic action of a plant rhamnogalacturonan enhancing antitumor cytotoxicity of human natural killer and lymphokine-activated killer cells: chemical specificity of target cell recognition. *Cancer Res* 1990; **50**: 3646-3651

11 **Mockel B**, Schwarz T, Zinke H, Eck J, Langer M, Lentzen H. Effects of mistletoe lectin I on human blood cell lines and peripheral blood cells. Cytotoxicity, apoptosis and induction of cytokines. *Arzneimittelforschung* 1997; **47**: 1145-1151

12 **Thies A**, Nugel D, Pfuller U, Moll I, Schumacher U. Influence of mistletoe lectins and cytokines induced by them on cell proliferation of human melanoma cells in vitro. *Toxicology* 2005; **207**: 105-116

13 **Harmsma M**, Gromme M, Ummelen M, Dignef W, Tusenius KJ, Ramaekers FC. Differential effects of Viscum album extract IscadorQu on cell cycle progression and apoptosis in cancer cells. *Int J Oncol* 2004; **25**: 1521-1529

14 **Lee HS**, Kim YS, Kim SB, Choi BE, Woo BH, Lee KC. Isolation and characterization of biologically active lectin from Korean mistletoe, Viscum album var. Coloratum. *Cell Mol Life Sci* 1999; **55**: 679-682

15 **Gong F**, Ma Y, Ma A, Yu Q, Zhang J, Nie H, Chen X, Shen B, Li N, Zhang D. A lectin from Chinese mistletoe increases gammadelta T cell-mediated cytotoxicity through induction of caspase-dependent apoptosis. *Acta Biochim Biophys Sin* (Shanghai) 2007; **39**: 445-452

16 **Gao Y**, Yang W, Pan M, Scully E, Girardi M, Augenlicht LH,

Craft J, Yin Z. Gamma delta T cells provide an early source of interferon gamma in tumor immunity. *J Exp Med* 2003; **198**: 433-442
17 **Yin Z**, Zhang DH, Welte T, Bahtiyar G, Jung S, Liu L, Fu XY, Ray A, Craft J. Dominance of IL-12 over IL-4 in gamma delta T cell differentiation leads to default production of IFN-gamma: failure to down-regulate IL-12 receptor beta 2-chain expression. *J Immunol* 2000; **164**: 3056-3064
18 **Jung ML**, Baudino S, Ribereau-Gayon G, Beck JP. Characterization of cytotoxic proteins from mistletoe (Viscum album L.). *Cancer Lett* 1990; **51**: 103-108
19 **Gao Y**, Tao J, Li MO, Zhang D, Chi H, Henegariu O, Kaech SM, Davis RJ, Flavell RA, Yin Z. JNK1 is essential for CD8+ T cell-mediated tumor immune surveillance. *J Immunol* 2005; **175**: 5783-5789
20 **Gorelik L**, Flavell RA. Immune-mediated eradication of tumors through the blockade of transforming growth factor-beta signaling in T cells. *Nat Med* 2001; **7**: 1118-1122
21 **Ikeda H**, Old LJ, Schreiber RD. The roles of IFN gamma in protection against tumor development and cancer immunoediting. *Cytokine Growth Factor Rev* 2002; **13**: 95-109
22 **deVere White RW**, Hackman RM, Soares SE, Beckett LA, Li Y, Sun B. Effects of a genistein-rich extract on PSA levels in men with a history of prostate cancer. *Urology* 2004; **63**: 259-263
23 **deVere White RW**, Hackman RM, Soares SE, Beckett LA, Sun B. Effects of a mushroom mycelium extract on the treatment of prostate cancer. *Urology* 2002; **60**: 640-644
24 **Khil LY**, Kim W, Lyu S, Park WB, Yoon JW, Jun HS. Mechanisms involved in Korean mistletoe lectin-induced apoptosis of cancer cells. *World J Gastroenterol* 2007; **13**: 2811-2818
25 **Dunn GP**, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* 2004; **21**: 137-148
26 **Li NL**, Nie H, Yu QW, Zhang JY, Ma AL, Shen BH, Wang L, Bai J, Chen XH, Zhou T, Zhang DQ. Role of soluble Fas ligand in autoimmune diseases. *World J Gastroenterol* 2004; **10**: 3151-3156
27 **Shankaran V**, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, Schreiber RD. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* 2001; **410**: 1107-1111
28 **Carding SR**, Egan PJ. Gammadelta T cells: functional plasticity and heterogeneity. *Nat Rev Immunol* 2002; **2**: 336-345
29 **Yin Z**, Chen C, Szabo SJ, Glimcher LH, Ray A, Craft J. T-Bet expression and failure of GATA-3 cross-regulation lead to default production of IFN-gamma by gammadelta T cells. *J Immunol* 2002; **168**: 1566-1571

S- Editor Zhong XY **L- Editor** Negro F **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5282

World J Gastroenterol 2008 September 14; 14(34): 5282-5289
World Journal of Gastroenterology ISSN 1007-9327

CLINICAL RESEARCH

Incidence of gallstone disease in Italy: Results from a multicenter, population-based Italian study (the MICOL project)

Davide Festi, Ada Dormi, Simona Capodicasa, Tommaso Staniscia, Adolfo F Attili, Paola Loria, Paolo Pazzi, Giuseppe Mazzella, Claudia Sama, Enrico Roda, Antonio Colecchia

Davide Festi, Giuseppe Mazzella, Claudia Sama, Enrico Roda, Antonio Colecchia, Department of Internal Medicine and Gastroenterology, University of Bologna, Bologna 40138, Italy
Ada Dormi, Department of Clinical Medicine and Biotechnology, University of Bologna, Bologna 40138, Italy
Simona Capodicasa, Tommaso Staniscia, Department of Medicine and Aging, University of Chieti, Chieti 66100, Italy
Adolfo F Attili, Department of Gastroenterology, University "La Sapienza", Roma 00010, Italy
Paola Loria, Department of Internal Medicine, University of Modena, Modena 41100, Italy
Paolo Pazzi, Department of Internal Medicine, S. Anna Hospital, Ferrara 44100, Italy
Author contributions: Festi D, Attili AF, Pazzi P, Loria P, Sama C, Roda E designed research; Festi D, Attili AF, Pazzi P, Loria P, Colecchia A performed research; Capodicasa S, Staniscia T, Dormi A analyzed data; Festi D wrote the paper.
Correspondence to: Davide Festi, MD, Dipartimento di Medicina Interna e Gastroenterologia, Policlinico S. Orsola-Malpighi, Via Massarenti 9, Bologna 40138, Italy. davide.festi@unibo.it
Telephone: +39-51-6364123 Fax: +39-51-6364123
Received: January 3, 2008 Revised: August 13, 2008
Accepted: August 20, 2008
Published online: September 14, 2008

Abstract

AIM: To evaluate gallstone incidence and risk factors in a large population-based study.

METHODS: Gallstone incidence and risk factors, were evaluated by structured questionnaire and physical examination, respectively, in 9611 of 11 109 (86.5%) subjects who were gallstone-free at the cross-sectional study.

RESULTS: Six centers throughout Italy enrolled 9611 subjects (5477 males, 4134 females, aged 30-79 years), 9517 of whom were included into analysis: 424 subjects (4.4%) had gallstones and 61 (0.6%) had been cholecystectomized yielding a cumulative incidence of 0.67% per year (0.66% in males, 0.81% in females). Increasing age, a high body mass index (BMI), a history of diabetes, peptic ulcer and angina, and low cholesterol and high triglyceride levels were identified as risk factors in men while, in females, the only risk factors were increasing age and a high BMI. Increasing age and pain in the right hypochondrium in men and increasing age in females were identified as predictors of gallstones. Pain in the epigastrium/right hypochondrium was the only symptom related to gallstones; furthermore, some characteristics of pain (forcing to rest, not relieved by bowel movements) were significantly associated with gallstones. No correlation was found between gallstone characteristics and clinical manifestations, while increasing age in men and increasing age and BMI in females were predictors of pain.

CONCLUSION: Increasing age and BMI represent true risk factors for gallstone disease (GD); pain in the right hypochondrium and/or epigastrium is confirmed as the only symptom related to gallstones.

Key words: Gallstone disease; Ultrasonography; Epidemiology; Prevalence; Incidence; Abdominal pain; Cholecystectomy; Body mass index

Peer reviewer: Serdar Karakose, PhD, Professor, Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey

Festi D, Dormi A, Capodicasa S, Staniscia T, Attili AF, Loria P, Pazzi P, Mazzella G, Sama C, Roda E, Colecchia A. Incidence of gallstone disease in Italy: Results from a multicenter, population-based Italian study (the MICOL project). *World J Gastroenterol* 2008; 14(34): 5282-5289 Available from: URL: http://www.wjgnet.com/1007-9327/14/5282.asp DOI: http://dx.doi.org/10.3748/wjg.14.5282

INTRODUCTION

Gallstone disease (GD) is a very common gastrointestinal disorder, presents mainly in the Western world[1,2]. Although this disease has a low mortality rate, its economic and health impact is significant due to its high morbidity. In fact, GD is one of the most common abdominal conditions for which patients in developed countries are admitted to hospitals[3], and this frequency has increased in Western countries since the 1950s[4]. GD is considered "a surgical disease"

since only a cholecystectomy is capable of definitively curing the disease[5]. However, since the introduction of laparoscopic cholecystectomy in the early 90s, which is considered a safe treatment for GD[6], a possible unjustified increase in surgical procedures has been observed[7]. Therefore, there is the need for more knowledge of the epidemiological characteristics of GD in order to better identify therapeutic strategies.

The availability of ultrasonography (US) as an accurate tool for gallstone diagnosis has allowed the evaluation of gallstone prevalence by means of epidemiological surveys of the general population, both in Eastern and Western countries[8,9]. Furthermore, these studies, as well as case-control studies, have allowed the identification of the factors most frequently associated with GD, i.e. increasing age, female sex, familial history of GD, number of pregnancies, obesity, or type 2 diabetes[10].

However, only a few prospective US surveys, mainly in Europe[11-14], have been carried out which aimed measuring the gallstone incidence rate and risk factors for the disease. The knowledge of disease risk factors is crucial to carrying out primary or secondary preventive programs.

The Multicenter Italian Study on Epidemiology of Cholelithiasis (the MICOL project) is a population-based cross-sectional study which began in 1985 and extended since 1998 and it was designed to obtain an overview of the distribution of GD in Italy according to different regions and ages[15]. Results on prevalence distribution, associated factors, and clinical manifestations have been extensively reported in previous articles[15-17].

This article reports the incidence rate and risk factors for GD which were evaluated in six units belonging to the MICOL project.

MATERIALS AND METHODS

Study design

The MICOL project is a population-based, cross-sectional study carried out in 8 Italian regions by different operative units. Complete details on the study protocol have been published elsewhere[15]. The project plan includes 2 cross-sectional surveys; the first began in 1985 and was completed in 1988 and the second survey was carried out on the same subjects 10 years later in order to estimate the incidence of GD as well as its natural history. Seven of the original operative units were able to complete the second survey.

Subjects

After 10 years, all participants in the first survey were invited to a follow-up examination. Preliminarily, using the electoral lists, subjects who had died or moved were identified. The remaining subjects received a standardized invitation for a general health examination and an US examination of the upper abdomen. Subjects who did not respond were invited again; those who refused to participate or those who did not respond at all were contacted by telephone.

Screening protocol

The re-examination took place between 1995 and 1998. Subjects who were found to have had gallstones or a previous cholecystectomy for gallstones at the first survey were studied separately, the major aim of this re-examination being evaluation of the natural history of gallstones.

Similarly to what had been carried out in the first survey[15], the screening protocol included upper abdominal US, physical examination, fasting blood specimen collection, and administration of a precoded questionnaire. This questionnaire inquired about family and personal history, dietary habits, past and current use of medications, and the presence of comorbidities (diabetes, chronic heart disease, liver cirrhosis, peptic ulcer); the questionnaire also included a specific section aimed at assessing the occurrence/presence of abdominal symptoms (details of the structure of the questionnaire as well as details on its items have been published elsewhere[17]). The questionnaire was administered by trained interviewers selected from the medical staff of each operative unit. Upper abdominal US was performed thereafter by independent physicians.

Statistical analysis

The incidence rate for new-onset gallstones was determined from baseline to the second examination, after adjustment for age and gender. Logistic regression analysis was carried out providing risk factors and Odd Ratios (OR) for developing gallstones; Cox proportional hazard regression was used to calculate hazard ratios.

According to previous results regarding associated factors obtained from the gallstone prevalence study[15] and from available literature data[8,10,18], different factors were evaluated by means of logistic regression analysis as possible risk factors for GD including age, gender, educational level, family history of gallstones, body mass index (BMI) (measured both at the prevalence and the incidence studies), number of pregnancies, history of diets, diabetes, peptic ulcer, inflammatory bowel disease, liver cirrhosis, smoking, coffee drinking, and serum levels of total cholesterol, HDL-cholesterol, and triglycerides.

To identify the possible role of the disease status on clinical presentation, patients were subsequently classified into 4 groups according to their disease status: gallstone-free subjects (GF), patients with gallstones not previously diagnosed (GNDP), patients with previously diagnosed gallstones (GPD), and patients with a history of cholecystectomy for gallstones (CC).

Logistic regression models were created for the latter 3 groups, using the gallstone-free subjects as the comparison group.

As a consequence of the results of the logistic regression models, patients referring pain located at the right hypochondrium or epigastrium were selected and further evaluated.

A multivariate analysis was subsequently carried out using logistic regression and, for each gallstone group, a model was created using the gallstone-free subjects

as the comparison group. SPSS (ver.9.0 for windows) statistical software was used. A probability of $P < 0.05$ was considered significant.

RESULTS

Subjects

Out of the 12709 potentially enrolled subjects belonging to the seven participating units, 502 died and 1098 transferred. Among the dead subjects, one case of gallbladder cancer was observed, which translates in a mortality rate for this cause of 2.5/10000 per year.

Of the 11109 enrolled subjects, 9611 (5477 males, 4134 females, aged 30-79 years) were evaluated (86.5%). The attendance rate was different in different age classes, greater in younger than in older subjects (83% in the 40-60 years old group and 74% in the > 60 years old group) while no difference was documented between the sexes (78% in males, 89% in females). The mean follow-up period was 8 years.

On the basis of US diagnosis, 9517 (5428 males, 4089 females) were suitable for analysis since, in 94 cases, the US diagnosis was biliary sludge (35 cases) or gallbladder polyps (40 cases); in 19 cases, the US diagnosis was uncertain. These 94 cases were excluded from the study, since no definitive data are available in literature regarding these aspects as true gallstones[5,10,11].

Four hundred and twenty-four subjects (206 males, 218 females) (4.4%) were found to have gallstones and 61 (26 males, 35 females) (0.6%) had been cholecystectomized for gallstones during the follow-up period.

The cumulative incidence rate of GD thus was 0.67% per year [0.66% per year in males, 0.81% per year in females (Table 1)]. Table 1 also shows the incidence rate among the different operative units.

Table 2 reports the morphological characteristics of gallstones; incident gallstones were more frequently small in size (77.9% less than 1.5 cm); no difference was observed between males and females in terms of gallstone number or size.

According to the disease status evaluation, of the 9517 subjects enrolled in the study, 9032 (94.5%) were GF; 312 (3.3%) were GNPD, 112 (1.2%) were GPD, and 61 (0.6%) were CC.

Table 1 Incidence of GD in the different operative units of the MICOL project

Operative unit	Overall % yr	Male	Female
Bologna Loiano	0.50	0.50	0.50
Bologna Brisighella	0.61	0.60	0.80
Bolzano	0.82	0.50	1.10
Castellana Grotte	0.75	0.70	0.80
Tivoli	0.64	0.50	0.70
Modena San Lazzaro	0.43	0.40	0.40
Modena Madonnina	0.86	0.60	1.20
Overall	0.67	0.66	0.81

Table 2 Characteristics of newly developed gallstones

Characteristics		%	*P*
Overall			
Number	Single	48.90	< 0.0001
	Multiple	51.10	
Size	≤ 1.5 cm	77.90	< 0.0001
	≥ 1.5 cm	22.10	
Male			
Number	Single	44.80	< 0.0001
	Multiple	55.20	
Size	≤ 1.5 cm	78.70	< 0.0001
	≥ 1.5 cm	21.30	
Female			
Number	Single	52.30	< 0.0001
	Multiple	47.70	
Size	≤ 1.5 cm	77.10	< 0.0001
	≥ 1.5 cm	22.90	

Table 3 Risk factors for GD

Factor	Coefficient	SE	*P*
Men			
Age	0.0405	0.0071	< 0.000
BMI	0.0536	0.0195	< 0.006
HDL cholesterol	-0.0118	0.0059	< 0.040
Cholesterol	-0.0034	0.0016	< 0.030
Triglycerides	0.0004	0.0001	< 0.007
Diabetes	0.5293	0.2220	< 0.010
Peptic ulcer	0.4378	0.1753	< 0.010
Women			
Age	0.0279	0.0073	< 0.000
BMI	0.0178	0.0147	< 0.000

Logistic regression analysis: regression coefficients, corresponding SE, and *P* values for single factors and interactions, significantly associated with gallstones.

Risk factors and predictors for GD

Risk factors for GD: The results of the logistic regression analysis carried out to identify the risk factors for GD are shown in Table 3.

Among the factors considered, in males, increasing age ($P < 0.0001$), a high BMI ($P < 0.006$), a history of diabetes ($P < 0.01$) and of peptic ulcer ($P < 0.01$), low levels of total ($P < 0.03$) and HDL ($P < 0.04$) cholesterol, and high levels of triglycerides ($P < 0.007$) were identified as risk factors.

In females, only increasing age ($P < 0.00001$) and a high BMI ($P < 0.0001$) were identified as risk factors for GD.

When evaluating the risk factor distribution according to the classification of the patient's clinical status (Table 4), no differences were observed in terms of age, BMI, and biochemical parameters while the presence of comorbidities increased among the three groups; in particular in males, diabetes, peptic ulcer, and myocardial infarction while, in females, peptic ulcer and myocardial infarction.

Predictors of GD: The evaluation of predictive factors for the presence of GD (Table 5) in patients with abdominal pain indicated that these were increasing age ($P < 0.04$) and pain in the right hypochondrium ($P < 0.03$) in males with gallstones and only increasing age

Table 4 Risk factors for GD

Factor OR (95% CI)	Gallstones not previously diagnosed (*n* = 312)	Gallstones previously diagnosed (*n* = 112)	Cholecystectomized (*n* = 61)
Men			
Age	1.03 (1.01-1.05)	1.05 (1.02-1.07)	1.01 (0.97-1.06)
BMI	1.06 (1.01-1.11)	1.03 (0.97- 1.10)	1.07 (0.96-1.06)
HDL cholesterol	0.99 (0.97-1.004)	0.98 (0.96-1.00)	1.00 (0.97-1.03)
Cholesterol	0.99 (0.99-1.00)	0.99 (0.98-0.99)	1.00 (0.99-1.01)
Triglycerides	1.00 (0.99-1.00)	1.00 (1.00-1.01)	0.99 (0.99-1.00)
Diabetes	1.75 (1.04-2.96)	1.60 (0.77-3.32)	2.72 (0.89-8.33)
Peptic ulcers	1.11 (0.69-1.77)	2.53 (1.52-4.20)	3.38 (1.48-7.72)
Myocardial infarction	0.99 (0.99-1.00)	1.27 (0.38-4.23)	1.43 (0.18-11.18)
Women			
Age	1.01 (1.00-1.03)	1.04 (1.02-1.07)	0.98 (0.94-1.02)
BMI	1.08 (1.04-1.12)	1.05 (1.00-1.11)	1.07 (0.99-1.15)
HDL cholesterol	1.00 (0.99-1.01)	0.98 (0.97-1.00)	0.98 (0.95-1.00)
Cholesterol	0.99 (0.99-1.00)	1.00 (0.99-1.00)	1.00 (1.00-1.01)
Triglycerides	1.00 (0.99-1.00)	0.99 (0.99-1.00)	0.99 (0.99-1.00)
Diabetes	1.10 (0.58-2.09)	0.48 (0.14-1.58)	1.00 (0.22-4.49)
Peptic ulcers	0.67 (0.34-1.34)	0.70 (0.28-1.76)	3.55 (1.57-8.04)
Myocardial infarction	1.83 (0.99-1.00)	3.15 (0.70-14.18)	12.82 (2.69-60.94)

OR calculated by logistic regression analysis; regression coefficients, corresponding standard errors, and *P* values for single factors and interactions, significantly associated with gallstones.

Table 5 Predictors of GD in patients with abdominal pain

Variables	Gallstones			Cholecystectomized		
	P	OR	95% CI	*P*	OR	95% CI
Male						
Age	0.04	1.033	1.00-1.06	0.02	1.05	1.00-1.10
Pain in the right hypochondrium	0.03	2.35	1.04-5.3	0.62		
Pain in the epigastrium	0.68	1.19		0.66		
Female						
Age	0.000	1.053	1.02-1.08	0.50		
Pain in the right hypochondrium	0.36			0.01	14.64	1.83-116.81
Pain in the epigastrium	0.09			0.002	24.19	3.18-183.92

Table 6 Predictors of GD according to the presence of concomitant diseases

Variables	Gallstones			Cholecystectomized		
	P	OR	95% CI	*P*	OR	95% CI
Male						
Age	0.0000	1.03	1.02-1.05	0.385		
Diabetes	0.0008	2.09	1.36-3.22	0.055		
Peptic ulce	0.04	1.44	1.01-2.05	0.004	3.28	1.45-7.41
Cirrhosis	0.002	1.79	1.08-2.97	0.000	5.73	2.13-15.42
Female						
Age	0.0000	1.03	1.01-1.04	0.409		
Diabetes	0.522			0.208		
Peptic ulcer	0.186			0.004	3.19	1.43-7.08
Cirrhosis	0.388			0.742		

($P < 0.0001$) in females with gallstones; in cholecystectomized male patients, the only factor was increasing age ($P < 0.02$) while, in cholecystectomized females, pain in the right hypochondrium ($P < 0.01$) and the epigastrium ($P < 0.002$) were the predictive factors.

Considering only the effect of comorbidities, the evaluation of predictive factors for the presence of GD indicated that, in males with gallstones, these factors were increasing age ($P < 0.0001$), the presence of diabetes ($P < 0.001$), peptic ulcer ($P < 0.04$), and liver cirrhosis ($P < 0.002$) while, in females with gallstones, only increasing age ($P < 0.0001$) (Table 6) was a predictor of GD; in cholecystectomized male patients peptic ulcer ($P < 0.004$) and liver cirrhosis ($P < 0.0001$) were predictors while, in female patients, only peptic ulcer ($P < 0.004$) appeared as a predictor of the disease.

Clinical manifestation of GD

The distributions of abdominal symptoms in the four groups are reported in Table 7. No differences among the groups were documented in terms of frequency for non-specific symptoms, with the exception of nausea, while pain in the epigastrium and the right hypochondrium were related to GD and varied according to the disease categories.

In Table 8, the characteristics of abdominal pain in the considered groups are reported: some characteristics resulted significantly associated with GD, showing a progressive increase in the ORs throughout the disease categories; pain necessitating rest, pain not relieved by bowel movements and the presence of clinical signs of gallstone complications (jaundice, fever, dark urine).

Predictive factors for biliary pain

No correlation was found between US gallstone characteristics (number and size) and their presence or clinical manifestation.

In males with GD, increasing age was the only predictor of biliary pain (mainly pain in the right hypochondrium) while, in females, BMI was also related to pain (mainly pain in the epigastrium). No correlation was found between comorbidities and GD.

Table 7 Distribution of symptoms (and unadjusted ORs) in gallstone-free subjects, patients with gallstones not previously diagnosed, patients with a previous diagnosis of gallstones, and patients with a history of cholecystectomy for gallstones

Symptoms (%) OR (95% CI)	Gallstone-free subjects (n = 9001)	Gallstones not previously diagnosed (n = 312)	Gallstones previously diagnosed (n = 112)	Cholecystectomized (n = 61)
Belching	19.1	19.5	27.2	23.1
		1.03 (0.75-1.39)	1.27 (0.86-1.89)	0.92 (0.48-1.75)
Heartburn	32.2	33.1	37.5	44.2
		1.12 (0.85-1.39)	0.89 (0.61-1.30)	1.29 (0.72-2.03)
Nausea	12.3	14.3	18.4	26.9
		1.22 (0.82-1.82)	1.48 (0.90-2.42)	2.40 (1.19-4.83)
Vomiting	7.2	7.7	9.6	9.6
		0.99 (0.59-1.64)	0.79 (0.41-1.53)	0.97 (0.40-2.33)
Bloated feeling after meals	37.2	38.6	44.9	48.1
		1.24 (0.95-1.61)	1.12 (0.78-1.63)	1.24 (0.69-2.21)
Epigastric discomfort	33.8	33.1	40.4	38.5
		0.96 (0.72-1.26)	1.26 (0.87-1.83)	0.87 (0.48-1.59)
Bitter taste in the morning	36.4	35.4	41.2	44.2
		0.93 (0.71-1.21)	0.88 (0.61-1.26)	1.08 (0.61-1.88)
Heavy feeling on the right side	19.7	18.8	25.7	28.8
		0.94 (0.68-1.29)	1.14 (0.77-1.71)	1.09 (0.58-2.05)
Heavy feeling in the epigastrium	22.7	21.3	25.0	26.9
		0.83 (0.60-1.15)	0.81 (0.53-1.24)	0.67 (0.34-1.33)
Pain in the epigastrium	11.2	24.7	39.0	51.2
		1.5 (1.17-2.4)	3.0 (2.6-5.7)	29.9 (7.0-221.3)
Pain in the right hipocondrium	15.8	23.7	39.0	46.5
		1.7 (1.3-2.8)	3.9 (3.1-7.4)	32.1 (9.3-238.9)
Intolerance to fatty or fried foods	24.0	5	4.4	3.4
		0.99 (0.63-1.55)	0.94 (0.19-1.79)	1.04 (0.5-2.14)

DISCUSSION

The present study evaluated incidence and risk factors for GD in a large population from various Italian regions, thus providing a more detailed picture of the epidemiological characteristics of this disease. Incidence was higher in females than in males and increased with age. In our population, the risk factors for GD in males were increasing age, BMI, concomitant diseases such as diabetes, liver cirrhosis, peptic ulcer, coronary disease, HDL and total cholesterol, and high levels of triglycerides while, in females, only increasing age and BMI. Increasing age, pain in the right hypochondrium/epigastrium, and the presence of concomitant diseases are predictors of GD. Pain in the right hypochondrium or epigastrium was the only symptom associated with GD; symptom severity increased as a function of the natural history of the disease. Increasing age in men and aging and BMI in females were the only predictive factors for the eventual presence of symptoms.

GD is a very common gastrointestinal disorder mainly in the Western world[1,2]; although this disease has a low mortality rate, its economic and health impact is significant due to its high morbidity. In fact, GD is one of the most common abdominal conditions for which patients are admitted to hospitals in developed countries[3]. Knowledge of disease epidemiology is therefore crucial in managing this disorder, not only for planning preventive programs, but also for the identification of the best therapeutic strategy. Several US-based surveys have been carried out in Europe[19-23] and in North[24,25] and South[26] America as well as in Asia[27,28], indicating prevalence rates for GD ranging from 5.9%[20] to 21.9%[22]. However, few studies[11-14] have been carried out to evaluate incidence and risk factors of GD, mainly due to the difficulties in following up large populations for several years. The MICOL study was designed to obtain a general overview of GD in Italy, investigating GD in terms of prevalence, incidence, risk factors, and natural history[15]. In the present study, a large general population was evaluated with the objective of identifying gallstone incidence and risk factors as well as the morphological and clinical characteristics of newly developed gallstones. Incidence was higher than that measured in previous Italian studies[12-14]; these differences could be related to the small population sample evaluated in the earlier studies. Furthermore, prevalence was also higher than in Denmark (4.5% and 5.8%, in males and females, respectively)[11]. Differences in research design may justify these differences even it is not possible to exclude the role of environmental factors.

In the present study, the response rate was higher (79%) than that reported in other GD incidence surveys performed in the north (63.7)[12] and in the centre (73.5%)[14] of Italy and similar to that observed in Denmark (82.8%)[11] and in southern Italy (87.7%)[13]; this percentage indicates a high adherence of the target population to the epidemiological study.

The participation rate was also higher in the present incidence study (79%) than in the previous study evaluating GD prevalence (64.4%)[15]; this difference could be related to a possible self-selection of patients or to an effect of dilution in the prevalence study since, in that study, 14 units participated while, in the present study, only 7 units were able to adhere to the protocol.

Table 8 Distribution of pain characteristics (and unadjusted ORs) in subjects reporting abdominal pain

Characteristics of Pain	Gallstone-free subjects	Gallstones not previously diagnosed (*n* = 312)	Gallstones previously diagnosed (*n* = 112)	Cholecystectomized (*n* = 61)
Radiation				
Not radiated (%)	53.0	55.4	43.9	53.5
Radiated (%)	37.4	41.5	45.6	46.5
OR (95% CI)		0.97 (0.63-1.50)	1.11 (0.74-1.66)	0.94 (0.57-1.53)
Duration[1]				
< ½ h (%)	30.3	35.5	7.8	16.3
½-1 h (%)	21.1	19.4	21.6	16.3
1-3 h (%)	16.0	19.4	31.4	18.6
> 3 h (%)	32.6	25.8	39.2	48.8
OR (95% CI)		0.89 (0.71-1.10)	1.35 (1.05-1.75)	1.27 (0.96-1.69)
Tollerability				
Not forced to rest (%)	80.5	68.3	62.0	25.6
Forced to rest (%)	19.5	31.7	38.0	74.4
OR (95% CI)		1.98 (1.11-3.51)	2.26 (1.22-4.18)	9.88 (4.82-20.27)
Relationship with meals				
Yes (%)	13.2	9.7	10.2	9.5
No (%)	86.8	90.3	89.8	90.5
OR (95% CI)		1.84 (0.64-5.23)	1.30 (0.47-3.63)	1.29 (0.37-4.47)
During meals				
Yes (%)	2.6	1.7	6.1	
No (%)	97.4	98.3	93.9	100
OR (95% CI)		0.94 (0.12-7.32)	0.38 (0.10-1.48)	129.09 (0.90-4.62)
Soon after meals				
Yes (%)	32.3	24.6	43.1	40.5
No (%)	67.7	75.4	56.9	59.5
OR (95% CI)		1.61 (0.84-3.09)	0.70 (0.37-1.31)	0.63 (0.31-1.25)
After heavy meals				
Yes (%)	24.5	23.8	26.0	23.8
No (%)	75.5	76.2	74.0	76.2
OR (95% CI)		1.02 (0.55-1.91)	0.73 (0.38-1.38)	1.82 (0.77-4.33)
Relieved by bowel movements				
Yes (%)	41	30	28	12.2
No (%)	59	70	72	87.8
OR (95% CI)		2.02 (1.07-3.82)	1.76 (0.99-3.10)	5.35 (2.07-13.76)
Clinical signs of gallstone complications				
Yes (%)	29.9	36.4	47.7	69.4
No (%)	70.1	63.6	52.3	30.7
OR (95% CI)		1.31 (0.69-2.46)	2.13 (1.16-3.94)	5.28 (2.56-10.9)

[1]The category "< ½ h" has been taken as baseline.

We documented variability between the different units in terms of incidence; this difference could be related to the role of environmental factors (life style, dietary habit, *etc*.) even if we were unable to identify any possible difference between the different operative units.

Gender, increasing age, and BMI were confirmed as true risk factors for GD; in males, low levels of cholesterol, high levels of triglycerides, and the presence of co-morbidities such as diabetes, peptic ulcer, angina, and liver cirrhosis represented additional risk factors. These results further confirm the importance of environmental factors in gallstone development, possibly related to an unhealthy life style. In fact, recent epidemiological studies have suggested that GD may be included in those disorders which characterize the metabolic syndrome[29,30]. In particular, a close relationship between obesity and cardiovascular disease (two of the more characteristic features of the metabolic syndrome) and GD has been identified[31,32].

We did not confirm a family history of gallstones, dieting, the number of pregnancies, and the use of contraceptive pills, which were found to be significantly associated with GD in the prevalence study as risk factors[16]. We are unable to interpret the significance of this result; however, the data available on some risk factors for GD are frequently conflicting[8,10]. The prospective cohort design of the present study and the use of US as the diagnostic tool have reduced the possibility of a recall and other information bias; furthermore, the high response rate makes selection bias unlikely.

An important result of the present study is related to the clinical manifestation of incident gallstones. In fact, we have confirmed the observation made in the prevalence study[15,17] that pain in the right hypochondrium and/or epigastrium is the only symptom significantly associated with gallstones while the so-called "non-specific biliary symptoms", i.e. dyspeptic symptoms, showed the same frequency in gallstone-free subjects and GD patients.

We have also confirmed our previous observation[17] concerning the usefulness of splitting the subjects

enrolled in the study into 4 categories reproducing the different stages of GD (absence of disease, silent disease, overt disease, severe disease); in fact, the frequency and severity of the clinical symptoms and the signs of GD increased throughout the 3 disease categories.

Furthermore, for those characteristics that are an expression of the degree of pain severity (pain forcing to rest, presence of clinical signs of gallstone complications) they progressively increased from silent gallstones to symptomatic gallstones to cholecystectomized patients for gallstones, suggesting that the natural history of GD moves from a silent to a clinically evident stage; this is also true for newly developed gallstones. This information may be useful in choosing the best therapeutic strategy in gallstone patients since surgical treatment is indicated only in true symptomatic gallstone patients[5,33].

Finally, we were unable to identify any predictive factor for the presence of biliary pain in terms of gallstone characteristics (number and size), while increasing age in males and a high BMI in females were related to the presence of biliary pain. These results are in agreement with some[34], but in disagreement with others[35,36].

In conclusion, this study provides data on the gallstone incidence and risk factors for GD in a large free-living population; the incidence rate is higher in females and it increases with age. Increasing age and BMI represent true risk factors for GD; pain in the right hypochondrium and/or epigastrium is confirmed as the only symptom related to GD; pain, as well as its characteristics of disease severity, increases in severity and frequency throughout the different stages of GD (from silent to severe disease). This information may help physicians in clinical decision making.

COMMENTS

Background

Gallstone incidence and risk factors are poorly understood and the relationship between gallstone presence and its clinical manifestations is debated.

Research frontiers

Gallstone incidence, risk factors, and clinical manifestations.

Innovations and breakthroughs

The present paper provides important information on gallstone incidence and risk factors and clearly identifies the clinical manifestations of the disease.

Applications

The results of the present paper can be useful for the early recognition of gallstones and for deciding the most appropriate therapeutic management according to the clinical presentation. Furthermore, preventive strategies can be identified and planned according to these results.

Peer review

This manuscript is a multicenter Italian study giving information about the incidence of gallstone disease in Italy. The results of this study can provide information for the comparison of Italian population results with the other nations' results.

REFERENCES

1 **Sandler RS**, Everhart JE, Donowitz M, Adams E, Cronin K, Goodman C, Gemmen E, Shah S, Avdic A, Rubin R. The burden of selected digestive diseases in the United States. *Gastroenterology* 2002; **122**: 1500-1511

2 **Aerts R**, Penninckx F. The burden of gallstone disease in Europe. *Aliment Pharmacol Ther* 2003; **18** Suppl 3: 49-53

3 **Russo MW**, Wei JT, Thiny MT, Gangarosa LM, Brown A, Ringel Y, Shaheen NJ, Sandler RS. Digestive and liver diseases statistics, 2004. *Gastroenterology* 2004; **126**: 1448-1453

4 **Bateson MC**. Gallstones and cholecystectomy in modern Britain. *Postgrad Med J* 2000; **76**: 700-703

5 **Roda E**, Festi D, Lezoche E, Leushner U, Paumgartner G, Sauerbruch T. Strategies in the treatment of biliary stones. *Gastroenterol Int* 2000; **13**: 7-15

6 **National Institutes of Health Consensus Development Conference Statement: Gallstones and Laparoscopic Cholecystectomy**. September 14-16, 1992. *J Laparoendosc Surg* 1993; **3**: 77-90

7 **Legorreta AP**, Silber JH, Costantino GN, Kobylinski RW, Zatz SL. Increased cholecystectomy rate after the introduction of laparoscopic cholecystectomy. *JAMA* 1993; **270**: 1429-1432

8 **Shaffer EA**. Epidemiology and risk factors for gallstone disease: has the paradigm changed in the 21st century? *Curr Gastroenterol Rep* 2005; **7**: 132-140

9 **Kratzer W**, Mason RA, Kachele V. Prevalence of gallstones in sonographic surveys worldwide. *J Clin Ultrasound* 1999; **27**: 1-7

10 **Diehl AK**. Epidemiology and natural history of gallstone disease. *Gastroenterol Clin North Am* 1991; **20**: 1-19

11 **Jensen KH**, Jorgensen T. Incidence of gallstones in a Danish population. *Gastroenterology* 1991; **100**: 790-794

12 **Barbara L**, Sama C, Morselli Labate AM, Taroni F, Rusticani G, Festi D. A 10-year incidence of gallstone disease: the Sirmione study. *J Hepatol* 1993; **18** Suppl 1: 104A

13 **Misciagna G**, Leoci C, Elba S, Petruzzi J, Guerra V, Mossa A, Noviello M, Coviello A, Capece-Minutolo M, Giorgiot T. The epidemiology of cholelithiasis in southern Italy. *Eur J Gastroenterol Hepatol* 1994; **6**: 937-941

14 **Angelico F**, Del Ben M, Barbato A, Conti R, Urbinati G. Ten-year incidence and natural history of gallstone disease in a rural population of women in central Italy. The Rome Group for the Epidemiology and Prevention of Cholelithiasis (GREPCO). *Ital J Gastroenterol Hepatol* 1997; **29**: 249-254

15 **Attili AF**, Carulli N, Roda E, Barbara B, Capocaccia L, Menotti A, Okoliksanyi L, Ricci G, Capocaccia R, Festi D. Epidemiology of gallstone disease in Italy: prevalence data of the multicenter Italian study on cholelithiasis (MICOL). *Am J Epidemiol* 1995; **141**: 158-165

16 **Attili AF**, Capocaccia R, Carulli N, Festi D, Roda E, Barbara L, Capocaccia L, Menotti A, Okolicsanyi L, Ricci G, Lalloni L, Mariotti S, Sama C, Scafato E. Factors associated with gallstone disease in the MICOL experience. Multicenter Italian Study on Epidemiology of Cholelithiasis. *Hepatology* 1997; **26**: 809-818

17 **Festi D**, Sottili S, Colecchia A, Attili A, Mazzella G, Roda E, Romano F. Clinical manifestations of gallstone disease: evidence from the multicenter Italian study on cholelithiasis (MICOL). *Hepatology* 1999; **30**: 839-846

18 **Misciagna G**, Leoci C, Guerra V, Chiloiro M, Elba S, Petruzzi J, Mossa A, Noviello MR, Coviello A, Minutolo MC, Mangini V, Messa C, Cavallini A, De Michele G, Giorgio I. Epidemiology of cholelithiasis in southern Italy. Part II: Risk factors. *Eur J Gastroenterol Hepatol* 1996; **8**: 585-593

19 **Barbara L**, Sama C, Morselli Labate AM, Taroni F, Rusticali AG, Festi D, Sapio C, Roda E, Banterle C, Puci A. A population study on the prevalence of gallstone disease: the Sirmione Study. *Hepatology* 1987; **7**: 913-917

20 **Loria P**, Dilengite MA, Bozzoli M, Carubbi F, Messora R, Sassatelli R, Bertolotti M, Tampieri A, Tartoni PL, Cassinadri M. Prevalence rates of gallstone disease in Italy. The Chianciano population study. *Eur J Epidemiol* 1994; **10**: 143-150

21 **Jorgensen T**. Prevalence of gallstones in a Danish population. *Am J Epidemiol* 1987; **126**: 912-921
22 **Glambek I**, Kvaale G, Arnesjo B, Soreide O. Prevalence of gallstones in a Norwegian population. *Scand J Gastroenterol* 1987; **22**: 1089-1094
23 **Heaton KW**, Braddon FE, Mountford RA, Hughes AO, Emmett PM. Symptomatic and silent gall stones in the community. *Gut* 1991; **32**: 316-320
24 **Everhart JE**, Khare M, Hill M, Maurer KR. Prevalence and ethnic differences in gallbladder disease in the United States. *Gastroenterology* 1999; **117**: 632-639
25 **Everhart JE**, Yeh F, Lee ET, Hill MC, Fabsitz R, Howard BV, Welty TK. Prevalence of gallbladder disease in American Indian populations: findings from the Strong Heart Study. *Hepatology* 2002; **35**: 1507-1512
26 **Miquel JF**, Covarrubias C, Villaroel L, Mingrone G, Greco AV, Puglielli L, Carvallo P, Marshall G, Del Pino G, Nervi F. Genetic epidemiology of cholesterol cholelithiasis among Chilean Hispanics, Amerindians, and Maoris. *Gastroenterology* 1998; **115**: 937-946
27 **Khuroo MS**, Mahajan R, Zargar SA, Javid G, Sapru S. Prevalence of biliary tract disease in India: a sonographic study in adult population in Kashmir. *Gut* 1989; **30**: 201-205
28 **Nomura H**, Kashiwagi S, Hayashi J, Kajiyama W, Ikematsu H, Noguchi A, Tani S, Goto M. Prevalence of gallstone disease in a general population of Okinawa, Japan. *Am J Epidemiol* 1988; **128**: 598-605
29 **Mendez-Sanchez N**, Chavez-Tapia NC, Motola-Kuba D, Sanchez-Lara K, Ponciano-Rodriguez G, Baptista H, Ramos MH, Uribe M. Metabolic syndrome as a risk factor for gallstone disease. *World J Gastroenterol* 2005; **11**: 1653-1657
30 **Grundy SM**. Cholesterol gallstones: a fellow traveler with metabolic syndrome? *Am J Clin Nutr* 2004; **80**: 1-2
31 **Tsai CJ**, Leitzmann MF, Willett WC, Giovannucci EL. Prospective study of abdominal adiposity and gallstone disease in US men. *Am J Clin Nutr* 2004; **80**: 38-44
32 **Mendez-Sanchez N**, Bahena-Aponte J, Chavez-Tapia NC, Motola-Kuba D, Sanchez-Lara K, Conciano-Rodriguez G, Ramos MH, Uribe M. Strong association between gallstones and cardiovascular disease. *Am J Gastroenterol* 2005; **100**: 827-830
33 **Guidelines for the treatment of gallstones**. American College of Physicians. *Ann Intern Med* 1993; **119**: 620-622
34 **Gruppo Romano per la Epidemiologia e la Prevenzione della Colelitiasi (GREPCO)**. Radiologic appearance of gallstones and its relationship with biliary symptoms and awareness of having gallstones. Observations during epidemiological studies. Rome Group for the Epidemiology and Prevention of Cholelithiasis (GREPCO). *Dig Dis Sci* 1987; **32**: 349-353
35 **Ros E**, Valderrama R, Bru C, Bianchi L, Teres J. Symptomatic versus silent gallstones. Radiographic features and eligibility for nonsurgical treatment. *Dig Dis Sci* 1994; **39**: 1697-1703
36 **Juvonen T**, Niemela O, Makela J, Kairaluoma MI. Characteristics of symptomatic gallbladder disease in patients with either solitary or multiple cholesterol gallstones. *Hepatogastroenterology* 1994; **41**: 263-266

S- Editor Li DL **L- Editor** Mihm S **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5290

World J Gastroenterol 2008 September 14; 14(34): 5290-5300
World Journal of Gastroenterology ISSN 1007-9327

CLINICAL RESEARCH

Ileal lesions in patients with ulcerative colitis after ileo-rectal anastomosis: Relationship with colonic metaplasia

Livia Biancone, Emma Calabrese, Giampiero Palmieri, Carmelina Petruzziello, Sara Onali, Giuseppe Sigismondo Sica, Marta Cossignani, Giovanna Condino, Kiron Moy Das, Francesco Pallone

Livia Biancone, Emma Calabrese, Carmelina Petruzziello, Sara Onali, Marta Cossignani, Giovanna Condino, Francesco Pallone, Cattedra di Gastroenterologia, Dipartimento di Medicina Interna, Università di "Tor Vergata", Roma 00133, Italy
Giampiero Palmieri, Cattedra di Anatomia ed Istologia Patologica, Università di "Tor Vergata", Roma 00133, Italy
Giuseppe Sigismondo Sica, Cattedra di Chirurgia Generale, Università di "Tor Vergata", Roma 00133, Italy
Kiron Moy Das, GI Unit, Department of Gastroenterology & Hepatology, University of Medicine & Dentistry of New Brunswick, New Brunswick NJ 08903, United States
Author contributions: Biancone L designed research, performed endoscopies and wrote the paper; Calabrese E, Petruzziello C, Onali S, Condino G performed the clinical assessment and recruitment of patients; Cossignani M performed research and collected the data; Palmieri G performed immunohistochemistry; Sica GS gave his surgical support; Pallone F contributed to writing the paper; Das KM designed research, provided reagents and contributed to wrote the paper.
Supported by The Fondazione Umberto Di Mario, Largo Marchiafava n. 1, ZIP code 00161, Roma, Italy
Correspondence to: Livia Biancone, MD, PhD, Cattedra di Gastroenterologia, Dipartimento di Medicina Interna, Università "Tor Vergata", Via Montpellier, 1, Rome 00133, Italy. biancone@med.uniroma2.it
Telephone: +39-6-20903737 Fax: +39-6-20903738
Received: May 2, 2008 Revised: June 20, 2008
Accepted: June 27, 2008
Published online: September 14, 2008

Abstract

AIM: To assess whether in ulcerative colitis (UC) patients with ileo-rectal anastomosis (IRA), ileal lesions may develop in the neo-terminal-ileum and their possible relation with phenotypic changes towards colonic epithelium.

METHODS: A total of 19 patients with IRA under regular follow up were enrolled, including 11 UC and 8 controls (6 Crohn's disease, CD; 1 familial adenomatous polyposis, FAP; 1 colon cancer, colon K). Ileal lesions were identified by ileoscopy with biopsies taken from the ileum (involved and uninvolved) and from the rectal stump. Staining included HE and immunohistochemistry using monoclonal antibodies against colonic epithelial protein CEP (Das-1) and human tropomyosin isoform 5, hTM5 (CG3). Possible relation between development of colonic metaplasia and ileal lesions was investigated.

RESULTS: Stenosing adenocarcinoma of the rectal stump was detected in 1 UC patient. The neo-terminal ileum was therefore investigated in 10/11 UC patients. Ileal ulcers were detected in 7/10 UC, associated with colonic metaplasia in 4/7 (57.1%) and Das-1 and CG3 reactivity in 3/4 UC. In controls, recurrence occurred in 4/6 CD, associated with colonic metaplasia in 3/4 and reactivity with Das-1 and CG3 in 2/3.

CONCLUSION: Present findings suggest that in UC, ileal lesions associated with changes towards colonic epithelium may develop also after IRA. Changes of the ileal content after colectomy may contribute to the development of colonic metaplasia, leading to ileal lesions both in the pouch and in the neo-terminal ileum after IRA.

Key words: Ulcerative colitis; Ileo-rectal anastomosis; Ileal lesions; Colonic metaplasia

Peer reviewer: Jay Pravda, MD, Inflammatory Disease Research Center, Gainesville, Florida 32614-2181, United States

Biancone L, Calabrese E, Palmieri G, Petruzziello C, Onali S, Sica GS, Cossignani M, Condino G, Das KM, Pallone F. Ileal lesions in patients with ulcerative colitis after ileo-rectal anastomosis: Relationship with colonic metaplasia. *World J Gastroenterol* 2008; 14(34): 5290-5300 Available from: URL: http://www.wjgnet.com/1007-9327/14/5290.asp DOI: http://dx.doi.org/10.3748/wjg.14.5290

INTRODUCTION

Inflammatory changes of the distal ileum in ulcerative colitis (UC) may be observed in backwash ileitis[1-3] and after total proctocolectomy with ileal pouch ("pouchitis")[4-8]. Chronic inflammation of the ileal pouch has been described not only in patients with UC[9-13], but also after total proctocolectomy for other conditions, including Familial Adenomatous Polyposis (FAP). The frequency of pouchitis is more frequent in UC (60%-70% at 1 years)[14-16] than in FAP (< 50% at 1 years), thus suggesting that the underlying UC may be involved in the pathogenesis of pouchitis[8,14,17]. Common bacterial flora also appears to play a major role in

pouchitis, as suggested by both experimental and clinical evidences, supporting the efficacy of probiotics in subsiding symptoms of pouchitis in UC[18-22]. Although the etiology of pouchitis is unknown, the development of changes of the ileal mucosa lining the pouch, including flattening, reduced number and/or complete villar atrophy has been involved in UC[4,23,24]. These changes of the ileum, becoming similar to the colonic epithelium ("colonic metaplasia") have been reported more frequently associated with pouchitis. Changes of the resident bacterial flora after proctocolectomy have therefore been involved in the development of both colonic metaplasia and pouchitis.

Proctocolectomy represented the procedure of choice in severe UC over the past 30 years[25], and permanent ileostomy[26,27] is still a valid option in an elderly patient in whom either an ileal pouch or an ileo-rectal anastomosis (IRA) is contraindicated. Nowadays, colectomy with IRA[28-30] must be compared with the more recent sphincter saving procedure, namely restorative proctocolectomy. Ravitch and Sabiston[31] are rightly given the credit for the development of total colectomy and ileo-anal pouch, representing the preferred surgical option in referral IBD surgical unit[32].

IRA for UC determines the persistence of the diseased rectal stump requiring local treatment and cancer surveillance, thus being rarely performed in UC, due to technical feasibility or clinical requirements. It is conceivable that not only after ileal pouch, but also after IRA, the neo-terminal ileum may develop inflammatory changes.

In order to investigate this issue, we aimed to assess whether inflammatory changes of the ileum in UC may also be observed after IRA. We also explored whether these changes of the neo-terminal ileum are associated with the development of colonic metaplasia of the ileum itself. For this purpose, colonic metaplasia of the ileum was investigated in biopsy samples taken from the neo-terminal ileum of patients with IRA for UC, by both conventional histological assessment and immunohistochemistry, using monoclonal antibodies (MoAb) against colonic epithelial antigens (CEP and hTM5)[23,33-36]. In one UC patient, ileal lesions were also assessed by wireless capsule endoscopy (WCE). As controls, a small group of patients with IRA for Crohn's disease (CD), FAP or colon cancer (colon K) were also investigated as patients with IRA for UC.

MATERIALS AND METHODS

Patients

A total of 19 patients with IRA in regular follow up at the University "Tor Vergata" of Rome, Italy, were studied. Clinical characteristics of each patient are reported in Table 1. The study population included patients with IRA for UC (n = 11), and, as control group, 6 patients with IRA for CD, 1 for FAP and 1 for adenocarcinoma of the sigmoid colon (colon K). In all UC patients and controls the diagnosis was confirmed by macroscopic and histological analysis of the surgical specimen. Clinical history, endoscopic, surgical and histological findings were reviewed for the possibility that endoscopic findings after surgery would suggest possible changes of previous diagnosis (i.e. ileal lesions in UC). The study group included 11 UC patients (5 M; median age 46; range 25-63), with a median UC duration of 7 years (range 1-20), a median time interval from surgery of 4 years (range 1-12). Indication for surgery was adenocarcinoma in the rectal stump (n = 1), medical intractability (n = 8), toxic megacolon (n = 2). Among the 8 controls, there were 6 patients with CD (3 M, median age 51, range 22-83), with a median CD duration of 3 years (range 0-24), a median time interval from surgery of 4 years (range 1-20). Indication was stenosis of the sigmoid colon (n = 2), recto-vaginal fistula (n = 1), obstruction (n = 1), pelvic abscess (n = 1), refractory CD (n = 1). Control group also included 2 patients with IRA for FAP (F, age 57) (n = 1) and adenocarcinoma of the sigmoid colon (F, age 68) (n = 1).

Table 1 Demographic and clinical characteristics of patients with IRA for UC, CD, FAP and colon cancer

Patient	Sex	Age	Disease duration before surgery (yr)	Indication for surgery
UC				
DLE	M	50	7	Refractory UC
LRA	F	25	7	CS-Refractory
DFML	F	49	2	CS-Refractory
ML	M	37	1	Refractory UC
BM	F	46	1	Toxic megacolon
TE	M	47	8	Refractory UC
SC	F	39	1	Refractory UC
CF	M	36	8	Toxic megacolon
LP	F	50	20	Refractory UC
NF	M	63	8	Refractory UC
TR	F	47	11	Toxic megacolon
CD				
DAD	F	37	5	Colonic stenosis
MA	M	83	0	Colonic stenosis
CP	F	51	24	Pelvic abscess
DCE	M	30	5	Obstruction
DLLV	M	22	1	Refractory CD
CRL	F	59	1	Recto-vaginal fistula
FAP				
ZG	F	57	16	FAP
Colon K				
ML	F	68	1	Colon cancer

CS-Refractory: Steroid-refractory disease.

Study protocol

Endoscopy: Endoscopy was performed according to clinical criteria, including possible treatment changes, cancer surveillance, or recurrence assessment (in CD). According to these criteria, 9 IBD patients underwent repeated endoscopic assessments of the rectum and ileum, in a median follow up of 4 years (range 1-20). The numbers of endoscopic assessment were: 21 in the 11 UC, 12 in the 6 CD, and 1 in the only FAP and colon cancer patients. Persistence or healing of the ileal lesions was searched in patients undergoing repeated endoscopies. All endoscopies were performed by the same gastroenterologist (LB), with pictures taken from

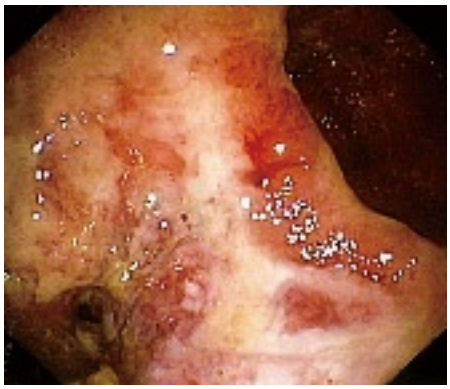


Figure 1 Endoscopic view of the rectal stump from one UC patient (TR) showing a hard and ulcerated mass inducing stenosis of the anastomosis, not passed by the colonoscope. Histological analysis of the lesion detected an adenocarcinoma of the rectal stump.

the rectum, anastomosis and neo-terminal ileum. In UC, the endoscopic degree of inflammation was graded according to the Mayo score (0-4)[37]. The presence and degree of ileal lesions visualized by the endoscope was searched and described as: erosions, aphthoid or deep ulcers, strictures and/or stenosis. The extent of the lesions (cm), the number of ulcerations (few or diffuse: < 5 or $\geqslant 5$) and macroscopic aspect of the ileum between ulcers were also reported. In CD, endoscopic recurrence was graded according to Rutgeerts *et al* (from 0 to 4)[38].

Histology: In all patients biopsies ($n = 2$) were taken from the neo-terminal ileum (uninvolved and involved area), anastomosis and rectal stump. In all patients, at least 10 cm of the neo-terminal ileum were visualized. When considering all biopsy samples taken during repeated endoscopies, 90 biopsy samples (180 biopsies) were taken from the ileum, anastomotic area and rectum. In UC, biopsies were taken from the neo-terminal ileum (macroscopically uninvolved $n = 17$, involved $n = 12$), anastomosis ($n = 3$) and rectum ($n = 18$). In controls, biopsies were taken: in CD from the ileum (uninvolved $n = 8$; involved $n = 8$), anastomosis ($n = 6$) and rectal stump ($n = 11$), in FAP ($n = 1$) and colon cancer patients ($n = 1$) from the uninvolved $n = 2$ or involved $n = 1$ ileum, anastomosis ($n = 2$) and rectum ($n = 2$). Biopsy samples were kept in 10% formalin. Paraffin blocks were used for routine histology by HE staining and for immunohistochemistry by immunoperoxidase staining. Histological assessment was made by one single histopathologist (GP), in order to: (1) Confirm the diagnosis of UC or CD; (2) Assess the presence and degree of inflammation; (3) Detect dysplasia/neoplasia in the rectal stump; (4) Detect changes of the epithelium lining the ileum towards colonic epithelium. Inflammatory changes were assessed according to conventional criteria[4,23,39]. Histologic elements of inflammatory and colonic metaplasia were assessed according to Fruin *et al*[24], including: (1) An inflammatory score considering histological characteristics of the villi epithelia, crypt epithelia, stroma and ulceration (A score: range 0-28); (2) Colonic metaplasia score considering characteristics of villous atrophy and crypt hyperplasia (B score: range 0-6).

Immunohistochemistry: In order to detect possible ileal changes towards colonic epithelium, the expression of colonic antigens was assessed by immunoperoxidase using 2 MoAbs against human tropomyosin isoform 5 (hTM5) (CG3) and against the > 200 kDa colonic epithelial antigen (Das-1)[33-36]. Sections were stained as reported previously[33-36].

Clinical assessment: Clinical assessment was made according to the Mayo score in UC[37] and to the CD Activity Index (CDAI) in CD[40].

WCE: In one compliant UC patient, the presence and extent of the lesions in the neo-terminal ileum was also investigated by WCE. WCE examination was performed using the Given M2A capsule (Given Imaging, Yoqneam, Israel[41,42]) as described[43], by using bowel preparation with PEG (2 L). Exclusion criteria included: low compliance, diverticula, blind loop, pace-maker, neurological disorders, intestinal strictures. WCE images were assessed by one gastroenterologist. The detection of the following lesions was reported: erosions, ulcers, strictures or stenosis. Any other lesion was also reported.

The study was approved by the Local Ethic committee.

RESULTS

UC patients

In one UC patient, an anastomotic stricture that could not be passed by the endoscope was detected. A hard and ulcerated area was present in the rectal stump, compatible with adenocarcinoma, confirmed by histology (Figure 1). The ileum was therefore visualized in 10/11 patients. Figure 2A shows the percentage of UC patients with endoscopic lesions in the neo-terminal ileum, together with changes towards colonic epithelium, as detected by both conventional histology and staining using CG3 and Das-1 MoAbs. As indicated, ileal lesions were detected at first endoscopy in 7/10 patients. Histological analysis of the macroscopically involved ileum detected changes towards colonic epithelium in 4/7 patients. CG3 and Das-1 MoAbs staining were observed in ileal samples from 3 out of these 4 patients. The endoscopic and histological views of the neo-terminal ileum and rectal stump from 2 UC patients with lesions associated or not with changes towards colonic epithelium are shown in Figures 3 and 4, respectively.

Clinical assessment: At first endoscopy, UC was clinically active (Mayo score) in 5 and inactive in 6 patients.

Endoscopy: As the neo-terminal ileum was not visualized in 1 UC patient due to an adenocarcinoma of

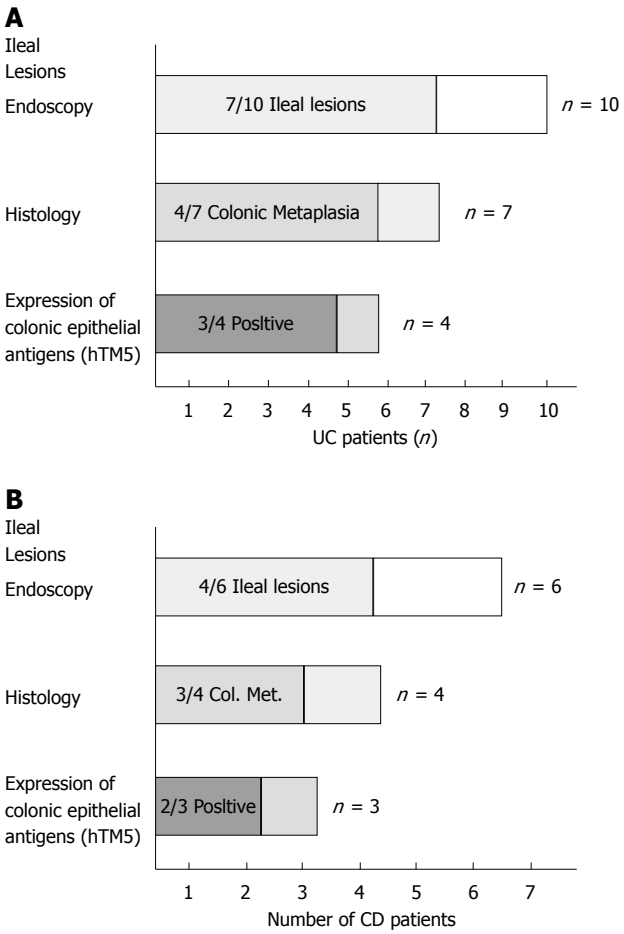


Figure 2 Histograms indications. **A**: The number of UC patients after IRA showing: Lesions in the neo-terminal ileum as assessed by endoscopy; Histological findings compatible with colonic metaplasia; Expression of hTM5-related colon epithelial antigens; **B**: The number of CD patients with IRA showing ileal recurrence at endoscopy, inflammatory changes at histology (including those compatible with colonic metaplasia) and expression of hTM5-related colonic epithelial antigens.

the rectal stump (Figure 1), the ileum was visualized in 10/11 patients. Among these 10 patients, macroscopic lesions were detected in the rectal stump in 8, in the anastomosis in 6, in the ileum in 7. Rectal lesions in the 8 patients included erosions (n = 2) and ulcers (n = 6). In 2/6 patients with anastomotic lesions, a stricture that could be passed by the endoscope was observed. Table 2 shows the endoscopic and histological findings in each of the 11 UC patients studied. Among the 10 patients, 7 showed macroscopic lesions in the ileum proximal to the IRA at first endoscopy. Lesions were localized within 15 cm above the anastomosis in all patients, including ulcers, sporadic (< 5) in 5 (apthoid, n = 3; deep, n = 2) and diffuse (> 5) in 2 UC. In all 7 patients, ileal lesions were surrounded by macroscopically uninvolved areas, while the rectal stump showed typical UC lesions. All 6 UC with anastomotic lesions also showed ileal lesions, while 1 patient with ileal lesions showed a normal anastomosis.

Histology: HE staining. Adenocarcinoma of the rectal stump was detected in 1 patient (Figure 1) and inflammatory changes in the remaining 10. In the macroscopically uninvolved ileum, biopsies were taken from 7/10 UC, showing inflammatory changes (n = 2), lamina propria oedema (n = 3) or no lesions (n = 2) (Table 2). In the macroscopically involved ileum, histology detected inflammation in all 7 UC (acute and chronic inflammation n = 3; villous atrophy n = 4). The anastomotic area showed inflammation in 3/6 UC.

Assessment of changes of the ileal epithelium toward colonic epithelium. Changes towards colonic epithelium are observed in the macroscopically uninvolved ileum from 7/10 UC, showing an inflammatory infiltrate in 3 (A1, n = 1; A2, n = 2), no inflammation in 4 and colonic metaplasia in 1 (B2) (Table 2). Changes toward colonic epithelium were found in the 7/10 UC with ileal lesions, showing inflammatory changes in 4 (A12, n = 1; A10, n = 1; A6, n = 2). Ileal changes towards colonic epithelium have been detected in the 4/7 UC with ileal lesions (A12, n = 1; A4, n = 1; A3, n = 1; A2, n = 1).

Figures 3A to C and 4A to C show the endoscopic, histologic and immunohistochemistry analysis of biopsy samples taken from 4 additional UC patients with IRA.

Immunohistochemistry: Expression of hTM5-related antigens was seen in the uninvolved ileum from 6/10 UC, showing CG3 staining in all 6 and Das-1 staining in 5/6 UC. The involved ileum showed CG3 staining in 5/6 UC and Das-1 staining in 3/6 UC.

Longitudinal study: Endoscopy. Among the 7 patients with ileal lesions, 6 underwent repeated endoscopies (n = 5 in 1; n = 2 in 4; n = 3 in 1). Ileal lesions were detected at all endoscopies in 3/6 UC, at first but not at second endoscopy in 2, healing at third endoscopy in 1 UC. Figure 5 shows the endoscopic, histologic and immunohistochemical analysis of the rectum, involved and uninvolved ileum from one UC patient at 3 consecutive endoscopies.

Histology. Ileal lesions at endoscopy were confirmed by histology in all UC patients. One patient showed ileal changes toward colonic epithelium at all endoscopies (Figure 3).

Immunohistochemistry. In UC, biopsy samples were taken during repeated endoscopies from the uninvolved (n = 11) and involved ileum (n = 10). In the uninvolved ileum, 10/11 biopsies showed CG3 staining (strong in 4) and 8/11 Das-1 staining (glandular n = 5; epithelial cells n = 3). In the involved ileum 9/10 biopsies showed CG3 staining (glandular n = 2; epithelial cells n = 7, strong in 3) and 6/10 showed Das-1 staining (glandular n = 5; epithelial cells, n = 1).

WCE. In the only UC patient studied by WCE, this procedure confirmed the endoscopic findings, showing multiple erosions and ulcers covered by fibrin in the distal 10 cm of the neo-terminal ileum (Figure 3D). These lesions appeared focal, being surrounded by macroscopically uninvolved mucosa. No other lesions, active bleeding or strictures were detected by WCE in the entire small bowel.

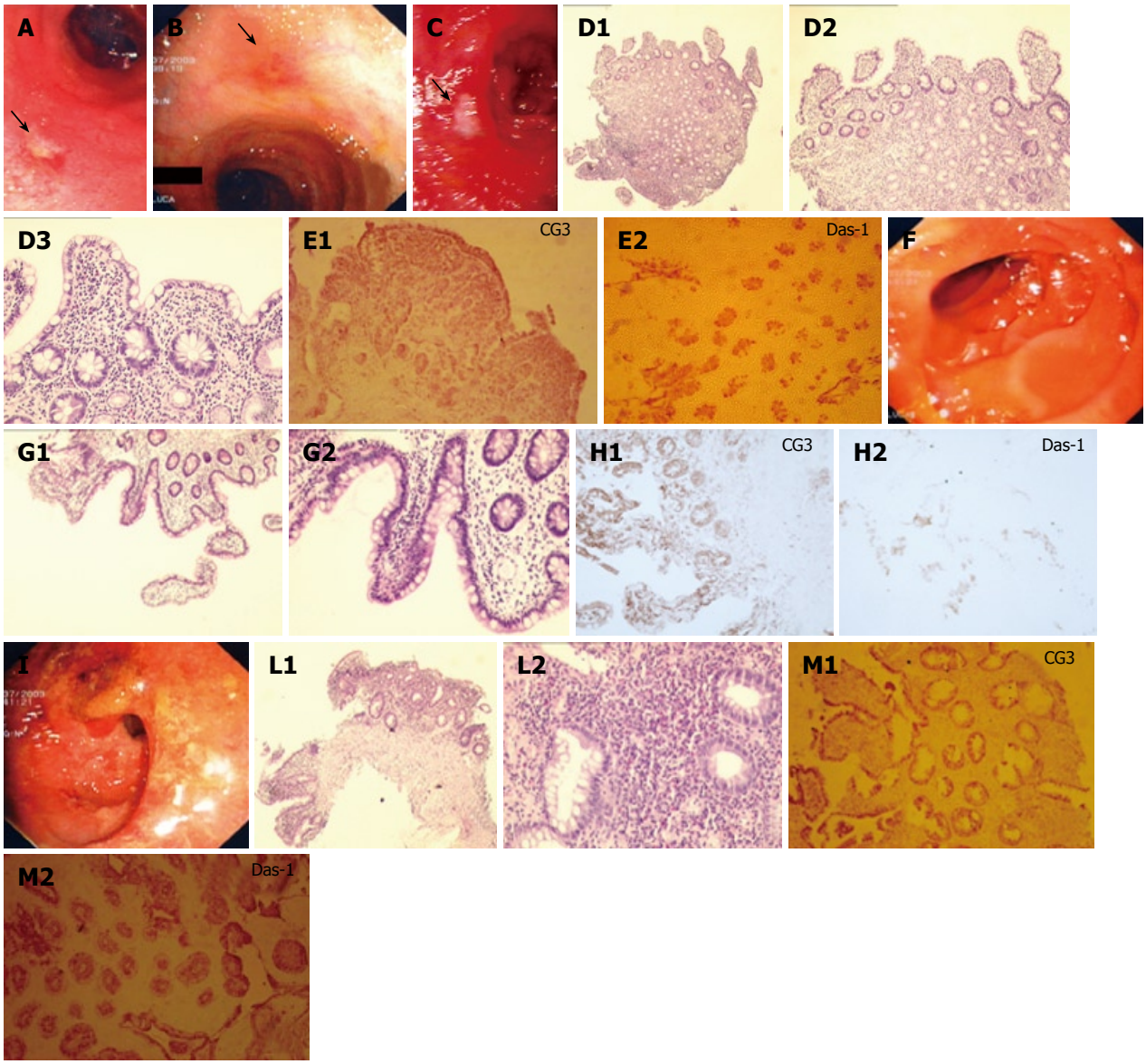


Figure 3 Endoscopic view, histological analysis and immunohistochemistry against CG3 and Das-1 MoAbs of biopsy samples taken from the neo-terminal ileum and rectal stump from a second UC patient (DLE) with IRA. **A-C**: Endoscopic views of the neo-terminal ileum at 3 endoscopies, showing focal erosions and ulcers at all times; **D**: Histological analysis of ileal biopsy samples, compatible with changes towards colonic epithelium, including villous atrophy; **E**: Immunostochemistry showing staining against CG3 (**E1**) and Das-1 (**E2**) MoAbs in the same ileal samples. **F-H**: Endoscopic view (**F**), histological (**G1, G2**) and immunohistochemical analysis against CG3(**H1**) and Das-1 MoAbs (**H2**) of the uninvolved ileum, showing no inflammation or colonic metaplasia. **I-M**: Endoscopic view (**I**), histological (**L1, L2**) and immunohistochemical analysis using CG3(**M1**) and Das-1 MoAbs (**M2**) of the rectal stump, showing typical UC lesions and staining against hTM5-related antigens.

Controls with IRA

CD patients: Figure 2B shows the percentage of CD patients with ileal recurrence detected by endoscopy and histology, together with changes towards colonic epithelium assessed by histology and immunohistochemistry. Recurrence was detected in 4 out of 6 patients: histology detected changes towards colonic epithelium in 3 out of these 4 patients, associated with the expression of colonic epithelial antigens in 2 out of 3 patients.

Clinical assessment. At first endoscopy, disease was active in 3 (CDAI > 150) and inactive in 3 patients.

Endoscopy. Lesions were detected in the rectum in 2/6 patients, in the anastomosis in 5/6 and in the ileum in 4/6 patients. Lesions included CD recurrence of grade 0 (n = 2), 2 (n = 2), 3 (n = 1) or 4 (n = 1) (Figures 6 and 7).

Histology. (1) HE staining: In rectal samples, inflammatory changes were detected in 3/6 CD. The macroscopically uninvolved ileum from 4/6 patients showed changes towards colonic metaplasia (n = 1), inflammation and villous atrophy (n = 2) or no lesions (n = 1) (Figures 6 and 7). Among the 4 patients with ileal recurrence, inflammation was detected in 3 (with villous atrophy in 2) and colonic metaplasia in 1. The anastomosis showed inflammation in 3 (glandular hyperplasia n = 1, villous atrophy n = 1, colonic metaplasia n = 1). (2) Assessment of changes of the ileal epithelium toward colonic epithelium: In 4/6 CD, biopsy samples were taken from the macroscopically uninvolved ileum, showing inflammation in 3/4 (A2, n = 1; A4, n = 2) and changes towards colonic metaplasia

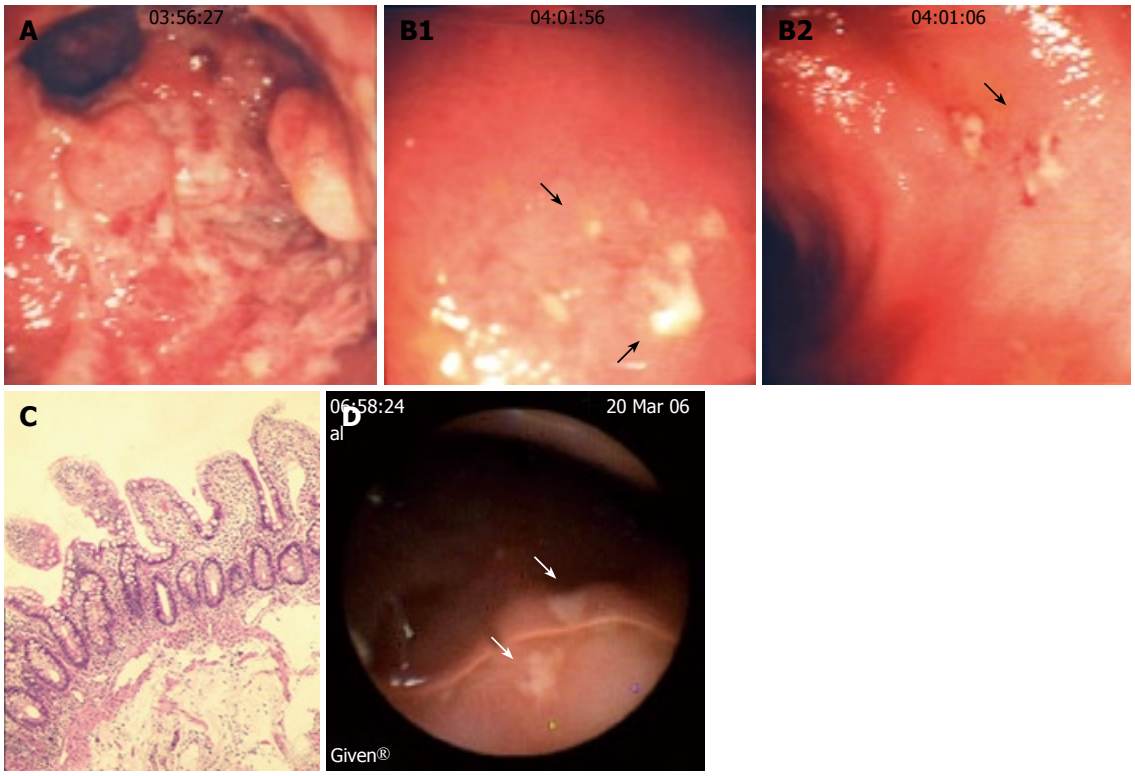


Figure 4 Endoscopic view, histological analysis and WCE images of the rectum and neo-terminal ileum from a third UC patient (LRA) with IRA. **A**: Endoscopic view of the rectal stump showing erosions and pseudopolyps; **B**: Endoscopic views of the ileum showing small focal ulcers above the anastomosis; **C**: Ileal biopsy sample from the same patient showing no villous atrophy or colonic metaplasia by histology; **D**: WCE images showing in the neo-terminal ileum above the anastomosis, 2 ulcers covered by fibrin (arrows) surrounded by macroscopically normal ileum.

Table 2 Endoscopic and histologic findings at different time intervals in each patient with IRA for UC

Patient	Endo (*n*)	Ileal lesions at endoscopy	Histology									
			Uninvolved ileum					Involved ileum				
			Conventional histology[23]			Immunohistochemistry		Conventional histology[23]			Immunohistochemistry	
			A	B	Colonic metaplasia	CG3 MoAb	Das-1 MoAb	A	B	Colonic metaplasia	CG3 MoAb	Das-1 MoAb
LRA	1	Y	1	0	N	-	-	ND	ND	ND	ND	ND
	2	Y	0	0	N	+	+	12	12	Y	+	+
	3	Y	2	0	N	+	+	6	1	N	+	+
	4	Y	0	0	N	+	+	6	0	N	+	+
	5	Y	0	0	N	+	+	8	0	N	+	+
DFML	1	Y	ND	ND	ND	ND	ND	0	0	N	+	-
	2	N	0	0	N	+	+	ND	ND	ND	ND	ND
SC	1	N	2	0	N	+	+	ND	ND	ND	ND	ND
ML	1	Y	0	0	N	+	+	10	4	Y	+	+
	2	N	0	0	N	ND	ND	ND	ND	ND	ND	ND
TE	1	Y	ND	ND	ND	ND	ND	0	0	Y	+	-
LP	1	N	0	0	N	ND	ND	ND	ND	ND	ND	ND
BM	1	N	0	0	N	ND	ND	ND	ND	ND	ND	ND
	2	N	1	0	N	+	-	ND	ND	ND	ND	ND
	3	N	0	0	N	ND	ND	ND	ND	ND	ND	ND
DLE	1	Y	0	0	N	+	-	6	2	Y	+	-
	2	Y	0	2	Y	+	+	0	2	Y	+	+
	3	N	0	0	N	ND	ND	ND	ND	ND	ND	ND
CF	1	Y	ND	ND	ND	ND	ND	2	0	N	-	-
	2	Y	0	0	N	ND	ND	ND	ND	ND	ND	ND
NF	1	Y	ND	ND	ND	ND	ND	6	3	Y	ND	ND

in 2/4 (B2, *n* = 1; B4, *n* = 1) (Table 3). Ileal lesions at endoscopy were confirmed by histology in all 4 CD (A2, *n* = 1; A8, *n* = 1; A10, *n* = 1; A12, *n* = 1) showing colonic metaplasia in 3/4 patients (B3, *n* = 1; B4, *n* = 2). (3) Immunohistochemistry: hTM5-related antigens expression in the uninvolved ileum was searched in 2 CD, showing staining against CG3 in both and against Das-1 in 1 patient. Biopsies from the involved ileum

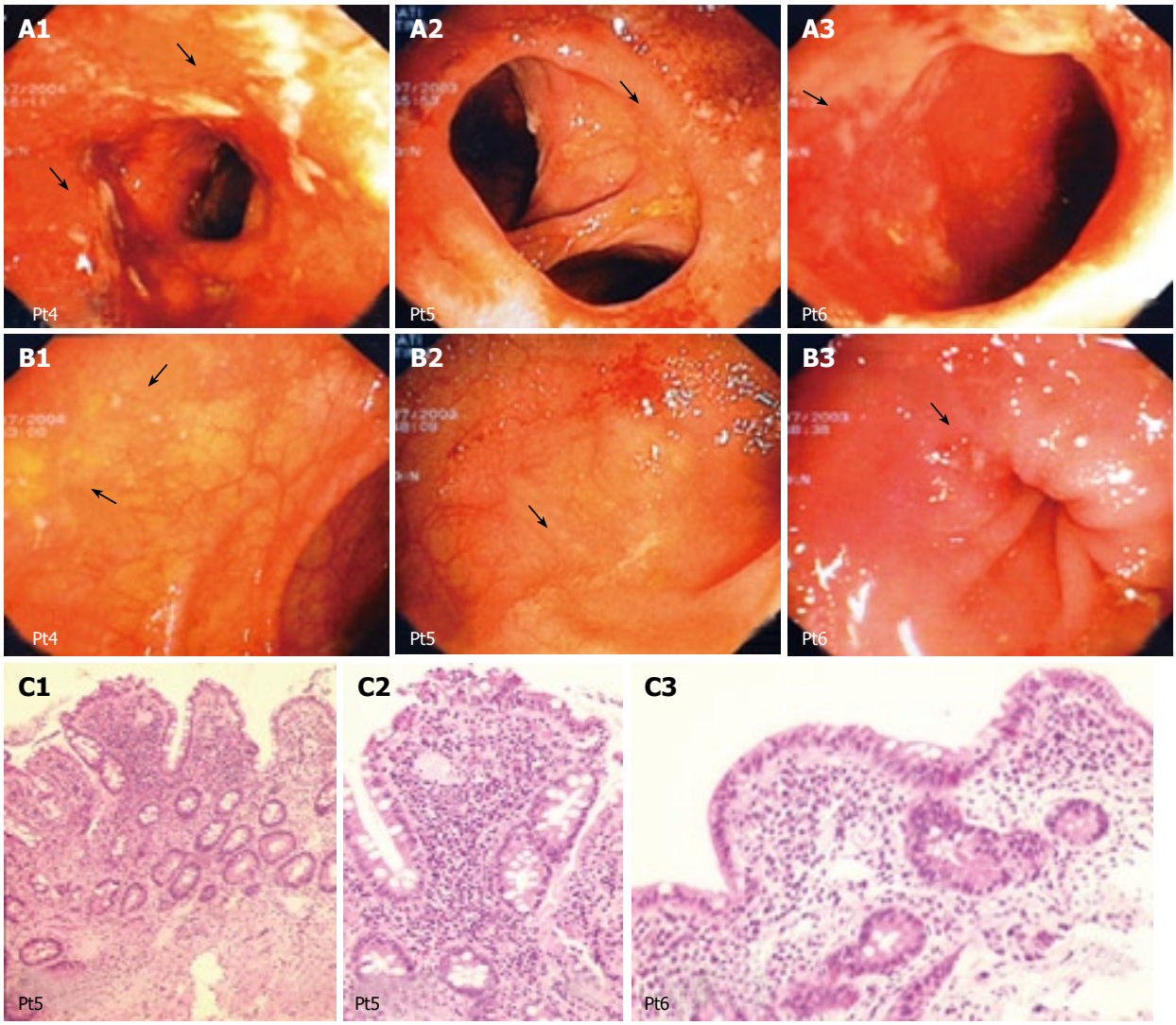


Figure 5 Endoscopic view and histological analysis of the anastomosis and neo-terminal ileum from 3 UC patients with IRA. **A**: Endoscopic views of the anastomosis from 3 UC patients showing focal small ulcers; **B**: Endoscopic views of the neo-terminal ileum from the same 3 UC patients, scattered ulcerations surrounded by macroscopically uninvolved ileum; **C**: Histological analysis of biopsy samples taken from the involved neo-terminal ileum from patients 5 and 6, showing changes towards colonic metaplasia.

were taken from 3 CD, showing CG3 staining in all 3, and Das-1 staining in 2/3 patients.

Longitudinal study: Endoscopy: Repeated endoscopies (n = 4 in 1; n = 3 in 2) were performed in 3/6 patients, showing persistent lesions. Histology: In 5/6 patients, ileal inflammatory changes were detected at all endoscopies. Immunohistochemistry: The only patient with repeated immunohistochemical analysis showed persistent CG3 positivity and Das-1 negativity.

Patient with FAP

Endoscopy: One anastomotic ulcer was detected. Histology: Inflammation were detected only in the anastomosis (A = 0; B = 2).

Patient resected for colon cancer

Endoscopy: No lesions were detected. Histology: Mild inflammation was detected in the rectum only.

DISCUSSION

In UC, inflammatory changes of the ileum in UC may be observed in pouchitis[4-8] and in backwash ileitis[1-3]. In pouchitis, ileal lesions have been related to the development of changes of the epithelium towards colonic epithelium ("colonic metaplasia")[4,23,24]. Although the etiology of pouchitis remains unknown, the resident bacterial flora is involved in the pathogenesis of this condition[15,18,19,21,22]. In particular, the efficacy "*in vivo*" of probiotic preparations in UC patients with pouchitis suggests a pathogenetic role for changes of the resident bacterial flora after proctocolectomy[18,19,21]. As proctocolectomy is required for both ileal pouch and IRA, we aimed to assess whether UC patients may develop inflammatory changes of the ileum not only in the ileal pouch, but also above the IRA. We also explored whether these changes are associated with the development of colonic metaplasia of the epithelium lining the ileum. Ileal changes have been examined by

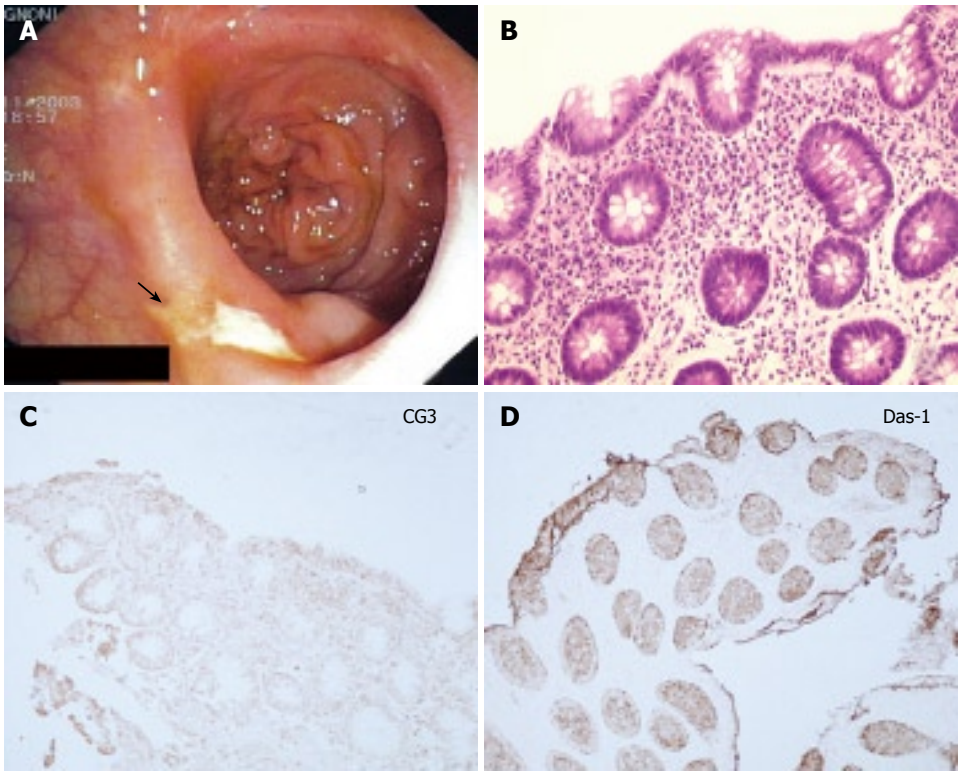


Figure 6 Endoscopic view (**A**), histological (**B**) and immunohistochemical analysis using CG3 (**C**) and Das-1 MoAbs (**D**) of the neo-terminal ileum form one CD patient. Endoscopy shows CD recurrence (grade 2), histology lesions compatible with changes of the ileum towards colonic epithelium and immunohistochemistry staining against CG3 and Das-1 MoAbs.

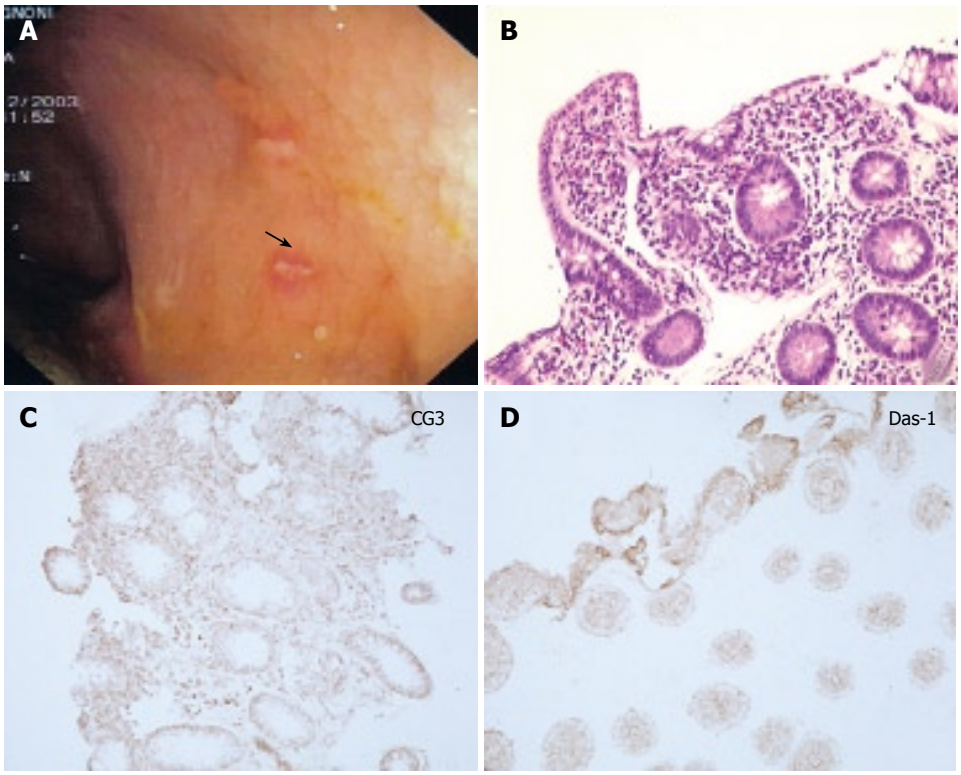


Figure 7 Endoscopic view (**A**), routine histochemical (**B**) and immunohistochemistry analysis using CG3 (**C**) and Das-1 MoAbs (**D**) of the neo-terminal ileum from a second CD patient. Endoscopy detected recurrence of the anastomosis and neo-terminal ileum (grade 3), histology lesions compatible with changes of the ileal epithelium towards colonic epithelium and immunohistochemistry the expression of colonic epithelial antigens, compatible with colonic metaplasia.

histology and immunohistochemistry using MoAbs against the major cytoskeletal microfilament protein, tropomyosin isoform 5 (hTM5) in colon epithelium[33-36]. Evidence indicates that bacterial-host interactions may induce the expression of cryptic cytoskeletal proteins on human cells surface[23,44]. Cytoskeletal proteins include a family of intracytoplasmatic proteins (α-actinin, talin, ezrin, villin, F-actin, myosin Ⅱ, calpactin, gelsolin, laminin, tropomyosin), modulating the structure, shape, and motility of several cell types, including human colonic epithelial cells[45]. The enteropathogenic Escherichia coli binds to enterocytes by injecting a translocated intimin receptor in the host cells membrane, linking to the intimin receptor of the bacterium itself. This binding is followed by a rearrangement of the cytoskeletal proteins within the cytoplasm of the enterocytes (α-actinin, talin, ezrin, villin, F-actin, myosin Ⅱ, TMs), thus forming pedestals linking the bacterium

Table 3 Endoscopical and histologic findings of patients with IRA for CD, FAP or colonic K

Patient	Endo (*n*)	Ileal lesions at endoscopy	Histology									
			Uninvolved ileum					Involved ileum				
			Conventional histology[23]			Immunohistochemistry		Conventional histology[23]			Immunohistochemistry	
			Score A	Score B	Colonic metaplasia	CG3 MoAb	Das-1 MoAb	Score A	Score B	Colonic metaplasia	CG3 MoAb	Das-1 MoAb
DAD	1	Y	ND	ND	ND	ND	ND	4	0	N	+	-
(CD-1)	2	Y	ND	ND	ND	ND	ND	0	0	N	+	-
	3	Y	0	0	N	+	-	0	0	N	+	-
	4	Y	0	0	N	+	-	8	4	Y	+	-
MA	1	N	4	4	Y	+	+	0	3	Y	+	+
(CD-2)												
DCE	1	N	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
(CD-3)												
CP	1	N	0	0	N	ND	ND	ND	ND	ND	ND	ND
(CD-4)	2	N	2	0	N	ND	ND	ND	ND	ND	ND	ND
	3	Y	1	1	N	ND	ND	2	1	N	ND	ND
DLV	1	Y	ND	ND	ND	ND	ND	4	1	Y	+	+
(CD-5)	2	N	2	2	Y	ND	ND					
	3	Y	4	2	Y	ND	ND	12	4	Y	ND	ND
CRL	1	Y	ND	ND	ND	ND	ND	10	3	Y	ND	ND
(CD-6)												
ZG	1	N	0	0	N	ND	ND	8	2	N	ND	ND
(FAP)												
ML	1	N	0	0	N	ND	ND	ND	ND	ND	ND	ND
(K)												

itself to colonic epithelial cells[44,45]. These observations prompted us to also assess whether hTM5 may be expressed on the epithelial cells lining the neo-terminal ileum in UC patients with IRA, due to changes of the ileum towards colonic epithelium after colectomy and the possible relation between the development of colonic metaplasia and ileal lesions. Our findings from a limited number of patients with IBD suggest that ileal lesions may be observed after IRA, although more frequently in UC (7 out of 10 patients). The presence of scattered erosions and ulcers in the neo-terminal ileum above the anastomosis was confirmed by WCE images, acquired as described[41-43], from the only patient performing this procedure. Despite the focal inflammation of the ileum, histological analysis of the surgical colonic specimen confirmed the diagnosis of UC in all 7 patients. Ileal lesions were associated with epithelial changes towards colonic epithelium in 4 out of these 7 patients, associated with the expression of hTM5-related antigens in 3 out of these 4 patients. The same findings were not detected in any of the 7 UC patients with no ileal lesions. Surprisingly, colonic metaplasia and the expression of colonic epithelial antigens were also observed in some of the few tested patients with IRA for CD. Although "colonization" of the ileum after total colectomy for any indication has been described, its possible relation with the development of ileal lesions is unknown. The score from Fruin *et al*[24] was used for assessing colonic metaplasia, as for pouchitis. Major limits of our study include the low number of patients and the cross-sectional study plan, not allowing comparisons before versus after surgery. Although it is said that colectomy cures UC, this study underscores the fact that surgery is not the final answer due to the high incidence of pouchitis and other functional problems experience by these patients. Of particular concern is the asymptomatic neoplasia that can arise in the residual rectal stump required for these surgical procedures, which was seen in one of the study patients.

Although not conclusive, our findings suggest that lesions may be observed in the neo-terminal ileum of UC and CD patients following IRA. These changes are towards colonic epithelium phenotype and with the expression of colonic epithelial antigens in some patients. Longitudinal studies are ongoing for further characterization of the molecular mechanisms leading to ileal changes in UC. Present findings suggest that changes of the ileal content after colectomy may contribute to the development of colonic type of metaplasia, leading to ileal lesions both in the pouch and in the neo-terminal ileum after IRA.

COMMENTS

Background

Inflammatory changes of the distal ileum in ulcerative colitis (UC) may be observed in backwash ileitis and after total proctocolectomy with ileal pouch ("pouchitis"). Although total proctocolectomy with ileal pouch currently represents the most frequently performed surgical procedure in UC, colectomy with ileo-rectal anastomosis (IRA) is still in these patients. The persistence of the diseased rectal stump after IRA requires endoscopical surveillance. Ileal inflammation may be observed in UC patients with pouchitis, being related to changes of the ileal epithelium towards colonic epithelium ("colonic metaplasia"). Whether the ileum above IRA in patients with IRA for UC may develop inflammatory changes as observed in pouchitis is unknown and this observation may be useful for proper follow up of patients after surgery.

Research frontiers

The etiology of pouchitis in patients with UC is unknown. However, the development of changes of the ileal mucosa lining the pouch, including flattening, reduced number and/or complete villar atrophy has been involved in the pathogenesis of pouchitis. These changes of the ileum, becoming similar

to the colonic epithelium ("colonic metaplasia") have been reported more frequently associated with pouchitis. Changes of the resident bacterial flora after proctocolectomy therefore have been involved in the development of both colonic metaplasia and pouchitis. It is conceivable that after total colectomy for UC and related changes of the common bacterial flora, ileal lesions may develop not only after ileal pouch, but also after IRA.

Innovations and breakthroughs

The present study showed that In UC, ileal lesions associated with changes towards colonic epithelium may develop after IRA. Changes of the ileal content after colectomy may contribute to the development of colonic metaplasia, leading to ileal lesions also after IRA.

Applications

Results from our study suggest that patients with IRA for UC need endoscopical assessment not only for cancer surveillance but also for assessing the possible development of ileal lesions above anastomosis. Present findings also add new insights regarding the natural history of UC after IRA.

Terminology

Tropomyosin isoform 5 (hTM5) is a cytoskeletal microfilament protein present in the epithelium from human colon, but not from the ileum. Mucosal and circulating antibodies against hTM5 have been demonstrated in patients with UC. Evidences indicate that bacterial-host interactions may induce the expression of cryptic cytoskeletal proteins on human cells surface. Cytoskeletal proteins include a family of intracytoplasmatic proteins (α-actinin, talin, ezrin, villin, F-actin, myosin Ⅱ, calpactin, gelsolin, laminin, tropomyosin), modulating the structure, shape, and motility of several cell types, including human colonic epithelial cells.

Peer review

This is a clinical and immunohistochemical study supporting the need of continued endoscopical follow up of UC patients after IRA.

REFERENCES

1 **Abdelrazeq AS**, Wilson TR, Leitch DL, Lund JN, Leveson SH. Ileitis in ulcerative colitis: is it a backwash? *Dis Colon Rectum* 2005; **48**: 2038-2046

2 **Gustavsson S**, Weiland LH, Kelly KA. Relationship of backwash ileitis to ileal pouchitis after ileal pouch-anal anastomosis. *Dis Colon Rectum* 1987; **30**: 25-28

3 **Haskell H**, Andrews CW Jr, Reddy SI, Dendrinos K, Farraye FA, Stucchi AF, Becker JM, Odze RD. Pathologic features and clinical significance of "backwash" ileitis in ulcerative colitis. *Am J Surg Pathol* 2005; **29**: 1472-1481

4 **Apel R**, Cohen Z, Andrews CW Jr, McLeod R, Steinhart H, Odze RD. Prospective evaluation of early morphological changes in pelvic ileal pouches. *Gastroenterology* 1994; **107**: 435-443

5 **Arai K**, Koganei K, Kimura H, Akatani M, Kitoh F, Sugita A, Fukushima T. Incidence and outcome of complications following restorative proctocolectomy. *Am J Surg* 2005; **190**: 39-42

6 **Brunel M**, Penna C, Tiret E, Balladur P, Parc R. Restorative proctocolectomy for distal ulcerative colitis. *Gut* 1999; **45**: 542-545

7 **Chambers WM**, McC Mortensen NJ. Should ileal pouch-anal anastomosis include mucosectomy? *Colorectal Dis* 2007; **9**: 384-392

8 **Madden MV**, Farthing MJ, Nicholls RJ. Inflammation in ileal reservoirs: 'pouchitis'. *Gut* 1990; **31**: 247-249

9 **Meagher AP**, Farouk R, Dozois RR, Kelly KA, Pemberton JH. J ileal pouch-anal anastomosis for chronic ulcerative colitis: complications and long-term outcome in 1310 patients. *Br J Surg* 1998; **85**: 800-803

10 **Meuwissen SG**, Hoitsma H, Boot H, Seldenrijk CA. Pouchitis (pouch ileitis). *Neth J Med* 1989; **35** Suppl 1: S54-S66

11 **Michelassi F**, Lee J, Rubin M, Fichera A, Kasza K, Karrison T, Hurst RD. Long-term functional results after ileal pouch anal restorative proctocolectomy for ulcerative colitis: a prospective observational study. *Ann Surg* 2003; **238**: 433-441; discussion 442-445

12 **Sagar PM**, Pemberton JH. Ileo-anal pouch function and dysfunction. *Dig Dis* 1997; **15**: 172-188

13 **Salemans JM**, Nagengast FM, Lubbers EJ, Kuijpers JH. Postoperative and long-term results of ileal pouch-anal anastomosis for ulcerative colitis and familial polyposis coli. *Dig Dis Sci* 1992; **37**: 1882-1889

14 **Sandborn WJ**, Tremaine WJ, Batts KP, Pemberton JH, Phillips SF. Pouchitis after ileal pouch-anal anastomosis: a Pouchitis Disease Activity Index. *Mayo Clin Proc* 1994; **69**: 409-415

15 **Shen B**, Fazio VW, Remzi FH, Lashner BA. Clinical approach to diseases of ileal pouch-anal anastomosis. *Am J Gastroenterol* 2005; **100**: 2796-2807

16 **Zuccaro G Jr**, Fazio VW, Church JM, Lavery IC, Ruderman WB, Farmer RG. Pouch ileitis. *Dig Dis Sci* 1989; **34**: 1505-1510

17 **Shen B,** Fazio VW, Remzi FH, Delaney CP, Bennett AE, Achkar JP, Brzezinski A, Khandwala F, Liu W, Bambrick ML, Bast J, Lashner B. Comprehensive evaluation of inflammatory and noninflammatory sequelae of ileal pouch-anal anastomoses. *Am J Gastroenterol* 2005; **100**: 93-101

18 **Gionchetti P**, Rizzello F, Venturi A, Brigidi P, Matteuzzi D, Bazzocchi G, Poggioli G, Miglioli M, Campieri M. Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo-controlled trial. *Gastroenterology* 2000; **119**: 305-309

19 **Gionchetti P**, Rizzello F, Helwig U, Venturi A, Lammers KM, Brigidi P, Vitali B, Poggioli G, Miglioli M, Campieri M. Prophylaxis of pouchitis onset with probiotic therapy: a double-blind, placebo-controlled trial. *Gastroenterology* 2003; **124**: 1202-1209

20 **Hurst RD**, Molinari M, Chung TP, Rubin M, Michelassi F. Prospective study of the incidence, timing and treatment of pouchitis in 104 consecutive patients after restorative proctocolectomy. *Arch Surg* 1996; **131**: 497-500; discussion 501-502

21 **Mimura T**, Rizzello F, Helwig U, Poggioli G, Schreiber S, Talbot IC, Nicholls RJ, Gionchetti P, Campieri M, Kamm MA. Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrent or refractory pouchitis. *Gut* 2004; **53**: 108-114

22 **Sandborn WJ**, McLeod R, Jewell DP. Medical therapy for induction and maintenance of remission in pouchitis: a systematic review. *Inflamm Bowel Dis* 1999; **5**: 33-39

23 **Biancone L**, Palmieri G, Lombardi A, Colantoni A, Tonelli F, Das KM, Pallone F. Tropomyosin expression in the ileal pouch: a relationship with the development of pouchitis in ulcerative colitis. *Am J Gastroenterol* 2003; **98**: 2719-2726

24 **Fruin AB**, El-Zammer O, Stucchi AF, O'Brien M, Becker JM. Colonic metaplasia in the ileal pouch is associated with inflammation and is not the result of long-term adaptation. *J Gastrointest Surg* 2003; **7**: 246-253; discussion 253-254

25 **Kettlewell MGW**. Proctocolectomy for ulcerative colitis. In: Allan RN, Keighley MRB, Alexander-Williams J & Hawkins C. Inflammatory Bowel Diseases. Edinburgh: Churchill Livingstone, 1991: 439-445

26 **Brooke BN**. Outcome of surgery for ulcerative colitis. *Lancet* 1956; **271**: 532-536

27 **Larson DW**, Pemberton JH. Current concepts and controversies in surgery for IBD. *Gastroenterology* 2004; **126**: 1611-1619

28 **Bulow C**, Vasen H, Jarvinen H, Bjork J, Bisgaard ML, Bulow S. Ileorectal anastomosis is appropriate for a subset of patients with familial adenomatous polyposis. *Gastroenterology* 2000; **119**: 1454-1460

29 **Lepisto A**, Jarvinen HJ. Fate of the rectum after colectomy with ileorectal anastomosis in ulcerative colitis. *Scand J Surg* 2005; **94**: 40-42

30 **Watts JM**, Hughes ES. Ulcerative colitis and Crohn's disease: results after colectomy and ileorectal anastomosis. *Br J Surg* 1977; **64**: 77-83

31 **Roggo A**, Brunner U, Ottinger LW, Largiader F. The

continuing challenge of aneurysms of the popliteal artery. *Surg Gynecol Obstet* 1993; **177**: 565-572

32 **Nicholls RJ**, Holt SD, Lubowski DZ. Restorative proctocolectomy with ileal reservoir. Comparison of two-stage vs. three-stage procedures and analysis of factors that might affect outcome. *Dis Colon Rectum* 1989; **32**: 323-326

33 **Kesari KV**, Yoshizaki N, Geng X, Lin JJ, Das KM. Externalization of tropomyosin isoform 5 in colon epithelial cells. *Clin Exp Immunol* 1999; **118**: 219-227

34 **Das KM**, Dasgupta A, Mandal A, Geng X. Autoimmunity to cytoskeletal protein tropomyosin. A clue to the pathogenetic mechanism for ulcerative colitis. *J Immunol* 1993; **150**: 2487-2493

35 **Lin JJ**, Hegmann TE, Lin JL. Differential localization of tropomyosin isoforms in cultured nonmuscle cells. *J Cell Biol* 1988; **107**: 563-572

36 **Takahashi F**, Das KM. Isolation and characterization of a colonic autoantigen specifically recognized by colon tissue-bound immunoglobulin G from idiopathic ulcerative colitis. *J Clin Invest* 1985; **76**: 311-318

37 **Tremaine WJ**, Sandborn WJ, Wolff BG, Carpenter HA, Zinsmeister AR, Metzger PP. Bismuth carbomer foam enemas for active chronic pouchitis: a randomized, double-blind, placebo-controlled trial. *Aliment Pharmacol Ther* 1997; **11**: 1041-1046

38 **Rutgeerts P**, Geboes K, Vantrappen G, Beyls J, Kerremans R, Hiele M. Predictability of the postoperative course of Crohn's disease. *Gastroenterology* 1990; **99**: 956-963

39 **Sandborn WJ**, Feagan BG, Hanauer SB, Lochs H, Lofberg R, Modigliani R, Present DH, Rutgeerts P, Scholmerich J, Stange EF, Sutherland LR. A review of activity indices and efficacy endpoints for clinical trials of medical therapy in adults with Crohn's disease. *Gastroenterology* 2002; **122**: 512-530

40 **Best WR**, Becktel JM, Singleton JW, Kern F Jr. Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study. *Gastroenterology* 1976; **70**: 439-444

41 **Costamagna G**, Shah SK, Riccioni ME, Foschia F, Mutignani M, Perri V, Vecchioli A, Brizi MG, Picciocchi A, Marano P. A prospective trial comparing small bowel radiographs and video capsule endoscopy for suspected small bowel disease. *Gastroenterology* 2002; **123**: 999-1005

42 **Iddan G**, Meron G, Glukhovsky A, Swain P. Wireless capsule endoscopy. *Nature* 2000; **405**: 417

43 **Biancone L**, Calabrese E, Petruzziello C, Onali S, Caruso A, Palmieri G, Sica GS, Pallone F. Wireless capsule endoscopy and small intestine contrast ultrasonography in recurrence of Crohn's disease. *Inflamm Bowel Dis* 2007; **13**: 1256-1265

44 **Vallance BA**, Finlay BB. Exploitation of host cells by enteropathogenic Escherichia coli. *Proc Natl Acad Sci USA* 2000; **97**: 8799-8806

45 **Luo Y**, Frey EA, Pfuetzner RA, Creagh AL, Knoechel DG, Haynes CA, Finlay BB, Strynadka NC. Crystal structure of enteropathogenic Escherichia coli intimin-receptor complex. *Nature* 2000; **405**: 1073-1077

S- Editor Li DL **L- Editor** Li M **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5301

World J Gastroenterol 2008 September 14; 14(34): 5301-5305
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Continuous wound infusion of local anaesthetic agents following colorectal surgery: Systematic review and meta-analysis

Alan Karthikesalingam, Stewart R Walsh, Sheraz R Markar, Umar Sadat, Tjun Y Tang, Charles M Malata

Alan Karthikesalingam, Stewart R Walsh, Sheraz R Markar, Umar Sadat, Tjun Y Tang, Department of General Surgery, Addenbrooke's Hospital, Cambridge University Hospitals NHS Foundation Trust, Cambridge CB2 2QQ, United Kingdom
Charles M Malata, Department of Plastic and Reconstructive Surgery, Addenbrooke's Hospital, Cambridge University Hospitals Foundation Trust, Cambridge CB2 2QQ, United Kingdom
Author contributions: Karthikesalingam A and Malata CM designed research; Karthikesalingam A, Markar SR, Walsh SR and Sadat U performed research; Walsh SR, Tang TY and Sadat U analysed data; and Karthikesalingam A, Walsh SR, Sadat U, Markar SR and Malata CM wrote the paper.
Correspondence to: Stewart R Walsh, Department of General Surgery, Addenbrooke's Hospital, Cambridge University Hospitals NHS Foundation Trust, Specialist Registrar in General Surgery, Box 201, Level 7, Hills Road, Cambridge CB2 2QQ, United Kingdom. srwalsh@doctors.org.uk
Telephone: +44-1223-217246 Fax: +44-1223-216015
Received: May 25, 2008 Revised: July 3, 2008
Accepted: July 10, 2008
Published online: September 14, 2008

Abstract

AIM: To provide a specific review and meta-analysis of the available evidence for continuous wound infusion of local anaesthetic agents following midline laparotomy for major colorectal surgery.

METHODS: Medline, Embase, trial registries, conference proceedings and article reference lists were searched to identify randomised, controlled trials of continuous wound infusion of local anaesthetic agents following colorectal surgery. The primary outcomes were opioid consumption, pain visual analogue scores (VASs), return to bowel function and length of hospital stay. Weighted mean difference were calculated for continuous outcomes.

RESULTS: Five trials containing 542 laparotomy wounds were eligible for inclusion. There was a significant decrease in post-operative pain VAS at rest on day 3 (weighted mean difference: -0.43; 95% CI: -0.81 to -0.04; P = 0.03) but not on post-operative day 1 and 2. Local anaesthetic infusion was associated with a significant reduction in pain VAS on movement on all three post-operative days (day 1 weighted mean difference: -1.14; 95% CI: -2.24 to -0.041; P = 0.04, day 2 weighted mean difference: -0.97, 95% CI: -1.91 to -0.029; P = 0.04, day 3 weighted mean difference: -0.61; 95% CI: 1.01 to -0.20; P = 0.0038). Local anaesthetic wound infusion was associated with a significant decrease in total opioid consumption (weighted mean difference: -40.13; 95% CI: -76.74 to -3.53; P = 0.03). There was no significant decrease in length of stay (weighted mean difference: -20.87; 95% CI: -46.96 to 5.21; P = 0.12) or return of bowel function (weighted mean difference: -9.40; 95% CI: -33.98 to 15.17; P = 0.45).

CONCLUSION: The results of this systematic review and meta-analysis suggest that local anaesthetic wound infusion following laparotomy for major colorectal surgery is a promising technique but do not provide conclusive evidence of benefit. Further research is required including cost-effectiveness analysis.

Key words: Colorectal surgery; Laparotomy; Local anaesthesia; Infusion; Wound healing

Peer reviewer: Conor P Delaney, MD, MCh, PhD, FRCSI, FACS, Professor of Surgery, Case Western Reserve University, Chief, Division of Colorectal Surgery, Vice-Chairman, Department of Surgery, Director, Institute for Surgery and Innovation, University Hospitals, Case Medical Center, 11100 Euclid Avenue Cleveland, Cleveland 44106-5047, United States

Karthikesalingam A, Walsh SR, Markar SR, Sadat U, Tang TY, Malata CM. Continuous wound infusion of local anaesthetic agents following colorectal surgery: Systematic review and meta-analysis. *World J Gastroenterol* 2008; 14(34): 5301-5305 Available from: URL: http://www.wjgnet.com/1007-9327/14/5301.asp DOI: http://dx.doi.org/10.3748/wjg.14.5301

INTRODUCTION

Open surgery comprising colonic resection and primary bowel anastomosis accounts for up to a third of elective general surgical admissions[1]. The control of pain following these operations represents a major challenge as highly complex nociceptive pathways are involved[2-4]. Pain control following abdominal laparotomy and bowel anastomosis is therefore not amenable to

pharmacological monotherapy and modern analgesic strategies following major colorectal surgery involve the combination of many agents including parenteral opiates, nonsteroidal anti-inflammatory drugs (NSAIDs), paracetamol and epidural infusion techniques[5].

Unfortunately, there is no ideal analgesic regimen - all current techniques have disadvantages in the form of important side-effects, cost, patient compliance, procedural complications and delays in discharge[6]. Suboptimal post-operative pain control is of great clinical consequence and has been associated with cardiovascular and respiratory complications and increased gastrointestinal paralysis[5].

A recent systematic review[7] has revealed the promise of continuous wound infusion of local anaesthetic agents to provide improved pain control following thoracic[8-10], abdominal[11-13], gynaecological[14-16], and orthopaedic[17-19] operations, but there is a need for a more focused review of the evidence specific to colorectal laparotomy.

MATERIALS AND METHODS

An electronic search was performed using the Embase and Medline databases from 1966 until 2007. The search terms "postoperative pain", "postoperative analgesia", "local anesthetics", "continuous", "infusion", "perfusion", "irrigation", "patient-controlled", and MeSH headings "Colorectal Surgery" (MeSH), "Laparotomy" (MeSH), were used in combination with the Boolean operators AND or OR. Two authors independently performed electronic searches in March 2008. The electronic search was supplemented by a hand search of published abstracts from meetings of the Surgical Research Society, the Society of Academic and Research Surgery, the American Society of Anesthesiologists, the Anaesthetic Research Society and the Association of Surgeons of Great Britain and Ireland from 1980 to 2007. The reference lists of articles obtained were also searched to identify further relevant citations. Finally, the search included the Current Controlled Trials Register (www.controlled-trials.com) and the Cochrane Database of Controlled Trials.

Abstracts of the citations identified by the search were then scrutinised by two observers (SRW and AK) in order to determine eligibility for inclusion in the meta-analysis. Studies were included if they met each of the following criteria: randomised controlled trial, patients undergoing midline laparotomy for colorectal surgery, randomisation to groups with or without continuous wound infusion of local anaesthetic.

The primary outcome measure for the meta-analysis was the opioid consumption in each arm. Data from eligible trials were entered into a computerized spreadsheet for analysis. The quality of each trial was assessed using the Jadad scoring system[20]. The statistical analysis was performed using Statsdirect 2.5.7 (Statsdirect Ltd., UK). Weighted mean difference were calculated for the effect of local anaesthetic infusion on opioid consumption and linear analogue pain scores on post-operative days 1, 2 and 3. Further pooled outcome measures were duration of hospital stay and time to return of bowel function. All pooled outcome measures were determined using random-effects models as described by Der Simonian and Laird[21]. Heterogeneity amongst the trials was assessed by Cochran's Q statistic, a null hypothesis test in which $P < 0.05$ is taken to indicate the presence of significant heterogeneity. The Egger test was used to assess the funnel plot for significant asymmetry, indicating possible publication or other biases.

RESULTS

The initial search identified 590 papers. After screening, 5 randomised controlled trials were identified[22-26]. The five trials included 542 laparotomy wounds, of which 259 were randomised to infusion of local anaesthetic agents.

Outcome measures

Opioid consumption: Four of the five trials reported total opioid consumption with or without local anaesthetic wound infusions[22-25] (Figure 1A). Local anaesthetic wound infusion was associated with a significant decrease in total opioid consumption (weighted mean difference: -40.13; 95% CI: -76.74 to -3.53; $P = 0.03$). This outcome measure was associated with significant statistical heterogeneity (Cochran's Q = 45.31, $P = 0.02$) but not significant bias (Egger Test = -4.69, $P = 0.27$).

Four of the five trials reported separate data for opioid consumption with or without local anaesthetic wound infusion on post-operative day 1[22,23,25,26] (Figure 1B). Local anaesthetic wound infusion was associated with a significant decrease in opioid consumption on post-operative day 1 (weighted mean difference: -8.34; 95% CI: -16.38 to -0.31; $P = 0.04$). There was significant statistical heterogeneity (Cochran's Q = 9.98, $P = 0.019$) but not significant bias (Egger test: -2.11, $P = 0.48$).

Three trials reported opioid consumption on post-operative days 2 and 3[22,23,26] (Table 1). There was no significant effect on opioid consumption (d 2 weighted mean difference: -9.49; 95% CI: -20.37 to 1.39; $P = 0.087$; day 3 weighted mean difference: -4.80; 95% CI: -11.72 to 2.13; $P = 0.17$). Two trials did not report this outcome measure rendering calculation of statistical heterogeneity or bias impossible.

Visual analogue pain scores at rest

Four of the five trials reported visual analogue scores (VASs) of pain on post-operative days 1, 2 and 3[22-24,26]. Post-operative pain was reduced with local anaesthetic infusion on d 1 and 2 but the difference was not significant (Table 1) (d 1 weighted mean difference: -0.18; 95% CI: -1.31 to 0.95; $P = 0.75$ and d 2 weighted mean difference: -0.20; 95% CI: -1.06 to 0.66; $P = 0.65$). However, these outcome measures were associated with significant statistical heterogeneity (Cochran's Q 18.15 and 15.42, $P < 0.05$). The use of local anaesthetic wound infusions was associated with a significant decrease in post-operative pain at rest on d 3 (Figure 1C) (weighted mean

Table 1 Results of meta-analyses

Outcome measure	Weighted mean difference	95% CI	*P*	Heterogeneity	Bias
Opioid consumption					
Total	-40.13	-76.74 to -3.53	0.03	$P = 0.02$	$P = 0.27$
Postoperative day 1	-8.34	-16.38 to -0.31	0.04	$P = 0.019$	$P = 0.48$
Postoperative day 2	-9.41	-20.37 to 1.39	0.087	NA	NA
Postoperative day 3	-4.8	-11.72 to 2.13	0.17	NA	NA
Visual analogue pain score at rest					
Postoperative day 1	-0.18	-1.31 to 0.95	0.75	$P < 0.05$	$P = 0.80$
Postoperative day 2	-0.20	-1.06 to 0.66	0.65	$P < 0.05$	$P = 0.47$
Postoperative day 3	-0.43	-0.81 to -0.044	0.029	NA	$P = 0.63$
Visual analogue pain score on coughing or movement					
Postoperative day 1	-1.14	-2.24 to -0.041	0.04	NA	NA
Postoperative day 2	-0.97	-1.91 to -0.029	0.04	NA	NA
Postoperative day 3	-0.61	1.01 to -0.20	0.0038	NA	NA
Duration of hospital stay	-20.87	-46.94 to 5.21	0.12	$P = 0.016$	$P = 0.30$
Time to return of bowel function	-9.4	-33.98 to 15.17	0.45	NA	NA

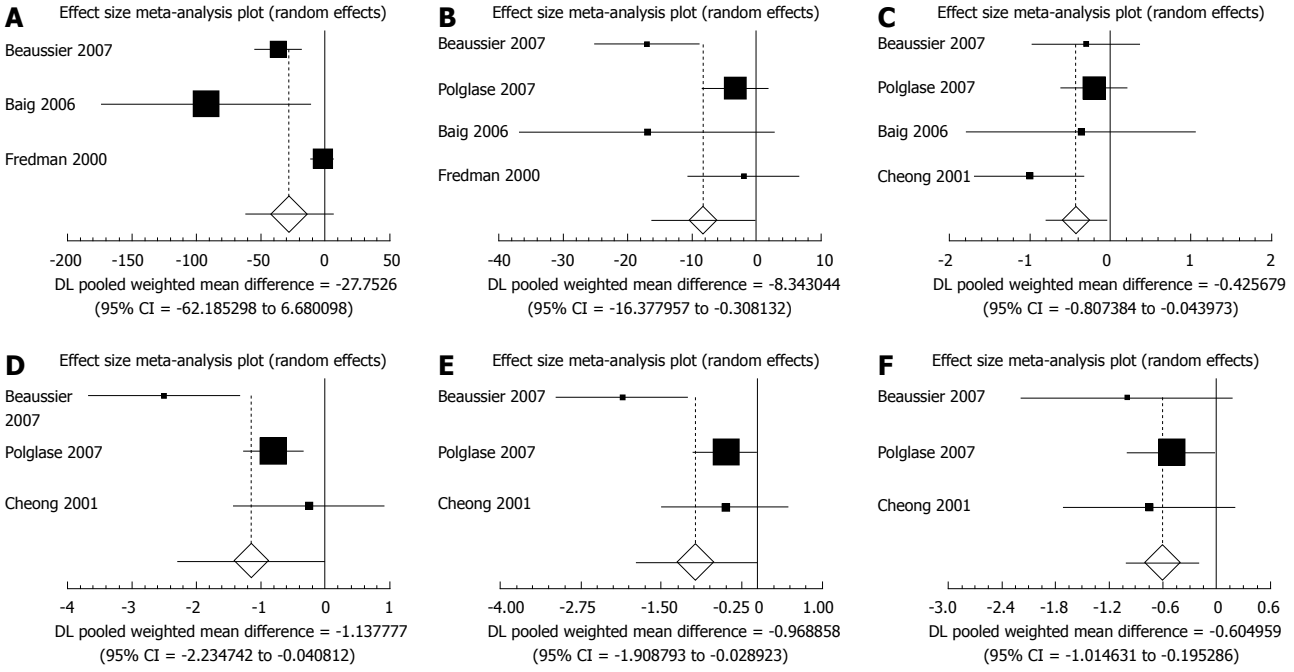


Figure 1 **A**: Forest plot for total postoperative opioid consumption with or without continuous wound infusion of local anaesthetic agent; **B**: Forest plot for opioid consumption on postoperative d 1 with or without continuous wound infusion of local anaesthetic agent; **C**: Forest plot for pain VAS at rest on postoperative d 3 with or without continuous wound infusion of local anaesthetic agent; **D**: Forest plot for pain VAS on coughing or movement on postoperative d 1 with or without continuous wound infusion of local anaesthetic agent; **E**: Forest plot for pain VAS on coughing or movement on postoperative d 2 with or without continuous wound infusion of local anaesthetic agent; **F**: Forest plot for pain VAS on coughing or movement on postoperative d 3 with or without continuous wound infusion of local anaesthetic agent.

difference: -0.43; 95% CI: -0.81 to -0.044; $P = 0.0288$). There was no evidence of bias for days 1, 2 or 3 (day 1 Egger test 0.99, $P = 0.80$; day 2 Egger test 2.75, $P = 0.47$; day 3 Egger test -1.00, $P = 0.63$).

Visual analogue pain scores on coughing or movement

Three of the five trials reported pain VAS on coughing or movement, grouped for this analysis as a composite endpoint[23,24,26]. Local Anaesthetic infusion was associated with a significant reduction in pain VAS on all three post-operative days (Figures 1D to F) (day 1 weighted mean difference: -1.14; 95% CI: -2.24 to -0.041; $P = 0.04$, day 2 weighted mean difference: -0.97, 95% CI: -1.91 to -0.029; $P = 0.04$, day 3 weighted mean difference: -0.61; 95% CI: 1.01 to -0.20; $P = 0.0038$). Two trials did not report this pain on movement, rendering calculation of statistical heterogeneity or bias impossible.

Duration of hospital stay

All five trials reported length of stay. There was no significant decrease in length of stay (Table 1) (weighted mean difference: -20.87; 95% CI: -46.96 to 5.21; $P = 0.12$). This outcome measure was associated with significant statistical heterogeneity (Cochran's Q: 12.20, $P = 0.016$) without significant bias (Egger test: -1.12, $P = 0.30$).

Time to return of bowel function

Mean time to production of faeces was reported by three trials[22-24]. There was no significant effect of local anaesthetic wound infusion (Table 1) (weighted mean difference: -9.40; 95% CI: -33.98 to 15.17; $P = 0.45$). Two trials did not report this outcome measure rendering calculation of statistical heterogeneity or bias impossible.

DISCUSSION

The results of our meta-analysis suggest that wound infusions are a promising adjunct to existing analgesic regimens following laparotomy for major colorectal surgery. The results do not, however, provide conclusive evidence of significant benefit conferred by this technique and it is doubtful whether the data gathered are sufficient to support generalisation of this conclusion to routine practice. The number of eligible trials (5) and total abdominal wounds (542) is small, and meta-analyses on small samples may be vulnerable to confounding if one or two of the eligible trials demonstrate a strong trend for or against the intervention under investigation.

For the purpose of this meta-analysis, the outcome measure "opioid consumption" was chosen to reflect opioid-sparing effect provided by local anaesthetic infusions. However, the significant statistical heterogeneity associated with this outcome measure reflects a variety of background analgesic regimens used in both control and treatment groups. Polglase *et al* utilized a multimodal analgesic regimen whereas the other trials studied used only patient controlled opioid analgesia to provide background analgesia. This degree of methodological heterogeneity between the trials may have influenced the meta-analysis.

Analysis of pain VAS may also have been affected by methodological heterogeneity between the trials studied. Furthermore, pain VAS is a non-parametric variable whereas the meta-analysis models used assume parametric distribution of the variables under study. The variable "length of stay" reflects a composite endpoint that may have been affected by several factors other than the presence of local anaesthetic infusions, and therefore it is not possible to draw causative inferences from the results of this pooled outcome measure with great validity. It was not possible to obtain sufficient data for all the trials under study to provide a reliable analysis of return to bowel function.

An economic analysis of local anaesthetic wound infusions is also needed - it seems likely that a greater amount of data is needed to clarify any trends in postoperative complications that may support the use of these infusions. Further large randomised controlled trials are required to investigate the promise of local anaesthetic wound infusions in major colorectal surgery, using standardized local anaesthetic agents, background analgesic regimens, experimental protocols, discharge criteria and anatomical site for wound infusion delivery.

In conclusion, Although suboptimal postoperative pain control is associated with cardiovascular, respiratory and gastrointestinal complications, many multimodal regimens for analgesia following major colorectal laparotomy provide inadequate pain relief. Although the number of trials available for meta-analysis is small, the available data demonstrate potential benefit in terms of reduction in opioid consumption following laparotomy for major colorectal surgery. Further large-scale studies will be needed to ascertain if any clear benefit or harm is conferred by the prophylactic use of local anaesthetic wound infusions in major colorectal surgery. Future research on this topic should also address the inaccuracies introduced by the methodological heterogeneity previously addressed in available trials, and provide a cost-effectiveness analysis of the use of continuous wound infusions in colorectal surgery.

COMMENTS

Background

Pain control following abdominal laparotomy and bowel anastomosis in colorectal surgery is a complex challenge not amenable to pharmacological monotherapy. Modern multimodal analgesic regimens may provide suboptimal postoperative pain control, which is associated with cardiovascular and respiratory complications and increased gastrointestinal paralysis.

Research frontiers

Continuous wound infusions of local anaesthetic agents have been suggested to provide improved pain control following a broad range of surgical incisions, both alone and as part of a multimodal analgesic regimen.

Innovations and breakthroughs

There is a need for a focused and quantitative review of the evidence for the analgesic benefit of continuous wound infusion of local anaesthetics specific to colorectal laparotomy, which is provided by this meta-analysis.

Applications

The meta-analysis demonstrates potential benefit in terms of reduction in opioid consumption following laparotomy for major colorectal surgery. The review highlights the need for future research on this topic and identifies that such future research should address the inaccuracies introduced by the methodological heterogeneity identified in available trials, and provides a cost-effectiveness analysis of the use of continuous wound infusions in colorectal surgery.

Terminology

Visual analogue scale (VAS) is a validated research tool used to quantitatively assess the subjective experience of patients' pain perception.

Peer review

The authors present a systematic analysis of local anaesthetics in wounds after open colorectal surgery. This is an area that has not been addressed by systematic analysis previously. It is a well-done and timely review.

REFERENCES

1 **Gendall KA**, Kennedy RR, Watson AJ, Frizelle FA. The effect of epidural analgesia on postoperative outcome after colorectal surgery. *Colorectal Dis* 2007; **9**: 584-598; discussion 598-600
2 **Kehlet H**. Surgical stress: the role of pain and analgesia. *Br J Anaesth* 1989; **63**: 189-195
3 **Stein C**. Peripheral mechanisms of opioid analgesia. *Anesth Analg* 1993; **76**: 182-191
4 **Dahl JB**, Kehlet H. Non-steroidal anti-inflammatory drugs: rationale for use in severe postoperative pain. *Br J Anaesth* 1991; **66**: 703-712
5 **PROSPECT working group**. Procedure specific postoperative pain management: open colonic resection. Available from: URL: http://www.postoppain.org/frameset.htm Accessed 28th January 2008
6 **Kehlet H**, Liu SS. Continuous local anesthetic wound infusion to improve postoperative outcome: back to the

periphery? *Anesthesiology* 2007; **107**: 369-371
7 **Liu SS**, Richman JM, Thirlby RC, Wu CL. Efficacy of continuous wound catheters delivering local anesthetic for postoperative analgesia: a quantitative and qualitative systematic review of randomized controlled trials. *J Am Coll Surg* 2006; **203**: 914-932
8 **Barron DJ**, Tolan MJ, Lea RE. A randomized controlled trial of continuous extra-pleural analgesia post-thoracotomy: efficacy and choice of local anaesthetic. *Eur J Anaesthesiol* 1999; **16**: 236-245
9 **Dowling R**, Thielmeier K, Ghaly A, Barber D, Boice T, Dine A. Improved pain control after cardiac surgery: results of a randomized, double-blind, clinical trial. *J Thorac Cardiovasc Surg* 2003; **126**: 1271-1278
10 **Francois T**, Blanloeil Y, Pillet F, Moren J, Mazoit X, Geay G, Douet MC. Effect of interpleural administration of bupivacaine or lidocaine on pain and morphine requirement after esophagectomy with thoracotomy: a randomized, double-blind and controlled study. *Anesth Analg* 1995; **80**: 718-723
11 **Chester JF**, Ravindranath K, White BD, Shanahan D, Taylor RS, Lloyd-Williams K. Wound perfusion with bupivacaine: objective evidence for efficacy in postoperative pain relief. *Ann R Coll Surg Engl* 1989; **71**: 394-396
12 **Lau H**, Patil NG, Lee F. Randomized clinical trial of postoperative subfascial infusion with bupivacaine following ambulatory open mesh repair of inguinal hernia. *Dig Surg* 2003; **20**: 285-289
13 **Levack ID**, Holmes JD, Robertson GS. Abdominal wound perfusion for the relief of postoperative pain. *Br J Anaesth* 1986; **58**: 615-619
14 **Fredman B**, Shapiro A, Zohar E, Feldman E, Shorer S, Rawal N, Jedeikin R. The analgesic efficacy of patient-controlled ropivacaine instillation after Cesarean delivery. *Anesth Analg* 2000; **91**: 1436-1440
15 **Gupta A**, Perniola A, Axelsson K, Thorn SE, Crafoord K, Rawal N. Postoperative pain after abdominal hysterectomy: a double-blind comparison between placebo and local anesthetic infused intraperitoneally. *Anesth Analg* 2004; **99**: 1173-1179, table of contents
16 **Kushner DM**, LaGalbo R, Connor JP, Chappell R, Stewart SL, Hartenbach EM. Use of a bupivacaine continuous wound infusion system in gynecologic oncology: a randomized trial. *Obstet Gynecol* 2005; **106**: 227-233
17 **Alford JW**, Fadale PD. Evaluation of postoperative bupivacaine infusion for pain management after anterior cruciate ligament reconstruction. *Arthroscopy* 2003; **19**: 855-861
18 **Barber FA**, Herbert MA. The effectiveness of an anesthetic continuous-infusion device on postoperative pain control. *Arthroscopy* 2002; **18**: 76-81
19 **Bianconi M**, Ferraro L, Ricci R, Zanoli G, Antonelli T, Giulia B, Guberti A, Massari L. The pharmacokinetics and efficacy of ropivacaine continuous wound instillation after spine fusion surgery. *Anesth Analg* 2004; **98**: 166-172, table of contents
20 **Jadad AR**, Moore RA, Carroll D, Jenkinson C, Reynolds DJ, Gavaghan DJ, McQuay HJ. Assessing the quality of reports of randomized clinical trials: is blinding necessary? *Control Clin Trials* 1996; **17**: 1-12
21 **DerSimonian R**, Laird N. Meta-analysis in clinical trials. *Control Clin Trials* 1986; **7**: 177-188
22 **Baig MK**, Zmora O, Derdemezi J, Weiss EG, Nogueras JJ, Wexner SD. Use of the ON-Q pain management system is associated with decreased postoperative analgesic requirement: double blind randomized placebo pilot study. *J Am Coll Surg* 2006; **202**: 297-305
23 **Beaussier M**, El'Ayoubi H, Schiffer E, Rollin M, Parc Y, Mazoit JX, Azizi L, Gervaz P, Rohr S, Biermann C, Lienhart A, Eledjam JJ. Continuous preperitoneal infusion of ropivacaine provides effective analgesia and accelerates recovery after colorectal surgery: a randomized, double-blind, placebo-controlled study. *Anesthesiology* 2007; **107**: 461-468
24 **Cheong WK**, Seow-Choen F, Eu KW, Tang CL, Heah SM. Randomized clinical trial of local bupivacaine perfusion versus parenteral morphine infusion for pain relief after laparotomy. *Br J Surg* 2001; **88**: 357-359
25 **Fredman B**, Zohar E, Tarabykin A, Shapiro A, Mayo A, Klein E, Jedeikin R. Bupivacaine wound instillation via an electronic patient-controlled analgesia device and a double-catheter system does not decrease postoperative pain or opioid requirements after major abdominal surgery. *Anesth Analg* 2001; **92**: 189-193
26 **Polglase AL**, McMurrick PJ, Simpson PJ, Wale RJ, Carne PW, Johnson W, Chee J, Ooi CW, Chong JW, Kingsland SR, Buchbinder R. Continuous wound infusion of local anesthetic for the control of pain after elective abdominal colorectal surgery. *Dis Colon Rectum* 2007; **50**: 2158-2167

S- Editor Li DL **L- Editor** Lutze M **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5306

World J Gastroenterol 2008 September 14; 14(34): 5306-5310
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

mRNA levels of TLR4 and TLR5 are independent of *H pylori*

Elvira Garza-González, Virgilio Bocanegra-García, Francisco Javier Bosques-Padilla, Juan Pablo Flores-Gutiérrez, Francisco Moreno, Guillermo Ignacio Perez-Perez

Elvira Garza-González, Francisco Moreno, Departmento de Microbiología, Facultad de Medicina, Universidad Autónoma de Nuevo León, Monterrey 64460, Mexico
Virgilio Bocanegra-García, Departmento de Biología Molecular y Bioingeniería, UAM Reynosa Aztlán, Universidad Autónoma de Tamaulipas, Reynosa, Tamaulipas 88740, Mexico
Francisco Javier Bosques-Padilla, Juan Pablo Flores-Gutiérrez, Hospital Universitario "Dr. José Eleuterio González", Universidad Autónoma de Nuevo León, Monterrey 64460, Mexico
Guillermo Ignacio Perez-Perez, Department of Medicine and Microbiology, New York University School of Medicine, New York NY 10010, United States
Author contributions: Garza-González E designed and coordinated the study, optimized PCR conditions, and drafted the manuscript; Bocanegra-García V and Moreno F extracted RNAs and performed molecular assays; Flores-Gutiérrez JP performed histological examination and interpretation of results; Bosques-Padilla FJ recruited patients and carried out endoscopies; Pérez-Perez GI participated in the design, coordination of the study and revised the drafted manuscript.
Correspondence to: Elvira Garza-González, Departmento de Microbiología, Facultad de Medicina, Universidad Autónoma de Nuevo León, Av. Madero y Dr. Aguirre s/n, Colonia Mitras Centro, Monterrey 64460,
Mexico. elvira_garza_gzz@yahoo.com
Telephone: +52-81-83294166 Fax: +52-81-83294166
Received: May 4, 2008 Revised: August 18, 2008
Accepted: August 25, 2008
Published online: September 14, 2008

Abstract

AIM: To determine if the presence *H pylori* or its virulence affect toll-like receptor 4 (TLR4) and TLR5 mRNA expression levels.

METHODS: For the *in vivo* assays, gastric biopsies were obtained from 40 patients and *H pylori* status was determined. For the *in vitro* assays, human gastric adenocarcinoma mucosal cells (AGS) were cultured in the presence or absence of twelve selected *H pylori* strains. *H pylori* strains isolated from culture-positive patients and selected strains were genotyped for *cagA* and *vacA*. The cDNA was obtained from mRNA extracted from biopsies and from infected AGS cells. TLR4 and TLR5 mRNA levels were examined by real-time PCR.

RESULTS: The presence of *H pylori* did not affect the mRNA levels of TLR4 or TLR5 in gastric biopsies. The mRNA levels of both receptors were not influenced by the *vacA* status ($P > 0.05$ for both receptors) and there were no differences in TLR4 or TLR5 mRNA levels among the different clinical presentations/histological findings ($P > 0.05$). In the *in vitro* assay, the mRNA levels of TLR4 or TLR5 in AGS cells were not influenced by the *vacAs1* status or the clinical condition associated with the strains ($P > 0.05$ for both TLR4 and TLR5).

CONCLUSION: The results of this study show that the mRNA levels of TLR4 and TLR5 in gastric cells, both *in vivo* and *in vitro*, are independent of *H pylori* colonization and suggest that *vacA* may not be a significant player in the first step of innate immune recognition mediated by TLR4 or TLR5.

Key words: *H pylori*; Toll-like receptor 4; Toll-like receptor 5; AGS cells; mRNA

Peer reviewer: Dr. Limas Kupcinskas, Professor, Gastroenterology of Kaunas University of Medicine, Mickeviciaus 9, Kaunas LT 44307, Lithuania

Garza-González E, Bocanegra-García V, Bosques-Padilla FJ, Flores-Gutiérrez JP, Moreno F, Perez-Perez GI. mRNA levels of TLR4 and TLR5 are independent of *H pylori*. *World J Gastroenterol* 2008; 14(34): 5306-5310 Available from: URL: http://www.wjgnet.com/1007-9327/14/5306.asp DOI: http://dx.doi.org/10.3748/wjg.14.5306

INTRODUCTION

H pylori is a Gram-negative, flagellated bacterium that colonizes the gastric mucosa of approximately two-thirds of the world's population and is the primary cause of peptic ulcers and gastric adenocarcinoma. The complex interactions between different *H pylori* strains, the host immune system or environmental factors (or combinations thereof) are responsible for the significant variability in disease presentation associated with *H pylori* infection[1].

Vacuolating cytotoxin (VacA) and the CagA protein are the two major virulence markers usually associated with *H pylori* pathogenicity. VacA induces the formation of intracellular vacuoles in epithelial cell lines. Aside from its direct cell-damaging effect *in vitro*, VacA also plays a major role in inducing cytoskeletal changes, apoptosis and suppression of epithelial cell prolifera-

tion[2]. The *vac*A gene is present in all *H pylori* strains and contains at least two variable domains. The s-region, which encodes the signal peptide, exists as either an s1 or s2 isoform. The *vacA* s1 genotype has been linked to increased disease severity[2,3].

Some *H pylori* strains contain a pathogenicity island, which carries a number of virulence factors, including *cagA*, which is considered to be a marker for this island. A type Ⅳ secretory system translocates the CagA protein into host epithelial cells where it is phosphorylated by host-cell kinases[4]. *H pylori cagA*-positive strains have been associated with more severe inflammation of the gastric mucosa and more severe disease manifestations[1,2].

Toll-like receptors (TLRs) are a family of mammalian homologs of the *Drosophila* Toll proteins and, in mammalian systems, TLR4 confers responsiveness to Gram-negative lipopolysaccharide (LPS), while TLR5 recognizes flagellin[5]. Previous studies have shown that gastric epithelia express both TLR4 and TLR5[6-9]. Here, we studied the mRNA levels of TLR4 and TLR5 in gastric epithelial cells to determine if distinctive changes in the levels of mRNA could be affected by the presence of toxigenic and non-toxigenic (in particular *vacA*+ strains) *H pylori* strains.

MATERIALS AND METHODS

Study population, RNA isolation, and H pylori status

Forty patients (mean age, 58.3 years; age range, 18-81 years; F/M, 25/15) with indications of upper gastroduodenal endoscopy were included and the study was approved by the local ethics committee. Eighteen biopsy specimens were obtained from each patient. Total RNA was extracted from four of the biopsies from each patient (two from the antrum and two from the corpus) using Trizol® reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Eight biopsies were used for histological evaluation; two from the lesser curvature, two from the greater curvature, two from the incisura angularis, and two from the prepyloric region. Biopsies were fixed, paraffin embedded and examined histologically after hematoxylin-eosin staining.

Patients' *H pylori* status was determined by histology, the rapid urease test performed by an in-house validated test on two biopsies (one from the antrum and one from the corpus)[10], and culture analysis performed by standard methods on four biopsies (two from the antrum and two from the corpus)[11]. Bacterial genomic DNA was extracted from *H pylori* strains and typing for *cagA* and *vacA* was performed using primers previously described[3,12]. Patients were considered *H pylori*-positive when at least two of the diagnostic tests were positive.

Infection of AGS cells and RNA isolation

From our collection, we selected 12 strains that were isolated from patients with gastritis, distal gastric cancer, and peptic ulcer disease (4 from each pathologic/histologic finding). All strains were genotyped for *vacA* and *cagA* as described above. Human gastric epithelial AGS CRL-1739 cells were grown in RPMI 1640 supplemented with 10% FBS. *H pylori* bacteria were added at a multiplicity of infection (MOI) of 100:1, followed by a phosphate buffered saline (PBS) pH 7.4 wash to remove non-adherent bacteria. After 24 h, total RNA was isolated using the RNA tissue kit (Gentra Systems, Minneapolis, MN). The *H pylori* J99 ATCC 700824 strain was used as a control in all experiments.

Table 1 Distribution of *H pylori* infection status and the *H pylori* genotype

	***H pylori* genotype (*n* = 20 strains)**		
Diagnosis	*vacA s1*	*vacA s2*	*cagA*
Gastritis	5/15	10/15	15/15
Intestinal metaplasia	4/4	0/4	4/4
Antral ulcer	0/1	1/1	1/1
Atrophic gastritis	0/0	0/0	0/0

Reverse transcription and real-time PCR

Five micrograms of RNA were reverse-transcribed using SuperScript Ⅲ (Invitrogen) in a 20 μL reaction volume using oligo dT primers. The resulting cDNA was real-time-amplified in a final volume of 25 μL. The mix contained 1 U of Hot-Start *Taq* DNA polymerase (Invitrogen), 1 × reaction buffer, 200 μmol/L of each deoxynucleoside triphosphate (dNTPs), 3 mmol/L $MgCl_2$, 0.3 μmol/L of each primer and 0.25 × SYBR Green (Molecular Probes, Eugene OR). PCR was performed in glass capillaries using a Light Cycler instrument (Roche Applied Science, Indianapolis, IN). All reactions were performed in duplicate, and the thermal cycling conditions were 1 min at 94℃, followed by 35 cycles of 94℃ for 10 s, 59℃ for 10 s and 72℃ for 10 s with a ramp of 5℃/s. Real time PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed as described previously[7]. The relative amounts of the PCR products were calculated as the ratio of each TLR mRNA to GAPDH mRNA.

Statistical analysis

Comparisons were performed using a non-paired Student's *t* test. Analyses were carried out using the Statistics software 7.0 (Melbourne, Australia).

RESULTS

H pylori infection status and histological findings in the study population

The majority, 57.5 % (*n* = 23), of the patients examined were *H pylori* positive (F/M, 14/9; 22-81 years; 53.8 ± 20.3). Of the 40 patients in the study, 29 had gastritis confirmed by histology, 5 had intestinal metaplasia (IM) and 4 had antral ulcers. Only two patients had atrophic gastritis. All of the *H pylori* strains that were isolated were *cagA*-positive (Table 1).

TLR4 and TLR5 mRNA levels in gastric biopsies

We plotted the TLR/GADPH ratios for both TLR4

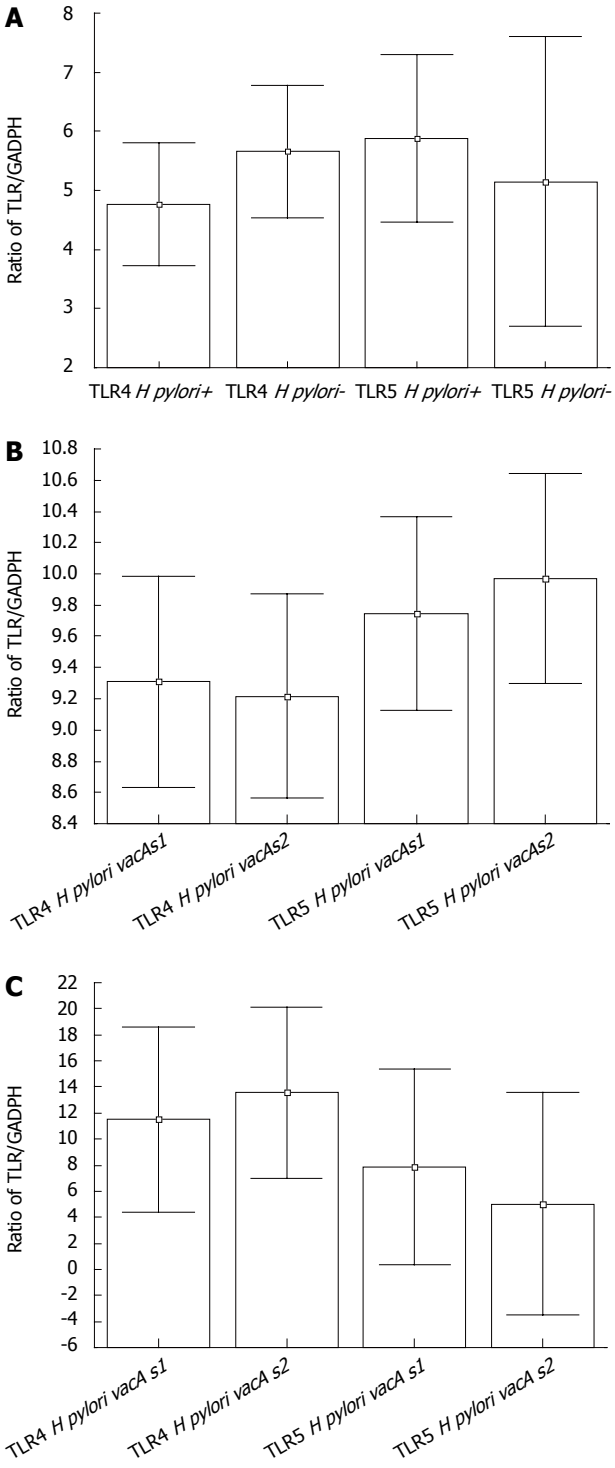


Figure 1 **A**: Mean (95% CI) ratio of TLR/GADPH mRNA levels for both TLR 4 and TLR5 in *H pylori*-positive and *H pylori*-negative patients; **B**: Mean (95%CI) TLR/GADPH mRNA ratios for both TLR 4 and TLR5 in patients infected with either *H pylori vacAs1* or *H pylori vacA s2* ($P > 0.05$ for both analysis); **C**: Mean (95% CI) TLR/GADPH mRNA ratios for both TLR 4 and TLR5 expression in AGS cells infected with *H pylori vacAs1* or *H pylori vacA s2*. All is $P > 0.05$.

and TLR5 in both infected and non-infected patients (Figure 1A). The presence of *H pylori* did not affect the mRNA levels of either Toll receptor. We did a similar analysis comparing patients infected with either *H pylori vacAs1* or *H pylori vacAs2* (Figure 1B). These data demonstrated that the mRNA levels of TLR4 and TLR5 were not influenced by the *vacA* status ($P > 0.05$ for both receptors). There were no differences in TLR4 or TLR5 mRNA levels between patients with different clinical presentations/histological findings ($P > 0.05$, data not shown). We were not able extract conclusions with respect to *cagA*, because all strains were *cagA* positive.

mRNA levels of TLR4 and TLR5 in AGS cells

Eleven (91.7%) of the selected strains were *cagA*-positive and there was an equal *vacA* s1 and s2 distribution between the strains examined. There were no differences in the mRNA levels of TLR4 or TLR5 regarding the *vacAs1* status or the clinical condition associated with the infecting strains ($P > 0.05$ for both TLR4 and TLR5, Figure 1C).

DISCUSSION

Several studies have addressed the mRNA levels and protein expression of TLR4 and TLR5 in gastrointestinal cells or in AGS cells[6-9,13]. In this study, we examined the mRNA levels of TLR4 and TLR5 in gastric epithelial cells (biopsies and AGS cells) in order to determine if significant changes in mRNA levels could be related to the presence of *H pylori* or differences between the virulence of the strains. Our examination of gastric biopsies from infected and non-infected patients showed that there were no quantitative differences in the mRNA levels of these receptors regardless of whether *H pylori* was present or of the patient's *H pylori vacA* status.

Gastritis is thought to precede the development of IM, antral ulcer or atrophic gastritis[1,2]. In this study, we included biopsies from 40 patients and among them, 29 had gastritis, 5 had IM, 4 had antral ulcers, and 2 had atrophic gastritis. When we compared the mRNA levels of both receptors between patients with gastritis and with each clinical condition/histological findings, we were unable to identify differences, suggesting that mRNA levels for both receptors may not be influenced by the infection process, or at least not at the time points selected for analysis.

Our analysis of TLR4 and TLR5 mRNA levels in AGS cells in the presence or absence of *H pylori* showed that the amounts of TLR4 and TLR5 mRNA in human gastric epithelial cells were independent of *H pylori vacA* status. In the *in vitro* assay, the analysis showed no differences in the amounts of TLR4 and TLR5 mRNA linked to different clinical conditions related to the *H pylori* strains selected.

Our results do not exclude the possibility of differential expression between TLR4 and TLR5 receptors since mRNA levels do not accurately reflect protein expression. In this study, we used quantitative real-time PCR, which is the most commonly used technique for studying mRNA expression levels[14]. This technique has some advantages, such as accuracy, sensitivity and reproducibility. Also, it allows for high throughput analyses and can be performed on very small samples. However, some problems associated with this

technique must be addressed, such as the effects of different amounts of starting material, especially when analyzing biopsies. To deal with this variation, an internal control (housekeeping gene) must be simultaneously amplified with the gene of interest for normalization purposes[15]. In this study, even though we included analysis of GADPH as an internal control we could not assume that protein expression levels of both receptors were equal based on the levels of detected mRNA.

An additional caveat was that the *in vivo* mRNA analysis of pooled biopsies from forty patients minimized the ability to identify biopsy-to-biopsy or patient-to-patient differences. Since RNA was extracted from all four antrum and corpus biopsies in the same vial, different gene expression profiles associated with different tissues would not have been detected. By analyzing four combined biopsies, it was possible that mRNA differences between corpus and antrum mucosal samples could have been missed. However, in the *in vitro* assay the mRNA levels obtained following infections with 12 different *H pylori* strains showed no differences in TLR expression. Although this is a uniform tissue, the observation that at least different *H pylori* strains did not affect TLR mRNA levels suggested that the same results would be observed in biopsy samples.

Some investigators have suggested that the subcellular distribution of receptors, rather than TLR expression level, could be relevant in the pathogenesis of inflammatory diseases because the expression of these receptors seems to be constitutive[7]. Our results are consistent with this view. Nonetheless, a study examining the cellular distribution of expression in relation to *H pylori vacA* status would be an interesting issue to address.

Schmausser *et al*[9], reported that gastric epithelium with intestinal metaplasia and dysplasia expressed TLR4 and TLR5. They demonstrated that 17 out of 22 patients strongly expressed TLR4 (77.27%) and all 22 patients with gastric carcinoma expressed TLR5. Our study confirmed the presence of TLR4 and TLR5 mRNA, which preceded the expression of both receptors.

It is likely that evaluating the roles of other Toll-like receptors would help elucidate differences in disease manifestation and severity of diseases caused by *H pylori* infections. Valuable information regarding the recognition of whole *H pylori* or its LPS by TLR2 has been reported. Smith *et al*[16] demonstrated that gastric epithelial cells recognized and responded to *H pylori* infection, at least in part, via TLR2 and that *H pylori* LPS was a TLR2 agonist.

Additionally, Mandell *et al*[17] demonstrated that cytokine responses to whole *H pylori* were mediated by TLR2. Based on these investigations, more work examining the role of TLR2 in relation to the *H pylori vacA* status is needed. The results of this study show that the TLR4 and TLR5 mRNA levels in gastric cells both *in vivo* and *in vitro* are independent of *H pylori* and suggest that *vacA* may not be involved in the first steps of innate immune-recognition of *H pylori*.

COMMENTS

Background

H pylori is the primary cause of peptic ulcers and gastric adenocarcinoma. The variability of clinical manifestations is associated with bacterial, host immune responses and environmental factors.

Research frontiers

Gastric epithelia express toll-like receptor 4 (TLR4) and TLR5. We studied the mRNA levels of TLR4 and TLR5 in gastric epithelial cells to determine if distinctive changes in mRNA levels could be influenced by the presence of toxigenic *H pylori* strains.

Innovations and breakthroughs

In this study, *we* analyzed the mRNA levels of both TLR4 and TLR5 in gastric biopsies from infected and non-infected patients and in AGS cells infected with *H pylori*. We correlated these results with the *vacA* status of the strains. There were no quantitative differences in the mRNA levels of these receptors regardless of *H pylori* presence or the *H pylori vacA* status both in gastric biopsies and in AGS cells.

Applications

The mRNA levels of TLR4 and TLR5 in gastric cells both *in vivo* and *in vitro* are independent of *H pylori* or their *vacA* status.

Peer review

The results show that the mRNA levels of TLR4 and TLR5 in gastric cells are not influenced by *H pylori vacA* status and suggest that *vacA* may not be a significant player in the first step of innate immune recognition mediated by TLR4 or TLR5. It seems innovative and very interesting.

REFERENCES

1 **Blaser MJ**, Berg DE. Helicobacter pylori genetic diversity and risk of human disease. *J Clin Invest* 2001; **107**: 767-773

2 **Figueiredo C**, Machado JC, Yamaoka Y. Pathogenesis of Helicobacter pylori Infection. *Helicobacter* 2005; **10** Suppl 1: 14-20

3 **Atherton JC**, Cao P, Peek RM Jr, Tummuru MK, Blaser MJ, Cover TL. Mosaicism in vacuolating cytotoxin alleles of Helicobacter pylori. Association of specific vacA types with cytotoxin production and peptic ulceration. *J Biol Chem* 1995; **270**: 17771-17777

4 **Odenbreit S**, Puls J, Sedlmaier B, Gerland E, Fischer W, Haas R. Translocation of Helicobacter pylori CagA into gastric epithelial cells by type IV secretion. *Science* 2000; **287**: 1497-1500

5 **Ferrero RL**. Innate immune recognition of the extracellular mucosal pathogen, Helicobacter pylori. *Mol Immunol* 2005; **42**: 879-885

6 **Su B**, Ceponis PJ, Lebel S, Huynh H, Sherman PM. Helicobacter pylori activates Toll-like receptor 4 expression in gastrointestinal epithelial cells. *Infect Immun* 2003; **71**: 3496-3502

7 **Schmausser B**, Andrulis M, Endrich S, Lee SK, Josenhans C, Muller-Hermelink HK, Eck M. Expression and subcellular distribution of toll-like receptors TLR4, TLR5 and TLR9 on the gastric epithelium in Helicobacter pylori infection. *Clin Exp Immunol* 2004; **136**: 521-526

8 **Backhed F**, Rokbi B, Torstensson E, Zhao Y, Nilsson C, Seguin D, Normark S, Buchan AM, Richter-Dahlfors A. Gastric mucosal recognition of Helicobacter pylori is independent of Toll-like receptor 4. *J Infect Dis* 2003; **187**: 829-836

9 **Schmausser B**, Andrulis M, Endrich S, Muller-Hermelink HK, Eck M. Toll-like receptors TLR4, TLR5 and TLR9 on gastric carcinoma cells: an implication for interaction with Helicobacter pylori. *Int J Med Microbiol* 2005; **295**: 179-185

10 **Flores-Orta D**, Bosques-Padilla F, Gomez-Leija G, Frederick F. Comparative study of rapid urease test (Hazell test) vs CLO-test in the diagnosis of Helicobacter pylori infection. *Gut* 1997; **41** Suppl 3: A160

11 **Perez-Perez GI**. Accurate diagnosis of Helicobacter pylori. Culture, including transport. *Gastroenterol Clin North Am*

2000; **29**: 879-884
12 **Rugge M**, Busatto G, Cassaro M, Shiao YH, Russo V, Leandro G, Avellini C, Fabiano A, Sidoni A, Covacci A. Patients younger than 40 years with gastric carcinoma: Helicobacter pylori genotype and associated gastritis phenotype. *Cancer* 1999; **85**: 2506-2511
13 **Su B**, Ceponis PJ, Lebel S, Huynh H, Sherman PM. Helicobacter pylori activates Toll-like receptor 4 expression in gastrointestinal epithelial cells. *Infect Immun* 2003; **71**: 3496-3502
14 **Bustin SA**, Nolan T. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. *J Biomol Tech* 2004; **15**: 155-166
15 **Bustin SA**. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 2000; **25**: 169-193
16 **Smith MF Jr**, Mitchell A, Li G, Ding S, Fitzmaurice AM, Ryan K, Crowe S, Goldberg JB. Toll-like receptor (TLR) 2 and TLR5, but not TLR4, are required for Helicobacter pylori-induced NF-kappa B activation and chemokine expression by epithelial cells. *J Biol Chem* 2003; **278**: 32552-32560
17 **Mandell L**, Moran AP, Cocchiarella A, Houghton J, Taylor N, Fox JG, Wang TC, Kurt-Jones EA. Intact gram-negative Helicobacter pylori, Helicobacter felis, and Helicobacter hepaticus bacteria activate innate immunity via toll-like receptor 2 but not toll-like receptor 4. *Infect Immun* 2004; **72**: 6446-6454

S- Editor Zhong XY **L- Editor** Negro F **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5311

World J Gastroenterol 2008 September 14; 14(34): 5311-5315
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Chemoradiotherapy with twice-weekly administration of low-dose gemcitabine for locally advanced pancreatic cancer

Hisato Igarashi, Tetsuhide Ito, Ken Kawabe, Terumasa Hisano, Yoshiyuki Arita, Toyoma Kaku, Ryoichi Takayanagi

Hisato Igarashi, Tetsuhide Ito, Ken Kawabe, Terumasa Hisano, Yoshiyuki Arita, Toyoma Kaku, Ryoichi Takayanagi, Department of Medicine and Bioregulatory Science, Graduate School of Medical Science, Kyushu University, Fukuoka 812-8582, Japan

Author contributions: Igarashi H analyzed the data and wrote the paper; Ito T designed the study and revised the paper; Kawabe K, Hisano T, Arita Y and Kaku T collected and analyzed the data; Takayanagi R revised the paper.

Correspondence to: Hisato Igarashi, MD, PhD, Department of Medicine and Bioregulatory Science, Graduate School of Medical Science, Kyushu University 3-1-1 Maidashi Higashi-ku Fukuoka 812-8582 Japan. igaras@intmed3.med.kyushu-u.ac.jp

Telephone: +81-92-6425285 Fax: +81-92-6425287

Received: May 13, 2008 Revised: July 28, 2008
Accepted: August 4, 2008
Published online: September 14, 2008

Abstract

AIM: To evaluate the chemoradiotherapy for locally advanced pancreatic cancer utilizing low dose gemcitabine as a radiation sensitizer administered twice weekly.

METHODS: We performed a retrospective analysis of chemoradiotherapy utilizing gemcitabine administered twice weekly at a dose of 40 mg/m^2. After that, maintenance systemic chemotherapy with gemcitabine, at a dose of 1000 mg/m^2, was administered weekly for 3 wk with 1-wk rest until disease progression or unacceptable toxicity developed.

RESULTS: Eighteen patients with locally advanced unresectable pancreatic cancer were enrolled. Three of those patients could not continue with the therapy; one patient had interstitial pneumonia during radiation therapy and two other patients showed liver metastasis or peritoneal metastasis during an early stage of the therapy. The median survival was 15.0 mo and the overall 1-year survival rate was 60%, while the median progression-free survival was 8.0 mo. The subgroup which showed the reduction of tumor development, more than 50% showed a tendency for a better prognosis; however, other parameters including age, gender and performance status did not correlate with survival. The median survival of the groups that died of liver metastasis and peritoneal metastasis were 13.0 mo and 27.7 mo, respectively.

CONCLUSION: Chemoradiotherapy with low-dose gemcitabine administered twice weekly could be effective to patients with locally advanced pancreatic cancer; however, patients developing liver metastases had a worse prognosis. Another chemoradiotherapy strategy might be needed for those patients, such as administrating one or two cycles of chemotherapy initially, followed by chemoradiotherapy for the cases with no distant metastases.

Key words: Advanced pancreatic cancer; Chemoradiotherapy; Gemcitabine; Radiosensitizer; Tumor marker

Peer reviewer: Michael E Zenilman, MD, Clarence and Mary Dennis Professor and Chairman, Department of Surgery, SUNY Downstate Medical Center, Box40, 450, Clarkson Avenue, Brooklyn, NY 11202, United States

Igarashi H, Ito T, Kawabe K, Hisano T, Arita Y, Kaku T, Takayanagi R. Chemoradiotherapy with twice-weekly administration of low-dose gemcitabine for locally advanced pancreatic cancer. *World J Gastroenterol* 2008; 14(34): 5311-5315 Available from: URL: http://www.wjgnet.com/1007-9327/14/5311.asp DOI: http://dx.doi.org/10.3748/wjg.14.5311

INTRODUCTION

Pancreatic cancer is one of the leading causes of cancer death in the world and in most patients the tumor is surgically unresectable at the time of diagnosis[1]. Even in the patient with complete surgical resection, both distant and local patterns of recurrence are common[2]. In approximately 50% of resected pancreatic tumors, the surgical margins are involved with tumor cells, so it can be assumed that most patients are harboring occult metastases at the time of diagnosis[3]. Recently, studies for adjuvant chemotherapy or chemoradiotherapy, and those for neoadjuvant chemotherapy or chemoradiotherapy have been investigated[3-6].

For patients with locally advanced pancreatic cancer, chemoradiotherapy has been accepted as a standard treatment[7]. The results of previous randomized trials

have indicated that external-beam radiation therapy and 5-fluorouracil (5-FU) therapy results in a significantly longer survival time than radiotherapy[8] or chemotherapy alone[9]. Gemcitabine, a deoxycytidine analog that functions as an antimetabolite, has been approved for use in patients with advanced pancreatic cancer[10,11]. In a randomized study, gemcitabine improved survival in inoperable pancreatic cancer in comparison with 5-FU[12]. Gemcitabine has also been shown to exert an effect in 5-FU-refractory pancreatic cancer[13]. Gemcitabine has also been shown to be a potent radiosensitizer, both *in vivo* and *in vitro*[14]. The vast majority of the reported phase I-Ⅲ clinical trials have used gemcitabine as a single agent given weekly in a single dose[7,15,16] (i.e. 250 mg/m^2).

Several preclinical data, including animal studies[17], would suggest that maximum radiation sensitization with gemcitabine is observed at a lower dose administered twice weekly[15,17]. Blackstock *et al*[14,15] and Magnino *et al*[18] reported on a phase Ⅱ study of chemoradiotherapy in which the patient's were treated with gemcitabine twice weekly at 40 mg/m^2 and 50 mg/m^2, respectively, associated with radiotherapy. Therefore, in the present study, we analyzed the results of retrospective analysis of chemoradiotherapy for locally advanced pancreatic cancer, utilizing gemcitabine as a radiation sensitizer administered twice weekly at a dose of 40 mg/m^2, followed by maintenance systemic chemotherapy with gemcitabine.

MATERIALS AND METHODS

Eligibility criteria included (1) locally advanced unresectable pancreatic cancer confirmed histologically or by imaging techniques including systemic computed tomography; (2) 20-74 years of age; (3) ECOG performance status of 0-2; (4) adequate hematological function, and adequate renal function, and (5) no prior anti-cancer treatment. A total dose of 40-50.4 Gy was delivered using 1.8-2.0 Gy daily fractions. Treatment planning was determined by a three-dimensional treatment planner. The targeted irradiation volume included the tumor, possible surrounding edema, and 1-cm margin. Gemcitabine, at a dose of 40 mg/m^2, was administered as a 30-min intravenous infusion twice weekly (80 mg/m^2 per week) for 4-5 wk. Gemcitabine was given within 2 h before radiation treatment. At 2 wk after the completion of chemoradiotherapy, maintenance systemic chemotherapy of gemcitabine at a dose of 1000 mg/m^2 was administered as a 30-min intravenous infusion weekly for 3 wk with 1-wk rest until disease progression or unacceptable toxicity. Both radiation therapy and chemotherapy were suspended for grade 3 hematological toxicities or grade 2 non-hematological toxicities (according to the National Cancer Institute Common Toxicity Criteria) during the treatment course, and treatment was resumed when toxicity was resolved. The objective tumor response, as defined by the WHO criteria, was assessed every 2 mo or 3 mo by computed tomography scan or earlier if clinically indicated.

The Kaplan-Meier method was used to estimate the distribution of overall survival and progression free survival. Progression free survival was calculated from the first day of treatment until there was evidence of clinical progression, tumor progression assessed by computed tomography scan measurement or death. Overall survival was calculated from the first day of treatment until the date of death. In this study, there is no control arm to treat the locally advanced pancreatic cancer.

Table 1 Patient characteristics

Number of patients completing the protocol	15
Gender	
Male	9 (60%)
Female	6 (40%)
Age (yr)	
Mean (range)	62.2 (50-73)
Tumor location	
Head	7 (46.7%)
Head-Body	1 (6.6%)
Body-Tail	7 (46.7%)
Total radiation dose	
40.0 Gy	12 (80%)
50.0 Gy	1 (6.6%)
50.4 Gy	2 (13.4%)
Response	
Complete response	1 (6.6%)
Partial response	4 (26.7%)
Stable disease	9 (60 %)
Progressive disease	1 (6.6%)
Cause of death	
Liver metastasis	10 (66.7%)
Peritoneal metastasis	3 (20%)

RESULTS

Clinical data

Eighteen patients were enrolled in this study. Three of those patients could not continue with the therapy under this protocol; one patient had interstitial pneumonia during radiation therapy and two other patients showed liver metastasis or peritoneal metastasis in an early stage of this protocol. Fifteen patients, including nine males and six females, completed therapy as planned and patient characteristics are shown in Table 1. The mean age was 62.2 years old (range, 50-73). The mean diameter of the tumor was 4.8-cm and the tumor was located in the pancreatic head in seven patients. Twelve patients received radiotherapy at a total of 40-Gy, two patients at a total dose of 50-Gy and one patient with 50.4-Gy. In general, therapy was well tolerated, one patient suffered AGML and another patient had an eruption. All the patients showed elevation of tumor markers, including CA19-9, Span-1 and DUPAN-2, at the enrollment for this study.

Survival

Regarding overall response, there was one complete response, 4 partial responses, 9 stable diseases and one progressive disease; the response rate was 33%. No patients could undergo tumor resection even after the completion of chemoradiotherapy, because of infiltration

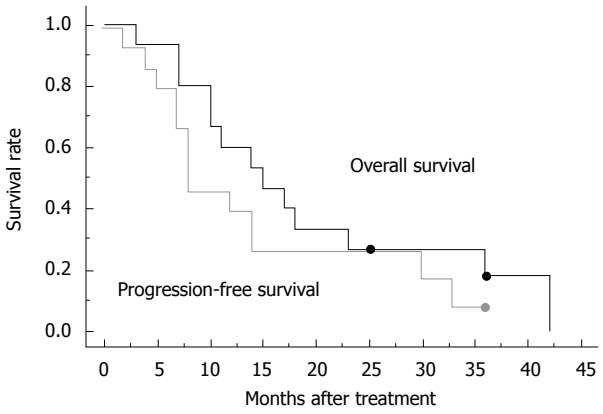


Figure 1 Overall survival curve and progression-free survival curve for 15 patients who received chemoradiotherapy under this study protocol. Dot indicates censored cases.

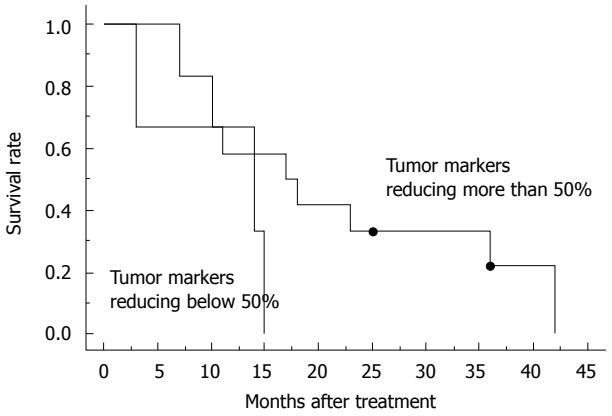


Figure 2 Overall survival curve for patients with tumor markers reducing more than 50% compared to that of pretreatment (n = 12) and those reducing below 50% (n = 3). Dot indicates censored cases.

of the adjacent large vessels. The median survival was 15.0 mo and the overall 1-year survival rate was 60%, while the median progression-free survival was 8.0 mo, estimated by the Kaplan-Meier method (Figure 1). In 80% of the patients, the level of tumor marker, including CA19-9, Span-1 and DUPAN-2, was reduced more than 50% compared to that of pretreatment. The subgroup where the tumor marker was reduced more than 50% had a tendency for a better prognosis (Figure 2), compared to the group with reduced tumor marker below 50% of pretreatment. Blackstock *et al*[15] postulated previously that the extended median survival observed in the CA19-9 responding patients might reflect the impact of the improved local control. However, a recent study demonstrated that pretreatment serum CA19-9 concentration was an independent prognostic factor for survival for advanced pancreatic cancer, but a decrease in concentration during chemotherapy was not significantly associated with lengthened survival compared with those who did not have a corresponding decrease[19]; therefore, the importance of decreasing in serum tumor marker concentration during therapy requires further discussion. In the subgroup with a tumor size less than 4-cm in diameter, median progression-free survival was 14.0 mo,

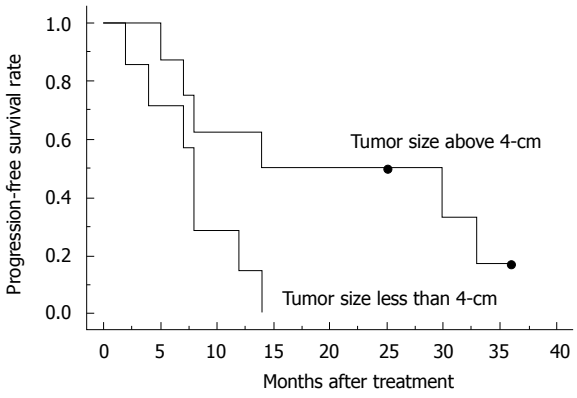


Figure 3 Progression free-survival curve for patients with tumor size less than 4-cm (n = 6) and those with a size above 4-cm (n = 9). Dot indicates censored cases.

which was better than those above 4-cm in diameter (8.0 mo, Figure 3). Other parameters, including age, gender and performance status, did not correlate with survival. The major causes of death were liver metastasis and peritoneal metastasis. The median survival of the groups that died of liver metastasis and peritoneal metastasis were 13.0 mo and 27.7 mo, respectively.

DISCUSSION

A recent retrospective comparison of the toxicity and efficacy of concurrent gemcitabine-based chemoradiotherapy with that of 5-FU based chemoradiotherapy for the patients with unresectable pancreatic cancer[20], showed a significantly higher toxicity rate in patients treated with gemcitabine and similar median survival times between the two arms. Investigators in Taiwan[21] reported favorable results for chemoradiotherapy with concurrent gemcitabine administration (600 mg/m^2 once a week); however, this needs further confirmation by larger multi-institutional clinical trials.

Although this study, using a twice weekly gemcitabine infusion schedule for locally advanced pancreatic cancer was not a controlled study, the results of the median survival time, median disease free survival time and overall 1-year survival rate was found to be preferable compared to previous studies[7-9,15]. Okusaka *et al*[7] presented data of a phase Ⅱ study for locally advanced pancreatic cancer treated with external-beam radiation (50.4 Gy) and weekly gemcitabine (250 mg/m^2 once a week) followed by maintenance chemotherapy using gemcitabine. The median survival time, median progression-free survival time and 1-year survival rate was 9.5 mo, 4.4 mo and 28%, respectively[7]. An expanded retrospective review of patients receiving gemcitabine-based chemoradiotherapy at the M. D. Anderson Cancer Center reflected the difficulties combining the systemic toxicities of 200-500 mg/m^2 doses of gemcitabine with the local-regional toxicities associated with chemoradiotherapy to the upper abdomen[22]. Furthermore, in the original GITSG trial of radiation and 5-FU based chemotherapy, 18% and 21% of the patients randomized into the

40-Gy and the 60-Gy treatment arms, respectively, were unable to complete all planned radiation[8]. For those patients completing the chemoradiotherapy, almost one-third were unable to initiate the planned maintenance 5-FU chemotherapy[8]. In this study, 15 of 18 patients could complete the planned protocol which might have come from the treatment with gemcitabine administered *via* a twice weekly infusion with radiation therapy and that most patients received radiation therapy at a total dose of 40-Gy. This might have resulted in the successful initiation in the maintenance of gemcitabine chemotherapy and to obtain a feasible survival rate in this trial. Some investigators did not propose maintenance chemotherapy after chemoradiotherapy[1]. Several studies of chemoradiotherapy used a therapeutic sequence with prior chemoradiotherapy and then chemotherapy until disease progression, but increased toxicity of chemotherapy after chemoradiotherapy limits this strategy[23,24], which might partially contribute to the total dose of radiation.

Two of the three patients enrolled initially who did not continue with the therapy under this protocol showed liver metastasis or peritoneal metastasis in the early stage of this protocol. Blackstock *et al*[15] pointed out in their study that the radiation sensitizing properties of twice weekly gemcitabine were important for improving the local control, and did not impact the survival for patients harboring micrometastatic disease at the initiation treatment. Hugutet *et al*[1] discussed that, an important concern about administrating chemoradiotherapy as first-line treatment in patients with locally advanced pancreatic cancer was that approximately 30% of them had occult metastatic disease at diagnosis and thus, they would clearly not benefit from this locoregional treatment. Furthermore, another investigator demonstrated that a fraction of patients with locally advanced pancreatic cancer developed metastases within a few weeks and died very quickly despite the type of treatment[25]. In this study, the patients who developed liver metastasis had a worse prognosis, which might owe to the miss-diagnosis of the staging of the disease at the initiation of the therapy, because of failure to detect micrometastasis by conventional imaging modalities. In this situation, we might need another strategy for the chemoradiotherapy for locally advanced pancreatic cancer, such as one in which the patients receive one or two cycles of systemic chemotherapy using gemcitabine at a dose of 1000 mg/m^2 weekly for 3 wk with 1-wk rest, and then re-evaluated the staging of the disease, initiating the chemoradiotherapy under the protocol in this study. A recent study suggested that after control of disease by initial chemotherapy for at least 3 mo using combination of leucovorin, fluorouracil and gemcitabine, or gemcitabine and oxaliplatin, chemoradiotherapy with 5-FU, could significantly improve survival in patients with locally advanced pancreatic cancer compared with chemotherapy alone[1].

In conclusion, chemoradiotherapy with low-dose gemcitabine given twice weekly could be effective to patients with locally advanced pancreatic cancer; however, patients developing liver metastases had a worse prognosis. We might need another strategy for the chemoradiotherapy for those patients. Further investigations are required in the near future.

COMMENTS

Background

Pancreatic cancer is the fifth most common cause of cancer death in Japan. The prognosis is extremely poor because it is difficult to detect this disease in the early stage and also the postoperative incidence of recurrence is still high. We do not have any effective treatment for inoperable patients. Recently, chemoradiotherapy has been regarded as one of the standard therapies for locally advanced pancreatic cancer and it has improved the survival and presented a clinical benefit.

Research frontiers

In the early 1980s, fluorouracil-based concomitant chemoradiotherapy was shown to be better than radiotherapy alone for patients with locally advanced pancreatic cancer. Gemcitabine has improved the outcome of patients with advanced disease by improving survival with a clinical benefit. Gemcitabine also has been shown to be a potent radiosensitizer, both *in vivo* and *in vitro*. The vast majority of the reported phase Ⅰ-Ⅲ clinical trials have used gemcitabine as a single agent given weekly in a single dose (i.e. 250 mg/m^2), and there is no consensus of the protocol of the administration of gemcitabine.

Innovations and breakthroughs

Several preclinical data, including animal studies, would suggest that maximum radiation sensitization with gemcitabine is observed at a lower dose administered twice weekly. In this study, we show that we could obtain the feasible results of survival compared to previous studies using our protocol. There existed some patients who could not continue the therapy, because of developing metastases. One reason could be the failure to detect micrometastasis by conventional imaging modalities at the beginning of chemoradiotherapy.

Applications

Chemoradiotherapy, with low-dose gemcitabine given twice weekly, could be effective to patients with locally advanced pancreatic cancer. To improve this survival data, we may need stricter selection of the cases suitable for this chemoradiotherapy; however, using conventional imaging modalities, it seems to be hard to diagnose the micrometastasis, especially in the liver before this chemoradiotherapy. Another strategy that may be useful is where patients receive one or two cycles of systemic chemotherapy using gemcitabine at a dose of 1000 mg/m^2 weekly for 3 wk with 1-wk rest, and then be re-evaluated for the staging of the disease, and then initiating the chemoradiotherapy under the protocol in this study.

Peer review

This is a nicely written paper that looks at the use of gemcitabine as a radiation sensitizer for pancreatic cancer. They report on twice-weekly doses. This dose contributes new information to the literature.

REFERENCES

1 **Huguet F**, Andre T, Hammel P, Artru P, Balosso J, Selle F, Deniaud-Alexandre E, Ruszniewski P, Touboul E, Labianca R, de Gramont A, Louvet C. Impact of chemoradiotherapy after disease control with chemotherapy in locally advanced pancreatic adenocarcinoma in GERCOR phase II and III studies. *J Clin Oncol* 2007; **25**: 326-331

2 **White RR**, Shah AS, Tyler DS. Pancreatic cancer since Halsted: how far have we come and where are we going? *Ann Surg* 2003; **238**: S132-S144; discussion S145-S147

3 **Takai S**, Satoi S, Yanagimoto H, Toyokawa H, Takahashi K, Terakawa N, Araki H, Matsui Y, Sohgawa M, Kamiyama Y. Neoadjuvant chemoradiation in patients with potentially resectable pancreatic cancer. *Pancreas* 2008; **36**: e26-e32

4 **Oettle H**, Post S, Neuhaus P, Gellert K, Langrehr J, Ridwelski K, Schramm H, Fahlke J, Zuelke C, Burkart C, Gutberlet K, Kettner E, Schmalenberg H, Weigang-Koehler K, Bechstein WO, Niedergethmann M, Schmidt-Wolf I, Roll L, Doerken B, Riess H. Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing

curative-intent resection of pancreatic cancer: a randomized controlled trial. *JAMA* 2007; **297**: 267-277

5 **Regine WF**, Winter KA, Abrams RA, Safran H, Hoffman JP, Konski A, Benson AB, Macdonald JS, Kudrimoti MR, Fromm ML, Haddock MG, Schaefer P, Willett CG, Rich TA. Fluorouracil vs gemcitabine chemotherapy before and after fluorouracil-based chemoradiation following resection of pancreatic adenocarcinoma: a randomized controlled trial. *JAMA* 2008; **299**: 1019-1026

6 **Vento P**, Mustonen H, Joensuu T, Karkkainen P, Kivilaakso E, Kiviluoto T. Impact of preoperative chemoradiotherapy on survival in patients with resectable pancreatic cancer. *World J Gastroenterol* 2007; **13**: 2945-2951

7 **Okusaka T**, Ito Y, Ueno H, Ikeda M, Takezako Y, Morizane C, Kagami Y, Ikeda H. Phase II study of radiotherapy combined with gemcitabine for locally advanced pancreatic cancer. *Br J Cancer* 2004; **91**: 673-677

8 **Moertel CG**, Frytak S, Hahn RG, O'Connell MJ, Reitemeier RJ, Rubin J, Schutt AJ, Weiland LH, Childs DS, Holbrook MA, Lavin PT, Livstone E, Spiro H, Knowlton A, Kalser M, Barkin J, Lessner H, Mann-Kaplan R, Ramming K, Douglas HO Jr, Thomas P, Nave H, Bateman J, Lokich J, Brooks J, Chaffey J, Corson JM, Zamcheck N, Novak JW. Therapy of locally unresectable pancreatic carcinoma: a randomized comparison of high dose (6000 rads) radiation alone, moderate dose radiation (4000 rads + 5-fluorouracil), and high dose radiation + 5-fluorouracil: The Gastrointestinal Tumor Study Group. *Cancer* 1981; **48**: 1705-1710

9 **Treatment of locally unresectable carcinoma of the pancreas: comparison of combined-modality therapy (chemotherapy plus radiotherapy) to chemotherapy alone**. Gastrointestinal Tumor Study Group. *J Natl Cancer Inst* 1988; **80**: 751-755

10 **Berlin JD**, Catalano P, Thomas JP, Kugler JW, Haller DG, Benson AB 3rd. Phase III study of gemcitabine in combination with fluorouracil versus gemcitabine alone in patients with advanced pancreatic carcinoma: Eastern Cooperative Oncology Group Trial E2297. *J Clin Oncol* 2002; **20**: 3270-3275

11 **Sakamoto H**, Kitano M, Suetomi Y, Takeyama Y, Ohyanagi H, Nakai T, Yasuda C, Kudo M. Comparison of standard-dose and low-dose gemcitabine regimens in pancreatic adenocarcinoma patients: a prospective randomized trial. *J Gastroenterol* 2006; **41**: 70-76

12 **Burris HA 3rd**, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, Nelson R, Dorr FA, Stephens CD, Von Hoff DD. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* 1997; **15**: 2403-2413

13 **Rothenberg ML**. New developments in chemotherapy for patients with advanced pancreatic cancer. *Oncology* (Williston Park) 1996; **10**: 18-22

14 **Blackstock AW**, Bernard SA, Richards F, Eagle KS, Case LD, Poole ME, Savage PD, Tepper JE. Phase I trial of twice-weekly gemcitabine and concurrent radiation in patients with advanced pancreatic cancer. *J Clin Oncol* 1999; **17**: 2208-2212

15 **Blackstock AW**, Tepper JE, Niedwiecki D, Hollis DR, Mayer RJ, Tempero MA. Cancer and leukemia group B (CALGB) 89805: phase II chemoradiation trial using gemcitabine in patients with locoregional adenocarcinoma of the pancreas. *Int J Gastrointest Cancer* 2003; **34**: 107-116

16 **Crane CH**, Varadhachary G, Pisters PW, Evans DB, Wolff RA. Future chemoradiation strategies in pancreatic cancer. *Semin Oncol* 2007; **34**: 335-346

17 **Fields MT**, Eisbruch A, Normolle D, Orfali A, Davis MA, Pu AT, Lawrence TS. Radiosensitization produced in vivo by once- vs. twice-weekly 2'2'-difluoro-2'-deoxycytidine (gemcitabine). *Int J Radiat Oncol Biol Phys* 2000; **47**: 785-791

18 **Magnino A**, Gatti M, Massucco P, Sperti E, Faggiuolo R, Regge D, Capussotti L, Gabriele P, Aglietta M. Phase II trial of primary radiation therapy and concurrent chemotherapy for patients with locally advanced pancreatic cancer. *Oncology* 2005; **68**: 493-499

19 **Hess V**, Glimelius B, Grawe P, Dietrich D, Bodoky G, Ruhstaller T, Bajetta E, Saletti P, Figer A, Scheithauer W, Herrmann R. CA 19-9 tumour-marker response to chemotherapy in patients with advanced pancreatic cancer enrolled in a randomised controlled trial. *Lancet Oncol* 2008; **9**: 132-138

20 **Crane CH**, Abbruzzese JL, Evans DB, Wolff RA, Ballo MT, Delclos M, Milas L, Mason K, Charnsangavej C, Pisters PW, Lee JE, Lenzi R, Vauthey JN, Wong AB, Phan T, Nguyen Q, Janjan NA. Is the therapeutic index better with gemcitabine-based chemoradiation than with 5-fluorouracil-based chemoradiation in locally advanced pancreatic cancer? *Int J Radiat Oncol Biol Phys* 2002; **52**: 1293-1302

21 **Li CP**, Chao Y, Chi KH, Chan WK, Teng HC, Lee RC, Chang FY, Lee SD, Yen SH. Concurrent chemoradiotherapy treatment of locally advanced pancreatic cancer: gemcitabine versus 5-fluorouracil, a randomized controlled study. *Int J Radiat Oncol Biol Phys* 2003; **57**: 98-104

22 **Crane CH**, Janjan NA, Evans DB, Wolff RA, Ballo MT, Milas L, Mason K, Charnsangavej C, Pisters PW, Lee JE, Lenzi R, Vauthey JN, Wong A, Phan T, Nguyen Q, Abbruzzese JL. Toxicity and efficacy of concurrent gemcitabine and radiotherapy for locally advanced pancreatic cancer. *Int J Pancreatol* 2001; **29**: 9-18

23 **Epelbaum R**, Rosenblatt E, Nasrallah S, Faraggi D, Gaitini D, Mizrahi S, Kuten A. Phase II study of gemcitabine combined with radiation therapy in patients with localized, unresectable pancreatic cancer. *J Surg Oncol* 2002; **81**: 138-143

24 **Schneider BJ**, Ben-Josef E, McGinn CJ, Chang AE, Colletti LM, Normolle DP, Hejna GF, Lawrence TS, Zalupski MM. Capecitabine and radiation therapy preceded and followed by combination chemotherapy in advanced pancreatic cancer. *Int J Radiat Oncol Biol Phys* 2005; **63**: 1325-1330

25 **Andre T**, Balosso J, Louvet C, Hannoun L, Houry S, Huguier M, Colonna M, Lotz JP, De Gramont A, Bellaiche A, Parc R, Touboul E, Izrael V. Combined radiotherapy and chemotherapy (cisplatin and 5-fluorouracil) as palliative treatment for localized unresectable or adjuvant treatment for resected pancreatic adenocarcinoma: results of a feasibility study. *Int J Radiat Oncol Biol Phys* 2000; **46**: 903-911

S- Editor Zhong XY **L- Editor** Rippe RA **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5316

World J Gastroenterol 2008 September 14; 14(34): 5316-5321
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Endoscopic findings can predict the efficacy of leukocytapheresis for steroid-naive patients with moderately active ulcerative colitis

Yasushi Umehara, Masatoshi Kudo, Masanori Kawasaki

Yasushi Umehara, Masatoshi Kudo, Masanori Kawasaki, Division of Gastroenterology and Hepatology, Department of Internal Medicine Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama 589-8511, Japan
Author contributions: Umehara Y, Kudo M and Kawasaki M contributed equally to this work; Umehara Y and Kudo M desined research; Umehara Y and Kawasaki M performed research; Umehara Y and Kudo M contributed new reagents/analytic tools; Umehara Y and Kudo M analyzed data and Umehara Y wrote the paper.
Correspondence to: Masatoshi Kudo, MD, PhD, Division of Gastroenterology and Hepatology, Department of Internal Medicine Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama 589-8511, Japan. m-kudo@med.kindai.ac.jp
Telephone: +81-723-660221-3525 Fax: +81-723-672880
Received: June 21, 2008 Revised: August 11, 2008
Accepted: August 18, 2008
Published online: September 14, 2008

Abstract

AIM: To investigate the therapeutic usefulness of leukocytapheresis (LCAP; Cellsoba) in steroid-naive patients with moderately active ulcerative colitis (UC).

METHODS: Eighteen steroid-naive patients with moderately active UC received one LCAP session every week for five consecutive weeks.

RESULTS: The remission rate 8 weeks after the last LCAP session was 61.1% (11/18). All three patients with deep ulcers showed worsening after LCAP. For the remaining 15 patients, who had erosions or geographic ulcers, the average clinical activity index (CAI) score dropped significantly from 9.4 to 3.8 eight weeks after the last LCAP session (t = 4.89, P = 0.001). The average C-reactive protein (CRP) levels before and after LCAP were 1.2 mg/dL and 1.0 mg/dL, respectively. Of the patients with erosions, geographic ulcers, and deep ulcers, 100% (9/9), 33.3% (2/6), and 0% (0/3) were in remission 8 weeks after the last LCAP session, respectively (χ^2 = 7.65, P < 0.005). Forty-eight weeks after the last LCAP session, the remission rates for patients with erosions and geographic ulcers were 44.4% (4/9) and 16.7% (1/6), respectively. Only one patient suffered a mild adverse event after LCAP (nausea).

CONCLUSION: LCAP is a useful and safe therapy for steroid-naive UC patients with moderate disease activity. Moreover, the efficacy of the treatment can be predicted on the basis of endoscopic findings.

Key words: Ulcerative colitis; Steroid-naive; Leukocytapheresis; Efficacy; Endoscopic findings

Peer reviewer: Alastair JM Watson, Professor, Department of Gastroenterology, University of Liverpool, the Henry Wellcome Laboratory, Nuffield Bldg, Crown St, Liverpool L69 3GE, United Kingdom

Umehara Y, Kudo M, Kawasaki M. Endoscopic findings can predict the efficacy of leukocytapheresis for steroid-naive patients with moderately active ulcerative colitis. *World J Gastroenterol* 2008; 14(34): 5316-5321 Available from: URL: http://www.wjgnet.com/1007-9327/14/5316.asp DOI: http://dx.doi.org/10.3748/wjg.14.5316

INTRODUCTION

Although the etiology of ulcerative colitis (UC) is still unknown, it is believed that an immune abnormality may be involved in its development[1,2]. It is characterized by chronic over-activation of the colonic mucosal immune system. Consequently, if remission cannot be achieved by salazosulfapyridine or mesalazine treatment, the second line of treatment has conventionally been to administer steroids[2-6]. However, steroid administration can increase susceptibility to infections, diabetes mellitus and osteoporosis. Recently, it was reported that steroid-refractory or steroid-dependent patients with UC can be effectively treated by cytapheresis[7-10]. To determine whether leukocytapheresis (LCAP) may also be useful with other UC patients, we administered LCAP to 18 steroid-naive UC patients. We also assessed whether the efficacy of LCAP can be predicted on the basis of endoscopic findings.

MATERIALS AND METHODS

From January 2005 to April 2007, 33 UC patients were treated with LCAP at our hospital. All patients were

Table 1 Steroid-naïve UC patient characteristics (mean ± SD)

Characteristics	Data
Male/Female	11/7
Age (yr)	46.1 ± 18.4
Duration of disease (yr)	6.0 ± 8.5
Clinical course	
First attack	4
Relapse-remitting	6
Chronic continuous	8
Extent of disease	
Entire	15
Left sided	2
Rectum	1
Endoscopic findings	
Erosions	9
Geographic ulcers	6
Deep ulcers	3

examined by colonoscopy before treatment and UC was diagnosed on the basis of established endoscopic and histological criteria[11]. At the time of diagnosis, infectious colitis (*Salmonella, Campylobacter, Vibrio, Yersinia* and *Shigella spp.*) was ruled out by stool culture and *Clostridium difficile* toxin testing. Moreover, we excluded Crohn's disease, ischemic colitis, radiation colitis and intestinal Behçet disease. None of patients were receiving drugs, including non-steroidal anti-inflammatory drugs or antibiotics. Patients with severe cardiovascular disease, severe cerebral disease, severe anemia (hemoglobin; less than 8 g/dL) and hypotension (less than 80 mmHg) were excluded. Of the 33 patients, 15 had severe activity and were treated with steroids along with LCAP. These patients were excluded from the study. The remaining 18 steroid-naive patients had moderate activity, as defined by a Lichtiger's clinical activity index (CAI) score[12] of < 12. These patients were enrolled in the study. Their characteristics are summarized in Table 1.

For all patients, LCAP sessions were performed once a week for five consecutive weeks by using Cellsorba (Asahi Medical Co., Ltd, Tokyo, Japan). Leukocyte removal in LCAP is effective because of its adherence to fibers in the filter. The throughput was 2-3 L of whole blood and the flow rate was 30-50 mL/min for approximately 60 min. The access and return lines were connected to cubital veins. Heparin was used as an anticoagulant for the extracorporeal circulation.

Patients were 46.1 ± 18.4 years old; there were 11 males and 7 females. Their duration of disease was 6.0 ± 8.5 years. With regard to their clinical course, four patients presented the "first attack" type, six the "relapse-remitting" type, and eight the "chronic continuous" type. We defined patients presenting with an activity phase lasting for 6 mo or longer from the first attack as belonging to the "chronic continuous" type. Fifteen patients had total colitis, two had left-sided colitis and one had proctitis. We performed endoscopy before and after LCAP. For the present study, we classified the patients into three groups on the basis of the endoscopic findings before LCAP treatment, namely, those with erosions, geographic ulcers, or deep ulcers (Figure 1). Nine patients had erosions in the large intestine, six had geographic ulcers, and three had deep ulcers. All patients were concomitantly treated with mesalazine (2250 mg/d) for at least 4 weeks prior to the initiation of LCAP therapy. There was no change in the dosage of mesalazine. Immunomodulators such as azathioprine, 6-mercaptopurine and cyclosporine were never administered.

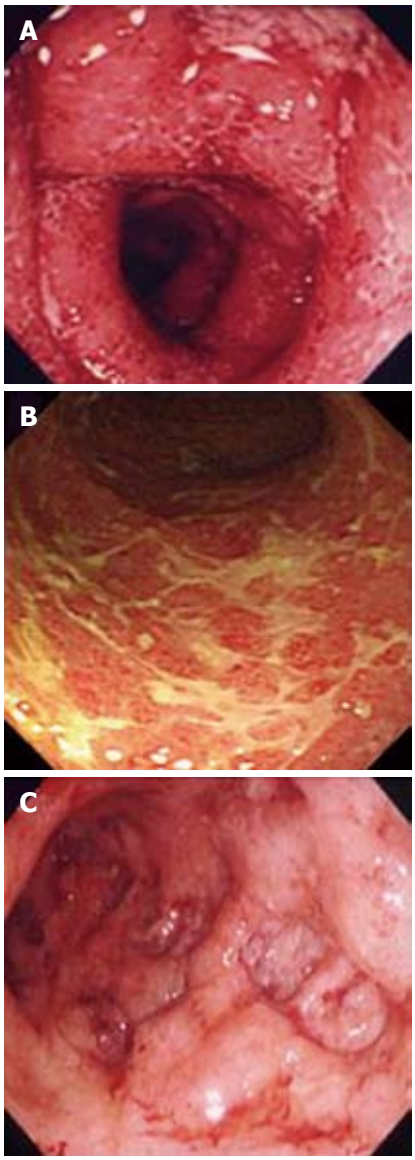


Figure 1 The patients were divided before LCAP into three groups according to whether their endoscopic findings revealed erosions (**A**), geographic ulcers (**B**), or deep ulcers (**C**). Representative findings are shown.

Disease activity was evaluated before and after LCAP by measuring the CAI. A CAI less than 4 indicates remission. Relapses were also identified when the patient needed another therapy, such as steroid or cyclosporine treatment and/or LCAP.

The endpoint of this study was to determine the factor related to remission by LCAP.

Statistical analysis

The Wilcoxon test was used to compare CAI scores

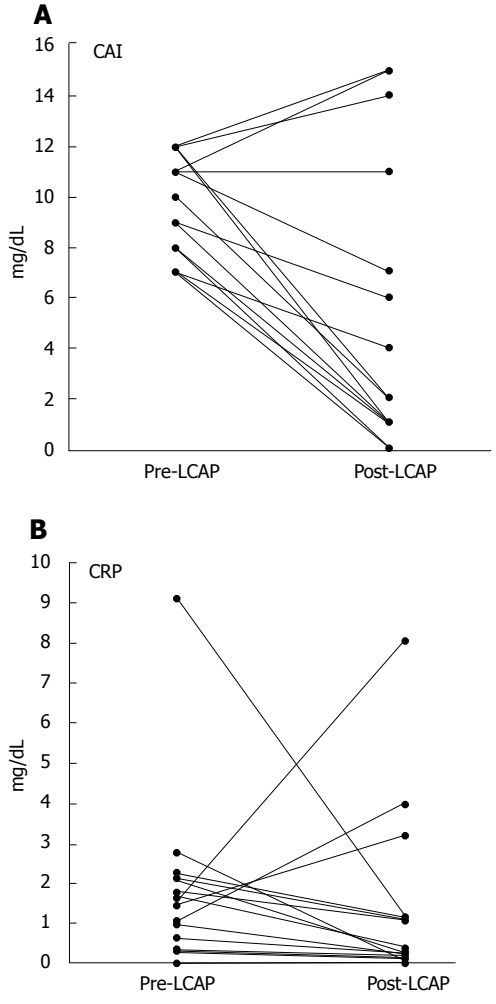


Figure 2 Change in average CAI score (**A**) and CRP levels (**B**) 8 wk after the last LCAP session. The data of the three patients with deep ulcers whose conditions worsened during LCAP are not included. The average CAI dropped from 9.4 ± 1.9 to 3.8 ± 4.8 (P = 0.001) while the average CRP levels before and after LCAP were 1.2 ± 0.8 mg/dL and 1.0 ± 2.0 mg/dL, respectively, and did not differ significantly.

and C-reactive protein (CRP) levels before and after treatment. The Mann-Whitney U-test was used to compare the age, duration of disease, pre-CAI, post-CAI, pre-CRP and post-CRP levels of two groups, while the χ^2 test was used to test the effect of sex. χ^2 test was used to compare the clinical course, extent of disease and endoscopic findings of two groups. $P < 0.05$ was considered to indicate statistical significance. Results were presented as mean ± SD.

RESULTS

Efficacy of LCAP

For all 18 patients, the remission rates 8 and 48 wk after the last LCAP session were 61.1% (11/18) and 27.7% (5/18), respectively. At 48 wk after remission, the relapse rate was 54.5% (6/11), and the duration to relapse was 8.7 ± 4.2 mo. Three patients with deep ulcers worsened during LCAP and required additional treatments such as steroids. However, the remaining 15 patients showed a significant drop in the CAI score from 9.4 ± 1.9 to 3.8 ± 4.8 eight weeks after the last LCAP session (P = 0.001) (Figure 2). The CRP levels before (1.2 ± 0.8 mg/dL) and after (1.0 ± 2.0 mg/dL) LCAP did not differ significantly (Figure 2). When the endoscopic findings obtained before LCAP were considered, we found that all nine patients with erosions had entered remission (100%) 8 wk after the last LCAP session. However, only two of six (33%) and none of three of the patients with geographic ulcers and deep ulcers, respectively, had entered remission at this time point ($P < 0.005$) (Table 2). The remission rates dropped over time as of the patients with erosions and geographic ulcers who were in remission at the 8-wk timepoint, 44.4% (4/9) and 16.7% (1/6) remained in remission 48 wk after LCAP.

Table 2 Comparison of responders and non-responders 8 wk after the last LCAP session (mean ± SD)

	Responders (n = 11)	Non-responders (n = 7)	P
Patient characteristic			
Male/Female	7/4	4/3	NS
Age (yr)	39.7 ± 15.9	56.1 ± 17.5	NS
Duration of disease (yr)	5.7 ± 7.7	6.3 ± 9.7	NS
Pre-CAI	9.0 ± 2.0	11.1 ± 0.9	NS
Pre-CRP	1.0 ± 0.8	2.4 ± 2.7	NS
Clinical course			
First attack	4	0	
Relapse-remitting	6	0	
Chronic continuous	1	7	< 0.001
Extent of disease			
Entire	8	7	
Left sided	2	0	
Proctitis	1	0	NS
Endoscopic findings			
Erosions	9	0	
Geographic ulcers	2	4	
Deep ulcers	0	3	< 0.005

LCAP: Leukocytapheresis; CAI: Clinical activity index; CRP: C-reactive protein levels (mg/dL).

Clinical characteristics of the patients who entered remission

Table 2 shows how the responders compare to the non-responders 8 wk after LCAP. These two groups did not differ significantly in patient characteristics (i.e., sex, age, duration of disease, pre-CAI levels and pre-CRP levels). However, all first attack and relapse-remitting type patients entered remission while seven of the eight chronic continuous type patients did not ($P < 0.001$). Whether there was total colitis, left-sided colitis, or proctitis was not significantly associated with the ability of the patient to enter remission after LCAP. As indicated above, with regard to the endoscopic findings, all patients with erosions entered remission after LCAP but it was more difficult to induce remission in patients with geographic ulcers or deep ulcers ($P < 0.005$).

Clinical characteristic of the patients who entered remission and then relapsed

Of the 11 patients who entered remission 8 wk after LCAP, six relapsed. Table 3 summarizes the

Table 3 Comparison of the remission and relapse groups 48 wk after the last LCAP session (mean ± SD)

	Remission (n = 5)	Relapse (n = 6)	P
Patient characteristic			
Male/Female	2/3	5/1	NS
Age (yr)	45.4 ± 13.5	35.0 ± 16.1	NS
Duration of disease (yr)	3.5 ± 3.2	7.6 ± 9.6	NS
Pre-CAI	8.8 ± 1.7	9.1 ± 2.2	NS
Post-CAI	0.6 ± 0.4	1.8 ± 1.0	< 0.05
Pre-CRP	1.0 ± 0.9	1.0 ± 0.8	NS
Post-CRP	0.18 ± 0.14	0.32 ± 0.39	NS
Clinical course			
First attack	3	1	
Relapse-remitting	2	4	
Chronic continuous	0	1	NS
Extent of disease			
Entire	3	5	
Left sided	1	1	
Proctitis	1	0	NS
Endoscopic findings			
Erosions	4	5	
Geographic ulcers	1	1	
Deep ulcers	0	0	NS

CAI: Clinical activity index; CRP: C-reactive protein levels (mg/dL).

characteristics of the five patients who remained in remission 48 wk after LCAP and the six relapsed patients. The two groups only differed significantly in terms of the post-CAI scores ($P < 0.05$). None of the other parameters, namely, the clinical course, extent of disease, or endoscopic findings, correlated with relapse.

Course of the cases who worsened during LCAP

Table 4 summarizes the courses of the three cases with deep ulcers that worsened during LCAP. As a result, two cases were given steroids and one case received cyclosporine. Two patients became infected with cytomegalovirus and received ganciclovir. After a transient improvement, two patients relapsed and underwent surgery. The remaining patient, who developed interstitial pneumonitis, died of aspiration pneumonitis. Aspiration pneumonitis developed 3 mo after LCAP, and therefore the two events were probably unrelated to each other. We think that interstitial pneumonitis was a cause of the development of aspiration pneumonitis.

Adverse effects

None of the patients experienced any severe adverse effects from LCAP. Only one patient reported a mild adverse event (nausea). However, this patient did not suffer from the same problem after subsequent LCAP sessions.

DISCUSSION

When UC patients fail to enter remission after salazosulfapyridine or mesalazine treatment, the conventional second-line therapy involves administration of steroids[13,14]. However, steroids can cause severe side effects in some patients[15,16]. When patients with severe

Table 4 Course of cases whose condition worsened during LCAP

	Case 1	Case 2	Case 3
Additional therapy	PSL	PSL	CsA
		GCV	GCV
Complication	-	CMV	CMV
			Aspiration pneumonitis
Result	Operation	Operation	Dead

PSL: Prednisolone; CsA: Cyclospoline; GCV: Ganciclovir; CMV: Cytomegalovirus.

activity fail to respond to steroids, they must undergo a colectomy, although cyclosporine can sometimes induce remission in these cases[17-20]. In 1995, Sawada *et al*[21] introduced LCAP therapy for patients with UC. This therapy is now a widely used treatment option for UC[22,23]. LCAP is a method where the blood is passed though a leukocyte removal filter before being returned to the body. On average, 1.6×10^{10} leukocytes are removed during one session. These leukocytes include granulocytes, lymphocytes and monocytes. Almost 100% of granulocytes and monocytes and 60% of lymphocytes are removed by removal filter[24,25]. It has been reported that 73.3% of steroid-refractory patients with UC enter remission after LCAP[26]. It is likely that this treatment is effective because it reduces the number of leukocytes available for transmigration and infiltration into the colonic mucosa.

In this study, we found that 61.1% of steroid-naive UC patients (11/18) had entered remission 8 wk after the last LCAP session. At this time point, the average CAI score had dropped significantly from 9.4 ± 1.9 to 3.8 ± 4.8 ($P = 0.001$) (the three cases who worsened during LCAP were excluded from this calculation). Since steroids can induce remission in 45% to 90% of salazosulfapyridine or mesalazine non-responders[15,27-29], it appears that LCAP is as efficacious as steroids as a second-line treatment. Given the low rate of adverse events suffered by patients treated with LCAP, we propose that patients with moderately active UC should be treated with LCAP before steroids are considered. It should be noted, however, that 54.5% of the patients in remission (6/11) relapsed 48 wk after the last LCAP session, and that the average duration to relapse was 8.7 mo. Thus, while LCAP is useful for inducing remission in steroid-naive UC patients, it does not maintain remission.

Analysis of the endoscopic findings of the patients revealed that while all nine patients with erosions had entered remission 8 wk after the last LCAP session, only two of six (33%) and none of three patients with geographic ulcers and deep ulcers, respectively, entered remission. Indeed, the three cases with deep ulcers worsened during LCAP and had to be given steroids (2 cases) or cyclosporine (1 case). Two of these cases became infected with cytomegalovirus and were administered ganciclovir. These observations suggest that patients with geographic ulcers and deep ulcers tend to be refractory to LCAP, particularly the latter. Indeed,

LCAP may not improve the situation for patients with deep ulcers given their higher risk of developing cytomegalovirus infections[30]. Such patients should perhaps be treated with an intensive therapy such as cyclosporine at a more early stage[31]. However, since all patients with erosion entered remission 8 wk after LCAP and many (44.4%) remained in remission at the 48-wk time point, LCAP is strongly recommended for patients with erosion.

The post-CAI was the only factor that predicted a relapse. In other words, if the post-CAI could be maintained at < 1 by LCAP, it may be possible to maintain long duration remission.

In conclusion, LCAP is a useful and safe therapy for steroid-naive UC patients with moderate activity. Moreover, endoscopic findings help to predict the efficacy of this treatment.

COMMENTS

Background

An immune dysfunction is believed to be involved in the development of ulcerative colitis (UC). For a long time, steroids have represented the second line therapy for the induction to remission in UC, if remission cannot be achieved by salazosulfapyridine or mesalazine treatment. However, steroid administration can cause several side effects. Leukocytapheresis (LCAP) have been reported to be effective for steroid-refractory or steroid-dependent patients with UC; however, the data of LCAP for steroid-naïve patient with UC is limited.

Research frontiers

To determine whether LCAP may also be useful in steroid-naive UC patients, these authors administered LCAP to steroid-naive UC patients. They also assessed whether the efficacy of LCAP can be predicted on the basis of endoscopic findings.

Innovations and breakthroughs

The authors found that 61.1% of steroid-naive UC patients (11/18) had entered remission 8 wk after the last LCAP session. Since steroids can induce remission in 45% to 90% of salazosulfapyridine or mesalazine non-responders, it appears that LCAP is as efficacious as steroids as a second-line treatment. Analysis of the endoscopic findings of the patients revealed that the remission rate of the patients with erosion was extremely high after LCAP, compared to the extremely low rate observed in patients with geographic ulcers and deep ulcers. None of the patients experienced any severe adverse effects from LCAP.

Applications

Given the low rate of adverse events suffered by patients treated with LCAP, authors propose that patients with moderately active UC should be treated with LCAP before steroids are considered.

Terminology

LCAP is a treatment procedure where leukocytes are removed through their adherence to fibers in the filter.

Peer review

LCAP is a useful and safe therapy for steroid-naive UC patients with moderate activity. Moreover, endoscopic findings help to predict the efficacy of this treatment. This is an interesting study.

REFERENCES

1 **Arai F**, Takahashi T, Furukawa K, Matsushima K, Asakura H. Mucosal expression of interleukin-6 and interleukin-8 messenger RNA in ulcerative colitis and in Crohn's disease. *Dig Dis Sci* 1998; **43**: 2071-2079

2 **Podolsky DK**. Inflammatory bowel disease (1). *N Engl J Med* 1991; **325**: 928-937

3 **Truelove SC**, Jewell DP. Intensive intravenous regimen for severe attacks of ulcerative colitis. *Lancet* 1974; **1**: 1067-1070

4 **Meyers S**, Sachar DB, Goldberg JD, Janowitz HD. Corticotropin versus hydrocortisone in the intravenous treatment of ulcerative colitis. A prospective, randomized, double-blind clinical trial. *Gastroenterology* 1983; **85**: 351-357

5 **Hanauer SB**. Inflammatory bowel disease. *N Engl J Med* 1996; **334**: 841-848

6 **Jarnerot G**, Rolny P, Sandberg-Gertzen H. Intensive intravenous treatment of ulcerative colitis. *Gastroenterology* 1985; **89**: 1005-1013

7 **Naganuma M**, Funakoshi S, Sakuraba A, Takagi H, Inoue N, Ogata H, Iwao Y, Ishi H, Hibi T. Granulocytapheresis is useful as an alternative therapy in patients with steroid-refractory or -dependent ulcerative colitis. *Inflamm Bowel Dis* 2004; **10**: 251-257

8 **Giampaolo B**, Giuseppe P, Michele B, Alessandro M, Fabrizio S, Alfonso C. Treatment of active steroid-refractory inflammatory bowel diseases with granulocytapheresis: Our experience with a prospective study. *World J Gastroenterol* 2006; **12**: 2201-2204

9 **Emmrich J**, Petermann S, Nowak D, Beutner I, Brock P, Klingel R, Mausfeld-Lafdhiya P, Liebe S, Ramlow W. Leukocytapheresis (LCAP) in the management of chronic active ulcerative colitis--results of a randomized pilot trial. *Dig Dis Sci* 2007; **52**: 2044-2053

10 **Domenech E**, Hinojosa J, Esteve-Comas M, Gomollon F, Herrera JM, Bastida G, Obrador A, Ruiz R, Saro C, Gassull MA. Granulocyteaphaeresis in steroid-dependent inflammatory bowel disease: a prospective, open, pilot study. *Aliment Pharmacol Ther* 2004; **20**: 1347-1352

11 **Garland CF**, Lilienfeld AM, Mendeloff AI, Markowitz JA, Terrell KB, Garland FC. Incidence rates of ulcerative colitis and Crohn's disease in fifteen areas of the United States. *Gastroenterology* 1981; **81**: 1115-1124

12 **Lichtiger S**, Present DH, Kornbluth A, Gelernt I, Bauer J, Galler G, Michelassi F, Hanauer S. Cyclosporine in severe ulcerative colitis refractory to steroid therapy. *N Engl J Med* 1994; **330**: 1841-1845

13 **Podolsky DK**. Inflammatory bowel disease. *N Engl J Med* 2002; **347**: 417-429

14 **Kornbluth A**, Sachar DB. Ulcerative colitis practice guidelines in adults (update): American College of Gastroenterology, Practice Parameters Committee. *Am J Gastroenterol* 2004; **99**: 1371-1385

15 **Lennard-Jones JE**. Toward optimal use of corticosteroids in ulcerative colitis and Crohn's disease. *Gut* 1983; **24**: 177-181

16 **Baron JH**, Connell AM, Kanaghinis TG, Lennard-Jones JE, Jones AF. Out-patient treatment of ulcerative colitis. Comparison between three doses of oral prednisone. *Br Med J* 1962; **2**: 441-443

17 **Rowe FA**, Walker JH, Karp LC, Vasiliauskas EA, Plevy SE, Targan SR. Factors predictive of response to cyclosporin treatment for severe, steroid-resistant ulcerative colitis. *Am J Gastroenterol* 2000; **95**: 2000-2008

18 **Stack WA**, Long RG, Hawkey CJ. Short- and long-term outcome of patients treated with cyclosporin for severe acute ulcerative colitis. *Aliment Pharmacol Ther* 1998; **12**: 973-978

19 **Sandborn WJ**. A critical review of cyclosporin therapy in inflammatory bowel disease. *Inframm Bowel Dis* 1995; **1**: 48-63

20 **Santos J**, Baudet S, Casellas F, Guarner L, Vilaseca J, Malagelada JR. Efficacy of intravenous cyclosporine for steroid refractory attacks of ulcerative colitis. *J Clin Gastroenterol* 1995; **20**: 285-289

21 **Sawada K**, Ohnishi K, Fukui S, Yamada K, Yamamura M, Amano K, Amano K, Wada M, Tanida N, Satomi M. Leukocytapheresis therapy, performed with leukocyte removal filter, for inflammatory bowel disease. *J Gastroenterol* 1995; **30**: 322-329

22 **Sawada K**, Shimoyama T. Therapeutic cytapheresis for inflammatory bowel disease. *Ther Apher* 1998; **2**: 90-92

23 **Sawada K**, Muto T, Shimoyama T, Satomi M, Sawada T, Nagawa H, Hiwatashi N, Asakura H, Hibi T. Multicenter

randomized controlled trial for the treatment of ulcerative colitis with a leukocytapheresis column. *Curr Pharm Des* 2003; **9**: 307-321
24 **Shirokaze J**. Leukocytapheresis using a leukocyte removal filter. *Ther Apher* 2002; **6**: 261-266
25 **Yamaji K**, Tsuda H, Hashimoto H. Current topics on cytapheresis technologies. *Ther Apher* 2001; **5**: 287-292
26 **Kohgo Y**, Hibi H, Chiba T, Shimoyama T, Muto T, Yamamura K, Popovsky MA. Leukocyte apheresis using a centrifugal cell separator in refractory ulcerative colitis: a multicenter open label trial. *Ther Apher* 2002; **6**: 255-260
27 **Lennard-Jones JE**, Longmore AJ, Newell AC, Wilson CW, Jones FA. An assessment of prednisone, salazopyrin, and topical hydrocortisone hemisuccinate used as out-patient treatment for ulcerative colitis. *Gut* 1960; **1**: 217-222
28 **Powell-Tuck J**, Bown RL, Lennard-Jones JE. A comparison of oral prednisolone given as single or multiple daily doses for active proctocolitis. *Scand J Gastroenterol* 1978; **13**: 833-837
29 **Faubion WA Jr**, Loftus EV Jr, Harmsen WS, Zinsmeister AR, Sandborn WJ. The natural history of corticosteroid therapy for inflammatory bowel disease: a population-based study. *Gastroenterology* 2001; **121**: 255-260
30 **Nishimoto Y**, Matsumoto T, Suekane H, Shimizu M, Mikami Y, Iida M. Cytomegalovirus infection in a patient with ulcerative colitis: colonoscopic findings. *Gastrointest Endosc* 2001; **53**: 816-818
31 **D'Haens G**, Lemmens L, Geboes K, Vandeputte L, Van Acker F, Mortelmans L, Peeters M, Vermeire S, Penninckx F, Nevens F, Hiele M, Rutgeerts P. Intravenous cyclosporine versus intravenous corticosteroids as single therapy for severe attacks of ulcerative colitis. *Gastroenterology* 2001; **120**: 1323-1329

S- Editor Li DL **L- Editor** Negro F **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5322

World J Gastroenterol 2008 September 14; 14(34): 5322-5326
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Halothane hepatitis in Iran: A review of 59 cases

Payam Eghtesadi-Araghi, Amir-Ali Sohrabpour, Homayoon Vahedi, Mehdi Saberi-Firoozi

Payam Eghtesadi-Araghi, Homayoon Vahedi, Digestive Disease Research Center, Shariati Hospital, Medical Sciences/University of Tehran, Tehran 14117, Iran
Amir-Ali Sohrabpour, Mehdi Saberi-Firoozi, Gastroenterology Research Center, Shiraz University of Medical Sciences, Shiraz 14117, Iran
Author contributions: Eghtesadi-Araghi P designed and performed research, analyzed data and wrote the paper; Vahedi H contributed in providing cases and critical review of the project; Sohrabpour A and Saberi-Firoozi M contributed in providing cases.
Supported by A grant from the Digestive Disease Research Center, Tehran University of Medical Sciences, Iran, No. 83.36
Correspondence to: Dr. Payam Eghtesadi-Araghi, Digestive Disease Research Center, Shariati Hospital, North Karegar Avenue, Medical Sciences/University of Tehran, Tehran 14117, Iran. payam_eghtesadi@yahoo.com
Telephone: +98-21-44454814 Fax: +98-21-44454814
Received: June 11, 2008 Revised: July 27, 2008
Accepted: August 3, 2008
Published online: September 14, 2008

Abstract

AIM: To study halothane hepatitis (HH) in Iran and its associated risk factors.

METHODS: We retrospectively studied files of all cases diagnosed with HH referred to three referral hospitals and four private centers in Iran from April 1994 to September 2006. Information on age at surgery, gender, medications history, obesity, history of previous exposure, previous reaction to halothane, familial history, type of surgery, perioperative hypoxia or sepsis, morbidity and mortality were recorded and analyzed.

RESULTS: A total of 59 cases were identified. Forty-eight (81%) were women. The median age at the time of surgery was 44 years (range, 18 to 80 years). Sixty percent of patients were above 40-year-old. Obesity was observed in 22.2%. Previous history of exposures to halothane was noted in 61% of which 50% had history of post-exposure reaction. Coronary artery bypass graft (CABG), cholecystectomy, and cosmetic surgeries (mainly weight reduction) were the most frequent surgeries. The mortality rate was 12.2%. In patients developing encephalopathy, it was as high as 50%.

CONCLUSION: HH remains an important cause of morbidity and mortality in centers still using this anesthetic. However, a large percentage of these cases could have been avoided. To lessen occurrence of further cases of HH, the authors suggest that in female patients having a history of surgery (or delivery) with general anesthesia, the use of halothane should be absolutely avoided. Utilization of proper substitutes in adults' anesthesia is advocated.

Key words: Anesthesia; Inhalation; Halothane; Hepatitis; Drug-induced

Peer reviewer: Jacques Bernuau, MD, Beaujon Hospital, 100, boulevard du Général Leclerc, Cedex 92118, France

Eghtesadi-Araghi P, Sohrabpour A, Vahedi H, Saberi-Firoozi M. Halothane hepatitis in Iran: A review of 59 cases. *World J Gastroenterol* 2008; 14(34): 5322-5326 Available from: URL: http://www.wjgnet.com/1007-9327/14/5322.asp DOI: http://dx.doi.org/10.3748/wjg.14.5322

INTRODUCTION

Halothane was first introduced to clinical practice in 1956 and was immediately recognized as a great advance in anesthesia[1]. First reports of postoperative liver necrosis with halothane began to appear in 1958[2,3] followed by further anecdotal reports[4-7]. By 1963, 7 years after the introduction of halothane, at least 350 cases of "halothane hepatitis" (HH) had been reported[8]. These reports led to the National Halothane Study, which estimated fatal hepatic necrosis following halothane anesthesia to be approximately 1 in 35 000 in the US[9]. Other retrospective studies confirmed that halothane was associated with severe liver dysfunction, with an incidence ranging from 1 in 6000 to 1 in 35 000[10].

Two major types of hepatotoxicity are associated with halothane administration: type I (mild) and type Ⅱ (fulminant). Type Ⅱ hepatotoxicity is associated with massive centrilobular liver cell necrosis that leads to fulminant liver failure and is clinically characterized by fever, jaundice, and grossly elevated serum transaminase levels. It appears to be immune mediated and initiated by oxidative halothane metabolism by cytochrome P450 to an intermediate compound. This compound binds to trifluoroacetylate proteins in the hepatic endoplasmic reticulum, thought to occur in genetically predisposed

individuals[11]. In this study, type Ⅱ hepatotoxicity was regarded as HH.

Concerns about its hepatotoxicity have virtually eliminated the use of halothane for adults in the United States and many other countries. Halothane was eventually replaced by safer newer volatile anesthetics such as isoflurane[12,13]. But in other countries with different medicolegal climates, halothane is still widely used because of its relatively low cost[11]. There are reports of HH from South Africa[14], Tunisia[15], Kenya[16], India[17], and Spain[18]. Halothane is being used as the main anesthetic in more than 80% of hospitals of Iran[19]. Unfortunately, increasing numbers of HH are being reported in Iran. In this retrospective study, we review cases reported within a 12-year period from 7 centers in Iran.

MATERIALS AND METHODS

Cases were recruited from three referral hospitals (Namazi Hospital of Shiraz University of Medical Sciences, Shariati Hospital of Tehran University of Medical Sciences, Mehr General Hospital of Tehran) and four referral GI clinics in Tehran, from April 1994 to September 2006.

There is no definite test for the diagnosis of HH and it is basically a diagnosis of exclusion. In our study, the diagnosis of HH was confirmed if the patient met the following criteria; clinical findings (jaundice, malaise), paraclinical findings (marked elevation of ALT, AST, total and direct bilirubin), recent exposure to halothane, exclusion of other causes of liver damage (viral hepatitis, *etc*) and confirmation by at least one gastroenterologist as fulminant HH.

Charts were reviewed to determine the age at surgery, weight, gender, medication history (including P450 inducing drugs), preoperative obesity (BMI $\geqslant$ 30), history of recurrent exposure to halothane, history of previous reaction to halothane (unexplained post-operative fever, jaundice, abnormal liver enzymes following earlier halothane anesthetics), positive family history (defined as reactions to halothane in first degree relatives), type of surgery, perioperative hypoxia, interval between anesthesia with halothane and symptoms heralding or attesting hepatitis, postoperative sepsis, morbidity and mortality.

Data were presented as simple count and percent, median and range, or mean ± SD and were compared by Fisher's exact test. Statistical calculations were performed using SPSS version 15.0. $P < 0.05$ was considered significant.

The study was approved by the institutional review board and ethics committee of the Digestive Disease Research Center (DDRC) of Tehran University of Medical Sciences.

RESULTS

Fifty-nine cases of HH were identified, 48 women (81%), 11 men (19%). The median age at the time of surgery was 44 years (range, 18 to 80 years). Sixty percent of patients were above 40 years old. The age distribution of patients is given in Figure 1.

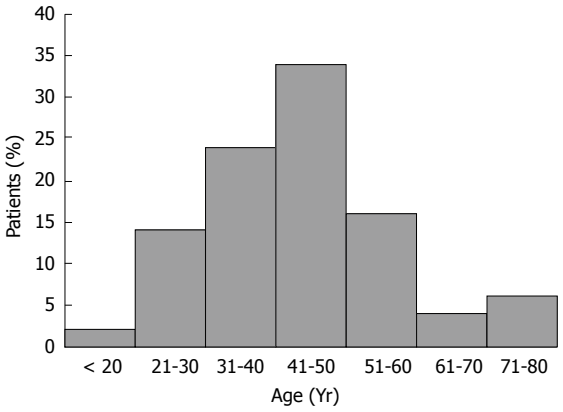


Figure 1 The age distribution of patients with HH.

Table 1 Characteristics and laboratory test results of 59 patients with HH

Variable	*n*	%
Characteristics		
Female gender	48	81.4
Obesity	6	22.2
Recurrent exposure history	22	61.2
Post-exposure reaction history	10	17
Encephalopathy	8	14.5
Requiring ICU care	7	12.5
Mortality	6	12.2
Laboratory test results		
ALT rise	48	100
AST rise	47	97.9
Total bilirubin rise	41	97.6
Direct bilirubin rise	37	100
Alkaline phosphates rise	35	100
PT rise	21	61.8

Percentages are relative to patients for whom data was available.

The mean of interval between anesthesia and hepatitis symptoms was 15.2 ± 13.6 d. The mean weight of patients was 76.1 ± 18.7 kg and 22.2% of patients were obese. Data on clinical findings, morbidity, and mortality are given in Table 1. No cases had previous history of liver disease or sepsis. One case had documented perioperative hypoxia, another one had a positive family history of HH, and two others had positive drug history (Phenobarbital). Table 1 shows the results of routine laboratory investigations including biochemistry and coagulation tests.

Coronary artery bypass graft (CABG), cholecystectomy and cosmetic operations (mainly weight reducing surgeries including partial gastrectomy and liposuction) were the most common operations. Information about different types of surgery is given in Table 2. The mortality rate was 12.2%. In patients who developed encephalopathy, it was as high as 50%. The mortality rate in male patients was higher than females (20% *vs* 12%), although this did not reach statistical significance.

Table 2 Surgery types and the percentage of cases of HH regarding each type of surgery

Variable	*n*	%
Cholecystectomy	5	8.5
CABG surgery	4	6.8
Aesthetic surgeries		
Partial gastrectomy	1	1.7
Liposuction	1	1.7
Face lifting	1	1.7
Mandible reconstruction	1	1.7
Others	3	3.4
General Surgeries		
Intra-abdominal surgeries	2	3.4
Extra-abdominal surgeries	4	6.8
Obstetrics & Gynecologic surgeries		
Cesarean section	2	3.4
Others	9	
Eye surgeries	3	5.1
Orthopedic surgeries	2	3.4
Urologic surgeries	2	3.4
Other types of surgery	5	8.5

Percentages are relative to patients for whom data was available.

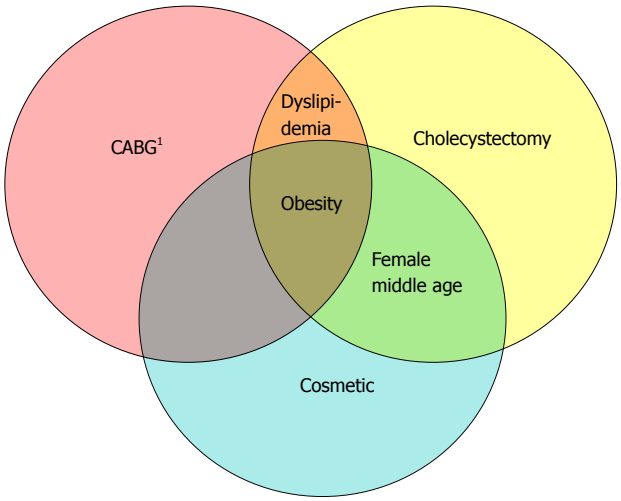


Figure 2 Common risk factors between three most common surgeries among patients with HH. [1]Coronary Artery Bypass Graft Surgery.

DISCUSSION

Because of the retrospective nature of the study, confirmation of the diagnosis with antibodies to halothane-altered protein antigens was not possible.

It has been shown that HH is more frequent in females, male-to-female ratio ranging from 1:1.6[11] to 1:2[20,21]. In our series, this ratio was 1:4.3. Additionally, it has been revealed that middle-aged patients have a greater propensity to develop liver damage than the young or elderly[11, 21-24]. Over 70% of patients are more than 40 years old with peak age of 50-60[10]. We also observed a greater prevalence among middle-aged patients, yet it appears that the patients in our series are younger compared to other series[14]. The younger age of our patients could be due to the fact that the most common surgery in our series, cholecystectomy, is frequently performed in young to middle-aged women. Obesity appears to be the common factor between most frequent operations associated with HH in our series. CABG, cholecystectomy and weight reducing-cosmetic surgeries are all linked with obesity (Figure 2). It has been showed that hepatic dysfunction is more common in obese than in non-obese patients[23-25]. As halothane accumulates in adipose tissue, this could delay its excretion and, theoretically, prolong exposure to potentially reactive halothane metabolites, resulting in increased risk in obese patients. In addition, obese patients metabolize halothane more extensively than do non-obese patients[26], further predisposing them to liver injury.

Other possible relationships between the most common surgeries are shown in Figure 2. Female gender and middle age are common risk factors for gall stone formation, cholecystitis, and coronary artery disease[27-30]. Dyslipidemia is also a risk factor for coronary artery disease and plays an important role in the pathogenesis of gallstones[27,28]. Furthermore, hypoperfusion is the possible explanation of hepatic injury in CABG and cholecystectomy surgeries. Imbalance between oxygen supply and demand predisposes the patient to halothane-induced liver damage[10,31]. Halothane has been shown to decrease both portal blood flow and hepatic arterial blood flow[32-35]. Furthermore, surgical manipulation of the splanchnic bed may reduce hepatic blood flow[36]. It has been demonstrated that CABG is accompanied with splanchnic hypoperfusion and hypoxia[37,38]. The ischemia is probably caused by hypoperfusion due to low cardiac output, hypotension due to blood loss, and intra-abdominal atheroemboli. As a result, ischemia may be the common mechanism of predisposing the patients to the HH in CABG and cholecystectomy surgeries.

Multiple exposures to halothane are the single greatest risk factor for HH[11]. The association between hepatotoxicity and repeated exposure to halothane might be explained by the fact that halothane anesthesia itself induces drug metabolizing enzymes[39-41]. The risk of HH is increased greatly when repeated halothane anesthetics are given over a short period[11,22,42,43], especially at intervals of < 6 wk[11]. In the present study, previous history of exposure to halothane was noted in 22 of 36 (61.1%) and obvious post-exposure reaction history in 10 of 22 (45.5%). Considering the fact that guidelines clearly elucidated that patients with previous reaction to halothane are among the high-risk groups[44] and should have not received halothane again, in addition to the results of this study, it is concluded that a large percentage of these cases could have been avoided. To lessen further occurrence of cases of HH, we suggest that the use of halothane in patients who are at risk of HH should be absolutely avoided. This group of patients consists of female patients having a history of post-anesthesia reactions following exposure to halogenated anesthetics. On the other hand, the item of "post-exposure reaction history" is often quite difficult to obtain with a reasonable certainty; therefore, its usefulness in preventing HH should be weak. As a result, a second set of criteria, which is female gender patients with a history of surgery (or delivery) with general anesthesia, is recommended. This is likely easier

to obtain and can be simply defined pre-operatively by the anesthesiologist *via* reviewing the "anesthesia sheet" of the previous general anesthesia. Strict adherence to this set of criteria will reduce, but not totally prevent, further cases from occurring in countries still utilizing halothane in adults' anesthesia.

Furthermore, the incidence of liver injury was greater in subjects treated with phenobarbitone before halothane anesthesia than in those not taking enzyme inducing medication[11,45]. There were two cases of chronic Phenobarbital use in our series among the 16 for which data was available.

Susceptibility to HH may be heritable and positive family history must be taken into consideration[11,46]. We had one case with positive family history of HH out of 7 cases that had data.

Type Ⅱ hepatotoxicity has a mortality rate of approximately 50%, which rises to 80% when hepatic encephalopathy is present[10,11,31,47]. Our data confirms this since the mortality was 50% in our patients with encephalopathy. Such patients have a very poor prognosis and should be referred to specialist centers where orthotopic liver transplantation is available[48]. Patients who survive the acute illness usually make a complete recovery[11]. Considering that none of our patients underwent liver transplantation, our mortality rate is considerably lower than other reports[14]. Improved intensive care, faster diagnosis and initiation of medical care may be involved. In consistence with former reports[22,24,49], mortality in male patients was higher than females in our series (20% *vs* 12.1%), although it did not reach statistical significance.

Although halothane is rarely associated with fulminant hepatic failure[48], it occupies the fifth place of suspected hepatic adverse drug reactions with a fatal outcome received by the WHO Collaborating Centre for International Drug Monitoring in Uppsala Sweden from 1968 to 2003[50]. Consequently, its usage was restricted in adults' anesthesia; however, it is still widely used in many countries, including Iran, mainly due to economic reasons[11].

Guidelines have been developed to reduce the probability of a patient developing HH; still, adverse reactions continue to occur. Even though it was clearly noted in guidelines that patients thought to be sensitized to halothane must never be re-exposed to the drug, we found ten cases of known previous reaction that were re-exposed to halothane. Based on the results of this study, to lessen further cases of HH to occur, the authors suggest that in female patients having a history of surgery (or delivery) with general anesthesia, the use of halothane should be absolutely avoided. However, the most effective preventive tool is to avoid the use of halothane in adults' anesthesia. Utilization of proper substitutes is advocated.

ACKNOWLEDGMENTS

This study was the postdoctoral project of Dr. Payam Eghtesadi-Araghi and supported by a grant from Digestive Disease Research Center of Tehran University of Medical Sciences. The authors wish to thank Professor Malekzadeh, Dr. Merat, Dr. Nasseri-Moghaddam, Dr. Ganji, and Dr. Mortezaei for their invaluable assistance.

COMMENTS

Background

Halothane is a volatile anesthetic, which was first introduced to clinical practice in 1956. After several years, concerns about its hepatotoxicity have virtually eliminated the use of halothane for adults in the United States and many other countries. It was replaced by safer newer volatile anesthetics such as isoflurane. However, in some countries with different medicolegal climates, halothane is still widely used because of its relatively low cost. In Iran, halothane is being used as the main anesthetic in more than 80% of hospitals. Unfortunately, increasing numbers of halothane hepatitis (HH) are being reported in Iran.

Research frontiers

Although the use of halothane was restricted in many countries, there are reports of HH occurring in South Africa, Tunisia, Kenya, India, and Spain in recent years.

Innovations and breakthroughs

In previous studies, guidelines have been developed to reduce the probability of a patient developing HH; however, the results of this study revealed that they are largely ignored in Iran. Furthermore, the results showed that a large percentage of these cases could have been avoided. In this study, we aimed to suggest a set of criteria that can easily define the high risk group of patients pre-operatively.

Applications

To reduce, but not totally prevent, further cases of HH from occurring in countries still utilizing halothane in adults' anesthesia, the authors suggest that the use of halothane should be absolutely avoided in female patients with a history of surgery (or delivery) with general anesthesia. However, the most effective preventive tool is to avoid the use of halothane in adults' anesthesia. Utilization of proper substitutes is advocated.

Terminology

Two major types of hepatotoxicity are associated with halothane administration: type Ⅰ (mild) and type Ⅱ (fulminant). Type Ⅱ hepatotoxicity is associated with massive centrilobular liver cell necrosis that leads to fulminant liver failure and is clinically characterized by fever, jaundice, and grossly elevated serum transaminase levels. In this study, type Ⅱ hepatotoxicity was regarded as HH.

Peer review

This series of 59 cases of HH collected in 12 years in a country with health security conditions lower than those found in the US or Western Europe is of high medical interest for the Iranian population and populations of other countries in the Middle East and, most likely, also in Africa and Asia. Therefore, the topic of the present paper is quite pertinent and extremely interesting for many physicians around the world.

REFERENCES

1 **Johnstone M**. The human cardiovascular response to fluothane anaesthesia. 1956. *Br J Anaesth* 1998; **80**: 396-405; discussion 395
2 **Burnap TK**, Galla SJ, Vandam LD. Anesthetic, circulatory and respiratory effects of fluothane. *Anesthesiology* 1958; **19**: 307-320
3 **Postoperative death after fluothane**. *Anesthesiology* 1958; **19**: 562-563
4 **Brody GL**, Sweet RB. Halothane anesthesia as a possible cause of massive hepatic necrosis. *Anesthesiology* 1963; **24**: 29-37
5 **Bunker JP**, Blumenfeld CM. Liver necrosis after halothane anesthesia. Cause or coincidence? *N Engl J Med* 1963; **268**: 531-534
6 **Lindenbaum J**, Leifer E. Hepatic necrosis associated with halothane anesthesia. *N Engl J Med* 1963; **268**: 525-530

7 **Temple RL**, Cote RA, Gorens SW. Massive hepatic necrosis following general anesthesia. *Anesth Analg* 1962; **41**: 586-592
8 **Brown BR Jr**, Gandolfi AJ. Adverse effects of volatile anaesthetics. *Br J Anaesth* 1987; **59**: 14-23
9 **Summary of the national Halothane Study**. Possible association between halothane anesthesia and postoperative hepatic necrosis. *JAMA* 1966; **197**: 775-788
10 **Ray DC**, Drummond GB. Halothane hepatitis. *Br J Anaesth* 1991; **67**: 84-99
11 **Peralta R**, Poterack KA, Guzofski S. Halothane Hepatotoxicity. Last modified date: May 25, 2006. Available from URL: http://www.emedicine.com
12 **Allan LG**, Hussey AJ, Howie J, Beckett GJ, Smith AF, Hayes JD, Drummond GB. Hepatic glutathione S-transferase release after halothane anaesthesia: open randomised comparison with isoflurane. *Lancet* 1987; **1**: 771-774
13 **Hussey AJ**, Aldridge LM, Paul D, Ray DC, Beckett GJ, Allan LG. Plasma glutathione S-transferase concentration as a measure of hepatocellular integrity following a single general anaesthetic with halothane, enflurane or isoflurane. *Br J Anaesth* 1988; **60**: 130-135
14 **Voigt MD**, Workman B, Lombard C, Kirsch RE. Halothane hepatitis in a South African population--frequency and the influence of gender and ethnicity. *S Afr Med J* 1997; **87**: 882-885
15 **Daghfous R**, el Aidli S, Sfaxi M, Daghfous M, Kastalli S, Srairi S, Loueslati MH, Belkahia C. [Halothane-induced hepatitis. 8 case reports] *Tunis Med* 2003; **81**: 874-878
16 **Otedo AE**. Halothane induced hepatitis: case report. *East Afr Med J* 2004; **81**: 538-539
17 **Kumar GP**, Bhat VJ, Sowdi V. Fulminant hepatic failure following halothane anaesthesia. *J Clin Forensic Med* 2005; **12**: 271-273
18 **Rodes J**, Bruguera M. The uses of error: iatrogenic hepatitis. *Lancet* 2001; **357**: 791
19 **The "Halothane" will be replaced with newer anesthetic drugs in Iran**. Date Accessed: July 17, 2008. Available from URL: http://www.reporter.ir/archives/86/10/005402.php
20 **Benjamin SB**, Goodman ZD, Ishak KG, Zimmerman HJ, Irey NS. The morphologic spectrum of halothane-induced hepatic injury: analysis of 77 cases. *Hepatology* 1985; **5**: 1163-1171
21 **Neuberger J**, Williams R. Halothane anaesthesia and liver damage. *Br Med J* (Clin Res Ed) 1984; **289**: 1136-1139
22 **Bottiger LE**, Dalen E, Hallen B. Halothane-induced liver damage: an analysis of the material reported to the Swedish Adverse Drug Reaction Committee, 1966-1973. *Acta Anaesthesiol Scand* 1976; **20**: 40-46
23 **Carney FM**, Van Dyke RA. Halothane hepatitis: a critical review. *Anesth Analg* 1972; **51**: 135-160
24 **Walton B**, Simpson BR, Strunin L, Doniach D, Perrin J, Appleyard AJ. Unexplained hepatitis following halothane. *Br Med J* 1976; **1**: 1171-1176
25 **Peters RL**, Edmondson HA, Reynolds TB, Meister JC, Curpey TJ. Hepatic necrosis associated with halothane anesthesia. *Am J Med* 1969; **47**: 748-764
26 **Bentley JB**, Vaughan RW, Gandolfi AJ, Cork RC. Halothane biotransformation in obese and nonobese patients. *Anesthesiology* 1982; **57**: 94-97
27 **Boland LL**, Folsom AR, Rosamond WD. Hyperinsulinemia, dyslipidemia, and obesity as risk factors for hospitalized gallbladder disease. A prospective study. *Ann Epidemiol* 2002; **12**: 131-140
28 **Grunhage F**, Lammert F. Gallstone disease. Pathogenesis of gallstones: A genetic perspective. *Best Pract Res Clin Gastroenterol* 2006; **20**: 997-1015
29 **Wild SH**, Byrne CD. ABC of obesity. Risk factors for diabetes and coronary heart disease. *BMJ* 2006; **333**: 1009-1011
30 **Franzosi MG**. Should we continue to use BMI as a cardiovascular risk factor? *Lancet* 2006; **368**: 624-625
31 **Cousins MJ**, Plummer JL, Hall PD. Risk factors for halothane hepatitis. *Aust N Z J Surg* 1989; **59**: 5-14
32 **Gelman S**, Fowler KC, Smith LR. Liver circulation and function during isoflurane and halothane anesthesia. *Anesthesiology* 1984; **61**: 726-730
33 **Hursh D**, Gelman S, Bradley EL Jr. Hepatic oxygen supply during halothane or isoflurane anesthesia in guinea pigs. *Anesthesiology* 1987; **67**: 701-706
34 **Gelman S**, Rimerman V, Fowler KC, Bishop SP, Bradley EL Jr. The effect of halothane, isoflurane, and blood loss on hepatotoxicity and hepatic oxygen availability in phenobarbital-pretreated hypoxic rats. *Anesth Analg* 1984; **63**: 965-972
35 **Hughes RL**, Campbell D, Fitch W. Effects of enflurane and halothane on liver blood flow and oxygen consumption in the greyhound. *Br J Anaesth* 1980; **52**: 1079-1086
36 **Seltzer JL**, Goldberg ME, Larijani GE, Ritter DE, Starsnic MA, Stahl GL, Lefer AM. Prostacyclin mediation of vasodilation following mesenteric traction. *Anesthesiology* 1988; **68**: 514-518
37 **D'Ancona G**, Baillot R, Poirier B, Dagenais F, de Ibarra JI, Bauset R, Mathieu P, Doyle D. Determinants of gastrointestinal complications in cardiac surgery. *Tex Heart Inst J* 2003; **30**: 280-285
38 **Zacharias A**, Schwann TA, Parenteau GL, Riordan CJ, Durham SJ, Engoren M, Fenn-Buderer N, Habib RH. Predictors of gastrointestinal complications in cardiac surgery. *Tex Heart Inst J* 2000; **27**: 93-99
39 **Nimmo WS**, Thompson PG, Prescott LF. Microsomal enzyme induction after halothane anaesthesia. *Br J Clin Pharmacol* 1981; **12**: 433-434
40 **St Haxholdt O**, Loft S, Clemmensen A, Hjortso E. Increased hepatic microsomal activity after halothane anaesthesia in children. *Anaesthesia* 1986; **41**: 579-581
41 **Neuberger JM**. Halothane and hepatitis. Incidence, predisposing factors and exposure guidelines. *Drug Saf* 1990; **5**: 28-38
42 **Inman WH**, Mushin WW. Jaundice after repeated exposure to halothane: an analysis of Reports to the Committee on Safety of Medicines. *Br Med J* 1974; **1**: 5-10
43 **Inman WH**, Mushin WW. Jaundice after repeated exposure to halothane: a further analysis of reports to the Committee on Safety of Medicines. *Br Med J* 1978; **2**: 1455-1456
44 **Neuberger J**, Williams R. Halothane hepatitis. *Dig Dis* 1988; **6**: 52-64
45 **Nomura F**, Hatano H, Ohnishi K, Akikusa B, Okuda K. Effects of anticonvulsant agents on halothane-induced liver injury in human subjects and experimental animals. *Hepatology* 1986; **6**: 952-956
46 **Hoft RH**, Bunker JP, Goodman HI, Gregory PB. Halothane hepatitis in three pairs of closely related women. *N Engl J Med* 1981; **304**: 1023-1024
47 **Bjornsson E**, Jerlstad P, Bergqvist A, Olsson R. Fulminant drug-induced hepatic failure leading to death or liver transplantation in Sweden. *Scand J Gastroenterol* 2005; **40**: 1095-1101
48 **Neuberger J**. Halothane hepatitis. *Eur J Gastroenterol Hepatol* 1998; **10**: 631-633
49 **Moult PJ**, Sherlock S. Halothane-related hepatitis. A clinical study of twenty-six cases. *Q J Med* 1975; **44**: 99-114
50 **Bjornsson E**, Olsson R. Suspected drug-induced liver fatalities reported to the WHO database. *Dig Liver Dis* 2006; **38**: 33-38

S- Editor Li DL **L- Editor** Li M **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5327

World J Gastroenterol 2008 September 14; 14(34): 5327-5330
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Anti-HBc screening in Indian blood donors: Still an unresolved issue

Hari Krishan Dhawan, Neelam Marwaha, Ratti Ram Sharma, Yogesh Chawla, Beenu Thakral, Karan Saluja, Sanjeev Kumar Sharma, Manish K Thakur, Ashish Jain

Hari Krishan Dhawan, Neelam Marwaha, Ratti Ram Sharma, Beenu Thakral, Karan Saluja, Manish K Thakur, Ashish Jain, Department of Transfusion Medicine PGIMER, Chandigarh 160012, India
Yogesh Chawla, Sanjeev Kumar Sharma, Department of Hepatology PGIMER, Chandigarh 160012, India
Author contributions: Marwaha N, Sharma RR and Chawla Y designed the research; Dhawan HK and Sharma SK performed the research; Thakral B, Saluja K, Thakur MK and Jain A analyzed data; Dhawan HK wrote the paper.
Correspondence to: Hari Krishan Dhawan, Department of Transfusion Medicine PGIMER, Chandigarh 160012, India. hkdpgimer@gmail.com
Telephone: + 91-172-2756481 Fax: + 91-172-2744401
Received: June 20, 2008 Revised: July 14, 2008
Accepted: July 21, 2008
Published online: September 14, 2008

Abstract

AIM: To study the seroprevalence of antibody to hepatitis B core antigen (anti-HBc) in healthy blood donors negative for HBsAg and to evaluate whether anti-HBc detection could be adopted in India as a screening assay for HBV in addition to HBsAg.

METHODS: A total of 1700 serum samples collected from HBsAg-negative healthy blood donors were tested for the presence of anti-HBc antibody (IgM + IgG). All samples reactive for anti-HBc antibody were then investigated for presence of anti-HBs and for liver function tests (LFTs). One hundred serum samples reactive for anti-HBc were tested for HBV DNA by PCR method.

RESULTS: Out of 1700 samples tested, 142 (8.4%) blood samples were found to be reactive for anti-HBc. It was significantly lower in voluntary (6.9%) as compared to replacement donors (10.4%, P = 0.011). Seventy-two (50.7%) anti-HBc reactive samples were also reactive for anti-HBs with levels > 10 mIU/mL and 70 (49.3%) samples were non-reactive for anti-HBs, these units were labeled as anti-HBc-only. These 142 anti-HBc reactive units were also tested for liver function test. HBV DNA was detected in only 1 of 100 samples tested.

CONCLUSION: Keeping in view that 8%-18% of donor population in India is anti-HBc reactive, inclusion of anti-HBc testing will lead to high discard rate. Anti-HBs as proposed previously does not seem to predict clearance of the virus. Cost effectiveness of introducing universal anti-HBc screening and discarding large number of blood units *versus* considering ID NAT (Individual donor nuclic acid testing) needs to be assessed.

Key words: Hepatitis B core antigen; Hepatitis B surface antigen; Hepatitis B virus; Transfusion-associated hepatitis B virus; Blood donors

Peer reviewer: Dr. Deepak Narayan Amarapurkar, Department Of Gastroenterology, Bombay Hospital & Medical Research Centre, D 401 Ameya Soc, New Prabhadevi Road, Prabhadevi, Mumbai 400025, India

Dhawan HK, Marwaha N, Sharma RR, Chawla Y, Thakral B, Saluja K, Sharma SK, Thakur MK, Jain A. Anti-HBc screening in Indian blood donors: Still an unresolved issue. *World J Gastroenterol* 2008; 14(34): 5327-5330 Available from: URL: http://www.wjgnet.com/1007-9327/14/5327.asp DOI: http://dx.doi.org/10.3748/wjg.14.5327

INTRODUCTION

Hepatitis B virus (HBV) infection is a serious global health problem affecting two billion people worldwide, and 350 million people suffer from chronic HBV infection[1]. Despite mandatory screening for HBsAg by ELISA for over 20 years, transfusion-associated HBV (TAHBV) continues to be a major problem in India, more so in patients receiving repeated transfusions[2]. The incidence of transfusion-associated hepatitis (TAH) after cardiac surgery (4 + 2.4 units transfusion) was estimated at 6.9%, of which TAHBV constituted 20%[2]. The incidence of TAHBV in patients receiving multiple transfusions, such as thalassemia, ranged from 17.9% in the first year to 69.2% by the third year. Patients on renal dialysis showed similar rates of infection with HBV[2]. It has been demonstrated that some HBsAg-negative individuals and those reactive for anti-HBc continue to replicate HBV[3,4]. Thus the absence of HBsAg in the blood of apparently healthy individuals may not be sufficient to ensure lack of circulating HBV. Blood containing anti-HBc with or without detectable presence of HBsAg might be infectious; therefore, routine blood donor screening for anti-HBc has been implemented

in some countries resulting in a decrease in the risk of post-transfusion HBV infection[5].

These findings suggest that recovery from acute hepatitis B virus infection may not result in complete virus elimination, but rather the immune system keeps the virus at a very low level called occult hepatitis B infection. Occult hepatitis B infection (OBI) is defined as the presence of HBV DNA in blood or tissues without detectable HBsAg, with or without antibody to hepatitis B core antigen (anti-HBc) or hepatitis B surface antigen (anti-HBs)[6]. Such occult hepatitis B infection may be detected in (1) individuals with resolving HBV infection reactive for both anti-HBc and anti-HBs, (2) "anti-HBc-only" carriers in a window period of infection who are seronegative for HBsAg, and (3) carriers in whom HBsAg is not detectable due to presence of escape mutants[7].

As of today, some countries have retained or adopted anti-HBc testing to decrease HBV transfusion risk, while others have not. Anti-HBc testing is still not mandatory in blood banks in India and only HBsAg testing by ELISA is used as screening test for HBV[8]. In this study, we aimed to evaluate whether anti-HBc detection could be adopted in India as a screening assay for HBV in addition to HBsAg to improve the safety of blood transfusion.

MATERIALS AND METHODS

Materials

This study was conducted by the Department of Transfusion Medicine in collaboration with Department of Hepatology, PGIMER, Chandigarh, India from July 2005 to December 2006, after obtaining an approval from the Ethics Committee of the Institute. Seventeen hundred HBsAg ELISA non-reactive blood donors were included, 998 of them were voluntary donors and 702 were replacement donors. These samples were then tested for anti-HBc (IgM + IgG), anti-HBs, LFTs and HBV DNA in the following sequence: (1) Blood units non-reactive for HBsAg and reactive for anti-HBc were tested for antibody to surface antigen (anti-HBs) and for liver function tests (LFTs); (2) the blood units non-reactive for HBsAg but reactive for anti-HBc with or without anti-HBs were tested for HBV DNA by PCR method.

Serological tests

HBsAg was tested by using commercial ELISA kit SURASE B-96 (TMB) (GBC, Taiwan, ROC); anti-HBc antibodies were tested using commercial ELISA kit HBcAb two-step incubation (MBS-SRL, Milano, Italy); anti-HBs antibodies were tested using commercial ELISA kit MONOLISA anti-HBs 3.0 (BIORAD).

HBV DNA detection

The DNA from serum was extracted using QiaAmp (Qiagen, Hilden, Germany), followed by in-house nested PCR, amplifying two different regions of the HBV genome using two sets of primers shown in Table 1. The procedure was standardized by positive control obtained from National Institute of Virology Pune, India.

Table 1 Primers used for HBV PCR

First PCR		
GP1	5'-YCCTGCTGGTGGCTCCAGTTC-3': sense	nt 3144-3164
GP2	5'-AAGCCANACARTGGGGGAAAGC-3': antisense	nt 583-604
Second PCR (nt 120-604)		
GP3	5'-GTCTAGACTCGTGGTGGACTTCTCTC-3': sense	nt 120-145
GP4	5'-AAGCCANACARTGGGGGAAAGC-3': antisense	nt 583-604

First PCR was carried out in 20 μL volume containing 1 × Taq buffer [100 mmol/L Tris HCL (pH 8.4), 25 mmol/L $MgCl_2$ and 500 mmol/L KCL], 20 pmol of reverse and forward primers (Table 1), 1 unit of Taq polymerase and 200 ng viral DNA. Thirty-five amplification cycles were performed in PCR machine (Stratagene, La Jolla, USA). Each cycle consisted of denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s and extension at 72℃ for 30 s. A second PCR was performed using the first PCR product as a template using the primers GP-3 and GP-4 which amplified a 485-bp product.

For the detection of PCR product, the second PCR product was run on 20 g/L agarose in TBE buffer containing 0.5 μg/mL ethidium bromide at 50 V for about 1 h and finally visualized under a UV transilluminator (UVP, Upland, USA) and then photographed. A 100-bp DNA ladder (MBI Fermentas, Opelstrasse, Germany) was also run in parallel and the predicted size of the PCR product, which was found to be 485 bp.

Biochemical tests

Liver function tests, including serum bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), were carried out by enzymatic method.

RESULTS

A total of 1700 HBsAg non-reactive blood donors (998 voluntary and 702 replacement donors) were screened for anti-HBc (IgM + IgG) using competitive ELISA, of them 142 turned out to be reactive, giving an overall seropositivity of 8.4%. It was markedly lower in voluntary (6.9%) as compared to replacement donors (10.4%, $P = 0.011$). Donors with age 18-30 years had minimum seropositivity (6.1%) with 50% donors contributing from this group. No significant difference was found in the seropositivity of first time *versus* repeat donors, male *versus* female donors and student *versuss* non-student donors, although the seropositivity was slightly less in female donors and student donors.

The anti-HBc reactive donors were tested for anti-HBs. Seventy-two (50.7%) anti-HBc reactive samples were also positive for anti-HBs with levels > 10 mIU/mL and 70 (49.3%) samples were non-reactive for anti-HBs, these units were labeled as anti-HBc-only. The anti-HBc-only reactivity was significantly lower in voluntary (34/998, 3.4%) than in replacement donors (36/702, 5.1%; $P = 0.38$) (Table 2). The anti-HBc reactive units were also tested for LFTs. All the samples had normal

Table 2 Prevalence of anti-HBc and anti-HBs in study population

Donor category	Number	Anti-HBc total	Anti-HBc-only	Anti-HBc and anti-HBs
Voluntary (%)	998 (58.7)	69 (6.9)	34 (3.4)	35 (3.5)
Replacement (%)	702 (41.3)	73 (10.4)	36 (5.1)	37 (5.3)
Total (%)	1700	142 (8.4)	70 (4.1)	72 (4.2)
P value		0.011	0.038	0.03

serum bilirubin levels, and 25 (18%) samples showed enzyme elevation. Out of these 25 samples with elevated enzyme levels, 14 (56%) were positive for anti-HBc-only and 11 (44%) were positive for anti-HBc + anti-HBs ($P = 0.460$).

One hundred samples from these 142 anti-HBc reactive (45 anti-HBc-only and 55 anti-HBc with anti-HBs) samples were tested for HBV DNA by using PCR. Only one sample was positive for HBV DNA. This sample had an anti-HBs level > 150 mIU/mL, LFTs for this sample were within normal limits. This was a 22-year-old male repeat replacement blood donor.

DISCUSSION

Despite mandatory screening of donor blood for HBsAg, transfusion-associated HBV (TAHBV) continues to be a major problem in India, more so in patients receiving repeated transfusions[2].

Literature worldwide shows presence of anti-HBc in HBsAg-negative blood donors. The incidence of anti-HBc in blood donors varies from 0.07% to 18%, and 0.3%-38% of these donors show presence of HBV DNA in their blood, depending on the type of blood donors and the endemicity of disease in the study population[2,4,6,9-12].

This study was conducted on 1700 HBsAg ELISA non-reactive blood donors. The study population belonged to Chandigarh and states of Punjab, Haryana, Himachal Pradesh, Jammu and Kashmir. The present study showed 8.4% anti-HBc positivity. Prevalence of anti-HBc was 6.9% in voluntary donors and 10.4% in replacement donors. A study reported from New Delhi (Northern India) by Chaudhuri *et al*[2] revealed that the prevalence of anti-HBc was 10.82% with distribution of 6.92% in voluntary donors and 12.53% in replacement donors. In contrast, a study from West Bengal (Eastern India) by Bhattacharya *et al*[7] showed an anti-HBc positivity as high as 18.3% in voluntary blood donors. Prevalence of anti-HBc reported by Behzad-Behbahani *et al*[10] in Iran was 6.55%, in this study only voluntary donors were included. High prevalence of anti-HBc (17.28%) was reported by Bhatti *et al*[6] from Pakistan, and all the donors in this study were replacement donors. The prevalence of anti-HBc in Europe and North America is quite low, an anti-HBc prevalence of 0.07% in the UK and 1.5% in Germany has been reported[6]. A study from Japan[4] reported anti-HBc prevalence of as 1.1%. The enormous variation in global seroprevalence of anti-HBc among blood donors is a reflection of difference in the type of blood donors and HBV endemicity of the study population. The low seroprevalence in US, UK and German blood donors may be due to 100% voluntary donor base, stringent donor screening, high literacy rates and self exclusion by high- risk donors. In our study, a significantly low seropositivity (6.1%) was seen in donors with age 18-30 years as compared to donors with age 31-40 years (11.6%, $P = 0.003$). Donors with age 18-30 years were 50% of the study population and were largely composed of young college students. The results are comparable with study from our department in 2004 by Sharma *et al*[13] that student donors have significantly lower incidence of all the markers for transfusion-transmitted infections. Therefore, efforts should be made to increase and retain these young motivated voluntary donors to maintain safe blood supply.

Our study revealed similar prevalence of anti-HBc positivity in first time donors (8.4%) and repeat donors (8.3%, $P = 0.94$), suggesting that lack of education among both our donor groups regarding minor modes of HBV transmission like tattooing, ear/nose piercing, sharing of shaving kits or a visit to a road side barber. It highlights the uniformity of donor behavior between the two groups. Our study is in accordance with recent three studies by Retrovirus Epidemiology Donor Study (REDS)[14-16] groups which showed that the incidence rate of viral infection is not lower in repeat blood donors than first time donors and abbreviated screening for repeat blood donors is not advisable.

Prevalence of anti-HBc-only was 4.1% in our study, out of which 3.2% was in voluntary and 5.1% in the replacement donors ($P = 0.038$). In a study from New Delhi (Northern India)[2], the reported prevalence of anti-HBc-only was 4.2%, out of which 2.72% was in voluntary and 4.85% in replacement donors[2]. Similarly, a study from Iran[10] reported anti-HBc-only prevalence of 2.3%.

Anti-HBc reactive samples were tested for LFTs. All the samples had normal serum bilirubin levels, and 25 (18%) samples showed elevation of liver enzymes. Elevated levels of liver enzymes in our donors indicates underlying hepatitis or some kind of liver injury which can be ruled out by additional investigations, such as hepatic ultrasound, liver biopsy and genetic testing which were not an objective of the present study.

HBV DNA was detected in 1 of 100 anti-HBc reactive donors tested. None of the anti-HBc-only samples were positive for HBV DNA. One sample was positive for HBV DNA and sample also contained anti-HBs levels > 150 mIU/mL, LFTs for this sample were within normal limits. Other studies from india[2,7] reported HBV DNA positivity of 20.87% in New Delhi (Northern India) and 21% in West Bengal (Eastern India). HBV DNA was detected among 12.2% of anti-HBc reactive donors in Iran, 2.8% in Lebanon, 2.9% in Pakistan[7]. A study from Japan[4] reported that HBV DNA was detected in 19 (38%) of 50 anti-HBc reactive samples. The viral load in these samples is usually low and their detection requires sensitive DNA amplification techniques.

In our study, low incidence of HBV DNA in anti-HBc reactive samples may be due to limitation of sensitivity of HBV DNA amplification technique. Another reason of low HBV DNA positivity in our study could be due to type of blood donors and the endemicity of disease in the study population, in our study 50% of donors were between 18-30 years, largely belong to young college students. This group being better educated can understand and cooperate in answering to the risk factors in acquiring HBV infection during pre-donation questionnaire. Another study from Chandigarh (Northwestern India) by Duseja *et al*[17] showed 0% prevalence of HBV DNA in 100 adult healthy blood donors, non-reactive for HBsAg.

Keeping in view that 8% to 18% of donor population in India is anti-HBc reactive, inclusion of anti-HBc testing will lead to high discard rate. Anti-HBs as proposed previously does not seem to predict clearance of the virus as the single donor, who tested reactive for HBV DNA in our study, had high anti-HBs titers. Cost effectiveness of introducing universal anti-HBc screening and discarding large number of blood units *versus* considering ID NAT (Individual donor nuclic acid testing) needs to be assessed. Awareness and education of donors is required regarding minor modes of HBV transmission, modification of the donor questionnaire to eliminate all donors with a history of jaundice in adult life and more stringent one-to-one donor screening to elicit such information should be implemented.

COMMENTS

Background

Despite mandatory screening of donor blood for HBsAg, transfusion-associated HBV (TAHBV) continues to be a major problem in India, more so in patients receiving repeated transfusions.

Research frontiers

The present study was undertaken to assess the seroprevalence of antibody to hepatitis B core antigen (anti-HBc) in serum samples of healthy blood donors negative for HBsAg and to evaluate whether anti-HBc detection could be adopted in India as a screening assay for HBV in addition to HBsAg.

Innovations and breakthroughs

There is high prevalence of anti-HBc in Indian blood donors. Voluntary donors have lower incidence of anti-HBc than replacement donors. Anti-HBs does not seem to predict clearance of the virus in the blood donors.

Applications

Keeping in view that 8%-18% of donor population in India is anti-HBc reactive, inclusion of anti-HBc testing will lead to high discard rate. Cost effectiveness of introducing universal anti-HBc screening and discarding large number of blood units *versus* considering ID NAT needs to be assessed.

Peer review

This is an important and timely paper assessing the role of anti-HBc testing to detect occult HBV infectivity for addressing the important issue of TAHBV in Indian blood donors. The research was done at PGIMER Chandigarh, India, one of the premier institutes in the country. This paper deserves publication.

REFERENCES

1 **Schmidt M**, Nubling CM, Scheiblauer H, Chudy M, Walch LA, Seifried E, Roth WK, Hourfar MK. Anti-HBc screening of blood donors: a comparison of nine anti-HBc tests. *Vox Sang* 2006; **91**: 237-243

2 **Chaudhuri V**, Nanu A, Panda SK, Chand P. Evaluation of serologic screening of blood donors in India reveals a lack of correlation between anti-HBc titer and PCR-amplified HBV DNA. *Transfusion* 2003; **43**: 1442-1448

3 **Nanu A**, Sharma SP, Chatterjee K, Jyoti P. Markers for transfusion-transmissible infections in north Indian voluntary and replacement blood donors: prevalence and trends 1989-1996. *Vox Sang* 1997; **73**: 70-73

4 **Yotsuyanagi H**, Yasuda K, Moriya K, Shintani Y, Fujie H, Tsutsumi T, Nojiri N, Juji T, Hoshino H, Shimoda K, Hino K, Kimura S, Iino S, Koike K. Frequent presence of HBV in the sera of HBsAg-negative, anti-HBc-positive blood donors. *Transfusion* 2001; **41**: 1093-1099

5 **Kleinman SH**, Kuhns MC, Todd DS, Glynn SA, McNamara A, DiMarco A, Busch MP. Frequency of HBV DNA detection in US blood donors testing positive for the presence of anti-HBc: implications for transfusion transmission and donor screening. *Transfusion* 2003; **43**: 696-704

6 **Bhatti FA**, Ullah Z, Salamat N, Ayub M, Ghani E. Anti-hepatits B core antigen testing, viral markers, and occult hepatitis B virus infection in Pakistani blood donors: implications for transfusion practice. *Transfusion* 2007; **47**: 74-79

7 **Bhattacharya P**, Chandra PK, Datta S, Banerjee A, Chakraborty S, Rajendran K, Basu SK, Bhattacharya SK, Chakravarty R. Significant increase in HBV, HCV, HIV and syphilis infections among blood donors in West Bengal, Eastern India 2004-2005: exploratory screening reveals high frequency of occult HBV infection. *World J Gastroenterol* 2007; **13**: 3730-3733

8 **Malik V**. Drugs and Cosmetics Act, 1940. 16th ed. Lucknow: Eastern Book Company, 2003: 279-303

9 **Kleinman SH**, Busch MP. HBV: amplified and back in the blood safety spotlight. *Transfusion* 2001; **41**: 1081-1085

10 **Behzad-Behbahani A**, Mafi-Nejad A, Tabei SZ, Lankarani KB, Torab A, Moaddeb A. Anti-HBc & HBV-DNA detection in blood donors negative for hepatitis B virus surface antigen in reducing risk of transfusion associated HBV infection. *Indian J Med Res* 2006; **123**: 37-42

11 **Allain JP**. Occult hepatitis B virus infection: implications in transfusion. *Vox Sang* 2004; **86**: 83-91

12 **Comanor L**, Holland P. Hepatitis B virus blood screening: unfinished agendas. *Vox Sang* 2006; **91**: 1-12

13 **Sharma RR**, Cheema R, Vajpayee M, Rao U, Kumar S, Marwaha N, Agnihotri SK. Prevalence of markers of transfusion transmissible diseases in voluntary and replacement blood donors. *Natl Med J India* 2004; **17**: 19-21

14 **Glynn SA**, Schreiber GB, Busch MP, Kleinman SH, Williams AE, Nass CC, Ownby HE, Smith JW. Demographic characteristics, unreported risk behaviors, and the prevalence and incidence of viral infections: a comparison of apheresis and whole-blood donors. The Retrovirus Epidemiology Donor Study. *Transfusion* 1998; **38**: 350-358

15 **Williams AE**, Thomson RA, Schreiber GB, Watanabe K, Bethel J, Lo A, Kleinman SH, Hollingsworth CG, Nemo GJ. Estimates of infectious disease risk factors in US blood donors. Retrovirus Epidemiology Donor Study. *JAMA* 1997; **277**: 967-972

16 **Schreiber GB**, Glynn SA, Busch MP, Sharma UK, Wright DJ, Kleinman SH. Incidence rates of viral infections among repeat donors:are frequent donors safer? *Transfusion* 2001; **41**: 730-735

17 **Duseja A**, Sharma S, Subramanian PG, Agnihotri SK, Chakraborti A, Chawla Y. Occult hepatitis B virus (HBV) infection in healthy blood donors. *Indian J Pathol Microbiol* 2003; **46**: 690-692

S- Editor Zhong XY **L- Editor** Kumar M **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5331

World J Gastroenterol 2008 September 14; 14(34): 5331-5335
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Characteristics of paraesophageal varices: A study with 64-row multidetector computed tomography portal venography

Li-Qin Zhao, Wen He, Guang Chen

Li-Qin Zhao, Wen He, Guang Chen, Department of Radiology, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China

Author contributions: He W and Zhao LQ designed the research; Zhao LQ, Chen G performed the data collection; Zhao LQ and He W performed the post-processing of the images and analyzed data; Zhao LQ wrote the manuscript; He W revised the paper.

Supported by The Science Technology Program of Beijing Education Committee, No. KM200810025002

Correspondence to: Wen He, MD, Department of Radiology, Beijing Friendship Hospital, Capital Medical University, 95 Yong'An Road, Xuan Wu District, Beijing 100050, China. hewen1724@sina.com
Telephone: + 86-10-63138470 Fax: + 86-10-63134411

Received: June 19, 2008 Revised: July 27, 2008
Accepted: August 3, 2008
Published online: September 14, 2008

Abstract

AIM: To identify the characteristics of morphology, location and collateral circulation involved in paraesophageal varices (para-EV) of portal hypertension patients with 64-row multidetector computed tomography (MDCT).

METHODS: Fifty-two of 501 patients with portal hypertensive cirrhosis accompanied with esophageal varices were selected for 64-row MDCT examination after the observation of para-EV. The CT protocol included unenhanced, arterial and portal phases with a slice thickness of 0.625 mm and a scanning field of 2 cm above the bifurcation to the lower edge of kidney. The CT portal venography (CTPV) was reformatted on AW4.3 workstation. The characteristics of origination, location, morphology and collateral circulation in para-EV were observed.

RESULTS: Among the 52 cases of para-EV, 50 showed the originations from the posterior branch of left gastric vein, while the others from the anterior branch. Fifty cases demonstrated their locations close to the esophageal-gastric junction, and the other two cases were extended to the inferior bifurcation of the trachea. The circuitous pattern was observed in 16 cases, while reticulated pattern was seen in 36 cases. Collateral circulation identified 4 cases of single periesophageal varices (peri-EV) communication, 3 cases of single hemiazygous vein, one case of single inferior vena cava, 41 cases of mixed type (collateral communications of at least 2 of above mentioned types) and 3 cases of undetermined communications. Among all the cases, 43 patients showed the communications between para-EV and peri-EV, while hemiazygous vein (43 cases) and inferior vena cava (5 cases) were also involved.

CONCLUSION: Sixty-four-row multidetector computed tomography portal venography could display the location, morphology, origin, and collateral types of para-EV, which provides important and referable information for clinical management and disease prognosis.

Key words: Computer tomography; Portal venography; Paraesophageal varices; Hepatic cirrhosis; Portal hypertension

Peer reviewers: Juan G Abraldes, MD, Hepatic Hemodynamic Laboratory, Liver Unit, Hospital Clinic, University of Barcelona, Villarroel 170, Barcelona 08036, Spain; Aydin Karabacakoglu, PhD, Assistant Professor, Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey

Zhao LQ, He W, Chen G. Characteristics of paraesophageal varices: A study with 64-row multidetector computed tomography portal venography. *World J Gastroenterol* 2008; 14(34): 5331-5335 Available from: URL: http://www.wjgnet.com/1007-9327/14/5331.asp DOI: http://dx.doi.org/10.3748/wjg.14.5331

INTRODUCTION

Esophageal varice is a common collateral circulation manifestation in portal hypertension, and may cause a severe complication if ruptured[1-3]. Endoscopic therapy is an effective approach that is being applied worldwide[4,5], although the recurrences have been reported in many cases[6]. Some authors reported that the existence of perforating veins, the communicating branches between periesophageal varices (peri-EV) and paraesophageal varices (para-EV), and their blood flow found by endoscopic color Doppler ultrasonography (ECDUS) affected the recurrences of hemorrhage caused by rupturing[7]. However, ECDUS is an invasive technique, requires specialized equipment

and technology, and is incapable of demonstrating the drainage types and morphologic features of esophageal varices. Multidetector computed tomography portal venography (MDCTPV) has been used widely in collateral circulation studies of esophageal and gastric varices[8-11]. The drainage veins of esophageal varices can be clearly displayed on MDCTPV[10]. Our study focuses on the utilization of MDCTPV in observing para-EV drainage types to provide referable information for clinical management selection and prognosis evaluation.

MATERIALS AND METHODS

Patient population

A total of 501 portal hypertensive cirrhotic patients with esophageal varices were investigated from April 2007 to May 2008. Among them, 52 patients with presence of para-EV were selected for this study, including 35 male and 17 female, aged 21-71 years, averaging 45.2 years. There were 27 cases of hepatitis B, 3 hepatitis C, 16 alcoholic, 2 primary biliary and 4 cases of cryptogenic cirrhosis. Based on Child's grading, 9 cases were Grade A, 28 cases Grade B and 15 cases of Grade C. Among the 52 patients, 50 were accompanied with gastric varices. Fifty patients were not treated with esophageal varice ligation (EVL), esophageal varice sclerotherapy (EVS) or any other endoscopic therapies, while 1 case received post-EVL therapy and 1 case received post-EVS therapy. Liver cirrhosis was confirmed in all patients through general consideration from clinical histories, laboratory findings, as well as CT, sonography or MRI examinations. Informed consent for CT scans and the use of contrast media were obtained from all patients before scanning procedures.

Imaging acquisition

A GE VCT 64-row MDCT scanner was applied to perform unenhanced, arterial and portal vein phase enhanced scans on all patients. The scanning range covers from 2 cm above the tracheal bifurcation to the lower edge of the kidney. One hundred mL of non-ionic contrast medium (Omnipaque 350) was introduced with an infusion rate of 4.0 mL/s. The arterial phase scanning time delay was determined with Smart technology, and portal phase scanning was initiated at 25 s after the beginning of arterial phase. Slice thickness was set at 5.0 mm, and reconstitution thickness was 0.625 mm.

Image analysis

Post-processing methods: vertebrae, costal bones and other structures with bony densities were removed using GE AW4.3 workstation, and then maximum intensity projection (MIP), multi-planner reformation (MPR) technique were applied to reveal the origin, location, morphology and communicating veins of para-EV. The 3D structures of esophageal varices were displayed with volume rendering (VR) technique.

Observation item and indications

Origin of para-EV: The relationship between para-EV and anterior or posterior branches of the left gastric vein were examined and determined.

Morphology and location of para-EV: The morphological patterns of para-EV were categorized into circuitous pattern and reticulated pattern, in which circuitous pattern was denoted as para-EV linear, near-parallel pathways, while reticulated pattern was denoted as prominent para-EV winding and distortion, forming meshwork or loops. In case of the presence of both circuitous and reticulated patterns, the categorization was determined according to their diameter ratios and then was defined as "mainly circuitous pattern" or "mainly reticulated pattern". Large-diameter vessels with a ratio of over 1 were defined as dominant type. The para-EV was divided into 3 sections according to the extent, including the upper section located at a level superior to the tracheal bifurcation; the middle section located at or near the tracheal bifurcation level, and the lower section located at the abdominal as well as the lower thoracic segment of the esophageal periphery.

Communicating veins of para-EV: The communications between para-EV and peri-EV, hemiazygous vein, subphrenic vein, inferior vena cava and other vessels were investigated. The criteria set for CTPV judgment on the communication of para-EV and its collaterals were as follows: (1) Para-EV was directly conjoint with the above-mentioned vessels; (2) Para-EV and the vessels mentioned above were conjoint with their peripheral circuitous and reticulated blood vessels, respectively.

RESULTS

Origination of para-EV

There were 50 patients with their varices originating from the posterior branch of the left gastric vein (50/52, 96.15%) (Figure 1A) while the varices of the other 2 patients originated from the anterior branch of the left gastric vein (2/52, 3.85%), in which the posterior branch of the left gastric vein was absent and the anterior branch of left gastric vein routes up and enters the fundus through the gastroesophageal junction to form gastric fundal varices. The latter routes up to form peri-EV, then penetrate through esophageal wall to form para-EV.

Morphology and location of para-EV

Sixteen cases of circuitous morphological pattern (16/52, 36.54%) (Figure 1B) and 36 cases of reticulated morphological pattern (36/52, 63.46%) (Figure 1C) were observed. The locations of para-EV were identified as follows: 50 cases in lower section (50/52, 96.15%) (Figure 1C), 2 cases in middle section (2/52, 3.85%), and none in upper section.

Communicating veins of para-EV

Among the 52 cases, 3 showed undetermined communicating vessels (3/52, 5.77%) with 1 of circuitous morphological pattern, while the others were of reticulated

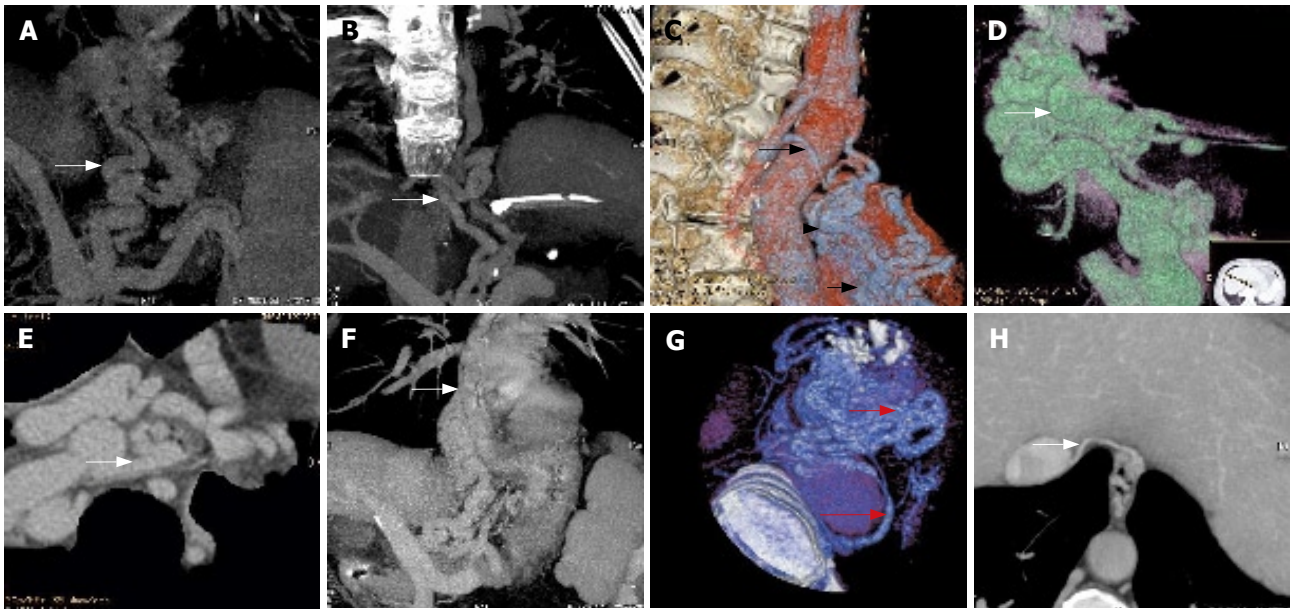


Figure 1 **A:** Para-EV originated from the posterior branch of the left gastric varices (arrow); **B:** Circuitous morphological para-EV (arrow); **C:** Para-EV in reticulatous pattern (short arrow), located at lower section of the esophagus (arrowhead), communicating to hemiazygous vein (long arrow); **D:** Para-EV communicating to peri-EV (arrow); **E:** Para-EV communicating to peri-EV directly (arrow); **F:** Para-EV communicating to peri-EV with peripheral circuitous appearance (arrow); **G:** Para-EV communicating to peri-EV (short arrow) and the hemiazygous vein (long arrow); **H:** Para-EV communicating to the subphrenic vein (arrow).

Table 1 Communicating varices and para-EV shapes

Drainage vein		Shape	Cases	Total
Single	Peri-EV	Reti	4	8
	Hemi	Cir	3	
	Sub	Cir	1	
	Peri-EV + Hemi	Cir	8	41
	Peri-EV + Hemi	Reti	29	
Mixed	Sub + Peri-EV	Cir	1	
	Sub + Hemi	Cir	2	
	Sub + Peri-EV + Hemi	Reti	1	
Uncertain		Cir	1	3
		Reti	2	
Total				52

Hemi: Hemiazygos vein; Sub: Subphrenic vein; Reti: Reticular; Cir: Circuitous.

morphological pattern. In the other 49 cases, 43 cases involved the peri-EV (43/49, 87.76%) (Figure 1D-G), and a similar number of patients also demonstrated the hemiazygous vein (Figure 1B, C and G). Inferior vena cava was included in 5 cases (5/49, 10.20%) (Figure 1H).

The specific communicating patterns found in this study were listed as follows (Table 1): 8 cases with single communication, 41 with mixed communications and, in 3 cases, the drainage veins of the para-EV remained uncertain. Among the 8 cases of single communication, the para-EV connecting to peri-EV (Figure 1E and F) with reticulated shape was found in 4 cases (4/49, 8.16%), connecting to Hemiazygos vein of circuitous appearance in 3 cases (3/49, 5.77%), and to the subphrenic vein draining circuitously in 1 case (1/49, 1.92%).

In the 41 cases of mixed type, the para-EV was found connecting to peri-EV and hemiazygous vein in 37 cases (37/49, 71.15%) (Figure 1G), with circuitous shape in 8 cases and reticulated in 29 cases. Three cases presented para-EV communicating to subphrenic vein: 1 to peri-EV (1/49, 1.92%) and 2 to Hemiazygos vein circuitously (2/49, 3.84%). In 1 case (1/49, 1.92%), all the drainage mentioned above was observed with reticulated appearance. The para-EV was circuitous in 1 case and reticular in 2 cases of uncertain drainage.

DISCUSSION

The prevalence rate of esophageal varices is high in patients with liver cirrhosis, and the mortality rate is very high imposed by massive hemorrhage due to rupture[2,12]. It is reported that more than 70% of portal hypertension patients with a variceal bleeding history may suffer from recurrent bleeding[13]. The esophageal varice was divided into three groups: peri-EV, para-EV and perforating veins[14]. The factors associated with the rupture of esophageal varices included the extent and collateral circulation of peri-EV and para-EV[15,16]. Therefore, the acquisition of reliable images for the above mentioned factors will provide valuable information for further treatment implementation and prognosis of the patients.

Angiography is one of the earliest investigations applied in hemodynamic evaluation of portal hypertension-induced esophageal varices by displaying esophageal varices and their drainage vessels through arterial portography[17]. However, the invasive procedure and the incapability to differentiate peri-EV and para-EV prevent it to be applied in a general clinical setting.

Color Doppler ultrasound has been widely used to investigate the relationship between EV and hemodynamics associated with portal hypertension and liver cirrhosis. But it could not show the para-EV and peri-EV clearly[18].

Endoscopy is a more popular approach for the observation of morphological patterns, extension and severity of esophageal submucotic varices with the limitations to intramural and para-EV[19].

ECDUS is gradually becoming a useful tool for esophageal varicose inspection, which is able to display peri-EV, para-EV, perforating veins and the blood flow directions of perforating veins[7,14,20]. However, it is unable to clearly display the pathway and collaterals of para-EV. On the other hand, endoscopic sonography is costly, and also an invasive procedure that requires a skillful operator, which limited its application in clinical settings.

MDCTPV has already been a matured investigation technique used to examine esophageal and gastric varices. It is competent in displaying the types of morphological pattern, origin and collateral circulations of esophageal and gastric varices[10,11]. Sixty-four-row MDCT consists of thin slice of 0.625 mm, and a high spatial resolution, which makes it capable of displaying the communicating patterns of para-EV and peri-EV. Among the 52 cases in our study, this pattern of collateral circulation was shown in 49 (94.23%) cases. Generally, it is a non-invasive technique, and hence, it can be used to evaluate para-EV conveniently. However, displaying of this sort of collaterals is relatively rough and unsatisfactory in detail if compared with EUS. In addition, it is incapable to assess the blood flow directions of paraesophageal-periesophageal collateral circulation due to the characters of CTPV.

According to our study, vast majority of para-EV originate from the posterior branch of left gastric vein, and only 2 cases originated from the anterior branch of left gastric vein. The latter is highly associated with gastric fundal varices. A previous report suggested that[21], in cases of coexisting gastric fundal varices and esophageal varices in lower esophageal segment, the extent of esophageal varices could be aggravated after imposing obliteration therapy for gastric fundal varices. In our study, there was one patient who experienced alleviation of gastric varices with aggravated para-EV and peri-EV after receiving gastroesophageal varices treatment. The origination of para-EV may be related directly to its anatomical formation.

In our study, para-EV was found more abundant near the superior part of the gastroesophageal junction, which is also the lower esophageal segment (96.15%) than in the middle segment (3.85%). There was no para-EV involved in the upper segment. This suggests that para-EV usually occurs in the middle and lower esophageal segment and drains into the vena cava system through collateral vessels. The involved drainage vessels that we have noted were peri-EV, hemiazygous veins and subphrenic veins.

The communicating branches of para-EV and hemiazygous vein manifest as drainage "detour" veins bypassing along the anterior descending aorta to the hemiazygous vein, and subsequently drain into the superior vena cava. Ibukuro *et al*[22] described the above anatomic changes as preaortic vein using CT and CTAP application. This "detour" is a commonly seen drainage route, and has an occurrence rate of 87.76%.

The hemodynamics of para-EV communicating peri-EV is relatively complicated. The communicating vessels of para-EV and peri-EV are mostly situated close to the top of the cardia level. Sato *et al*[7] observed the presence of perforating veins between peri-EV and para-EV with Levovist ECDUS, and meanwhile, he found the bidirectional blood flow of peri-EV and para-EV, which included the directions from para-EV to peri-EV and from peri-EV to para-EV, as well as mixed type that presented both blood flow directions. This may explain why previous para-EV studies about the recurrence risk of post-esophageal varices treatment remain controversial[20,23,24].

Para-EV can still drain into the inferior vena cava through the subphrenic vein, or directly communicate with the inferior vena cava. The subphrenic veins are the bilateral vessels which end up at inferior vena cava at the diaphragm level[25]. The communication between para-EV and subphrenic veins shown on CTPV was consistent to the precaval route and morphological pattern as reported by Ibukuro *et al*[25] from their CTAP and autopsy findings.

The degree of varices will be more severe if the morphological patterns of para-EV are reticulated. Under such condition, para-EV and peri-EV are individually communicated, or may be accompanied with hemiazygous vein drainage. They will mostly drain into the hemiazygous veins or subphrenic veins if the collaterals of para-EV are circuitous. Explanations about this correlation and its pathological significance require further studies.

Even though CTPV is incapable of displaying the blood flow directions of para-EV, it has a certain guidance value for EUS application. If para-EV is detected on CTPV, EUS should be further implemented to confirm the presence of specific communicating branches between peri-EV and para-EV as well as the location and hemodynamic characteristics of their communicating branches so as to provide relevant referable information for treatment selections.

COMMENTS

Background

The existence of para-EV and perforating veins and the direction of their blood flow can affect the recurrence of hemorrhage caused by rupturing.

Research frontiers

The morphology, location and collateral circulation characteristics of para-EV in portal hypertensive patients were studied with 64-row multidetector computer tomography portal venography (MDCTPV), which is a non-invasive method.

Innovations and breakthroughs

In majority cases of this study, the collateral circulation pattern of para-EV and the morphological characteristics of para-EV were revealed thanks to the high spatial resolution images of the advanced MDCT and the appropriate image post-processing.

Applications

MDCTPV is a noninvasive method to display the morphological characteristics of para-EV, which is useful in clinical management and disease prognosis.

Terminology

Paraesophageal varices are the varices that exist outside the esophagus. Peri-

EV is the varices that exist in the esophageal wall or the submucotic varices. Perforating vein is the communicating branch between peri-EV and para-EV. The subphrenic vein is the bilateral vessel that ends up at inferior vena cava at the diaphragm level. CTPV is the abbreviation of CT portal venography.

Peer review

This is a good review with excellent images. MDCTPV is a convenient method to display the morphological and collateral circulations of the para-EV. It is of important value in the selection of clinical therapy and evaluation of prognosis.

REFERENCES

1 **Bhasin DK**, Malhi NJ. Variceal bleeding and portal hypertension: much to learn, much to explore. *Endoscopy* 2002; **34**: 119-128

2 **Bratovic I**, Lacevic N. Management of esophageal varices. *Med Arh* 2002; **56**: 11-12

3 **Brandenburger LA**, Regenstein FG. Variceal Hemorrhage. *Curr Treat Options Gastroenterol* 2002; **5**: 73-80

4 **Gotoh Y**, Iwakiri R, Sakata Y, Koyama T, Noda T, Matsunaga C, Ogata SI, Ishibashi S, Sakata H, Tsunada S, Fujimoto K. Evaluation of endoscopic variceal ligation in prophylactic therapy for bleeding of oesophageal varices: a prospective, controlled trial compared with endoscopic injection sclerotherapy. *J Gastroenterol Hepatol* 1999; **14**: 241-244

5 **Mizumoto H**, Matsutani S, Fukuzawa T, Ishii H, Sato G, Maruyama H, Saisho H. Hemodynamics in the left gastric vein after endoscopic ligation of esophageal varices combined with sclerotherapy. *J Gastroenterol Hepatol* 2001; **16**: 495-500

6 **Grace ND**, Groszmann RJ, Garcia-Tsao G, Burroughs AK, Pagliaro L, Makuch RW, Bosch J, Stiegmann GV, Henderson JM, de Franchis R, Wagner JL, Conn HO, Rodes J. Portal hypertension and variceal bleeding: an AASLD single topic symposium. *Hepatology* 1998; **28**: 868-880

7 **Sato T**, Yamazaki K, Toyota J, Karino Y, Ohmura T, Akaike J, Kuwata Y, Suga T. Perforating veins in recurrent esophageal varices evaluated by endoscopic color Doppler ultrasonography with a galactose-based contrast agent. *J Gastroenterol* 2004; **39**: 422-428

8 **Kang HK**, Jeong YY, Choi JH, Choi S, Chung TW, Seo JJ, Kim JK, Yoon W, Park JG. Three-dimensional multi-detector row CT portal venography in the evaluation of portosystemic collateral vessels in liver cirrhosis. *Radiographics* 2002; **22**: 1053-1061

9 **Agarwal A**, Jain M. Multidetector CT portal venography in evaluation of portosystemic collateral vessels. *J Med Imaging Radiat Oncol* 2008; **52**: 4-9

10 **Zhang LQ**, He W. CT portal venography of collateral veins in esophageal varices. *Zhongguo Yixue Yingxiang Jishu* 2007; **23**: 242-245

11 **Zhao LQ**, He W, Zhao H, Yu YZ. The value of CT portalvenography in the diagnosis of collateral veins in patients with gastric varices. *Zhonghua Fangshexue Zazhi* 2006; **40**: 1175-1178

12 **Seewald S**, Seitz U, Yang AM, Soehendra N. Variceal bleeding and portal hypertension: still a therapeutic challenge? *Endoscopy* 2001; **33**: 126-139

13 **Grace ND**, Groszmann RJ, Garcia-Tsao G, Burroughs AK, Pagliaro L, Makuch RW, Bosch J, Stiegmann GV, Henderson JM, de Franchis R, Wagner JL, Conn HO, Rodes J. Portal hypertension and variceal bleeding: an AASLD single topic symposium. *Hepatology* 1998; **28**: 868-880

14 **Irisawa A**, Shibukawa G, Obara K, Saito A, Takagi T, Shishido H, Odajima H, Abe M, Sugino T, Suzuki T, Kasukawa R, Sato Y. Collateral vessels around the esophageal wall in patients with portal hypertension: comparison of EUS imaging and microscopic findings at autopsy. *Gastrointest Endosc* 2002; **56**: 249-253

15 **Irisawa A**, Obara K, Sato Y, Saito A, Takiguchi F, Shishido H, Sakamoto H, Kasukawa R. EUS analysis of collateral veins inside and outside the esophageal wall in portal hypertension. *Gastrointest Endosc* 1999; **50**: 374-380

16 **Irisawa A**, Saito A, Obara K, Shibukawa G, Takagi T, Yamamoto G, Sakamoto H, Takiguchi F, Shishido H, Hikichi T, Oyama H, Sato N, Katakura K, Kasukawa R, Sato Y. Usefulness of endoscopic ultrasonographic analysis of variceal hemodynamics for the treatment of esophageal varices. *Fukushima J Med Sci* 2001; **47**: 39-50

17 **Watanabe K**, Kimura K, Matsutani S, Ohto M, Okuda K. Portal hemodynamics in patients with gastric varices. A study in 230 patients with esophageal and/or gastric varices using portal vein catheterization. *Gastroenterology* 1988; **95**: 434-440

18 **Li FH**, Hao J, Xia JG, Li HL, Fang H. Hemodynamic analysis of esophageal varices in patients with liver cirrhosis using color Doppler ultrasound. *World J Gastroenterol* 2005; **11**: 4560-4565

19 **Chikamori F**, Kuniyoshi N, Shibuya S, Takase Y. Correlation between endoscopic and angiographic findings in patients with esophageal and isolated gastric varices. *Dig Surg* 2001; **18**: 176-181

20 **Choudhuri G**, Dhiman RK, Agarwal DK. Endosonographic evaluation of the venous anatomy around the gastro-esophageal junction in patients with portal hypertension. *Hepatogastroenterology* 1996; **43**: 1250-1255

21 **Cho SK**, Shin SW, Lee IH, Do YS, Choo SW, Park KB, Yoo BC. Balloon-occluded retrograde transvenous obliteration of gastric varices: outcomes and complications in 49 patients. *AJR Am J Roentgenol* 2007; **189**: W365-W372

22 **Ibukuro K**, Tsukiyama T, Mori K, Inoue Y. Preaortic esophageal veins: CT appearance. *AJR Am J Roentgenol* 1998; **170**: 1535-1538

23 **Dhiman RK**, Choudhuri G, Saraswat VA, Agarwal DK, Naik SR. Role of paraoesophageal collaterals and perforating veins on outcome of endoscopic sclerotherapy for oesophageal varices: an endosonographic study. *Gut* 1996; **38**: 759-764

24 **Hino S**, Kakutani H, Ikeda K, Uchiyama Y, Sumiyama K, Kuramochi A, Kitamura Y, Matsuda K, Arakawa H, Kawamura M, Masuda K, Suzuki H. Hemodynamic assessment of the left gastric vein in patients with esophageal varices with color Doppler EUS: factors affecting development of esophageal varices. *Gastrointest Endosc* 2002; **55**: 512-517

25 **Ibukuro K**, Tsukiyama T, Mori K, Inoue Y. Precaval draining vein from paraesophageal varices: radiologic-anatomic correlation. *AJR Am J Roentgenol* 1999; **172**: 651-654

S- Editor Li DL **L- Editor** Ma JY **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5336

World J Gastroenterol 2008 September 14; 14(34): 5336-5343
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Effect of music on procedure time and sedation during colonoscopy: A meta-analysis

Wilson WS Tam, Eliza LY Wong, Sheila F Twinn

Wilson WS Tam, Sheila F Twinn, Nethersole School of Nursing, 8/F Esther Lee Building, The Chinese University of Hong Kong, Shatin, Hong Kong, China
Eliza LY Wong, School of Public Health, 4/F Postgraduate Education Centre, Prince of Wales Hospital, Shatin, Hong Kong, China
Author contributions: Tam WWS, Wong ELY and Twinn SF designed the study; Tam WWS and Wong ELY performed the search and data (papers) collection; Tam WWS performed the analysis; Tam WWS, Wong ELY and Twinn SF wrote the paper.
Correspondence to: Wilson WS Tam, Nethersole School of Nursing, Room 830, Esther Lee Building, The Chinese University of Hong Kong, Shatin, Hong Kong, China. wilsontam@graduate.hku.hk
Telephone: +86-852-26098172 Fax: +86-852-26035935
Received: July 10, 2008 Revised: August 25, 2008
Accepted: September 2, 2008
Published online: September 14, 2008

Abstract

AIM: To integrate results from different studies in examining the effectiveness of music in reducing the procedure time and the amount of sedation used during colonoscopic procedure.

METHODS: An electronic search in various databases was performed to identify related articles. Study quality was evaluated by the Jadad's scale. The random effect model was used to pool the effect from individual trials and the Cohen Q-statistic was used to determine heterogeneity. Egger's regression was used to detect publication bias.

RESULTS: Eight studies with 722 subjects were included in this meta-analysis. The combined mean difference for the time taken for the colonoscopy procedure between the music and control groups was -2.84 with 95% CI (-5.61 to -0.08), implying a short time for the music group. The combined mean difference for the use of sedation was -0.46 with 95%CI (-0.91 to -0.01), showing a significant reduction in the use of sedation in the music group. Heterogeneity was observed in both analyses but no publication bias was detected.

CONCLUSION: Listening to music is effective in reducing procedure time and amount of sedation during colonoscopy and should be promoted.

Key words: Colonoscopy; Colon cancer; Meta-analysis; Music therapy; Screening

Peer reviewer: Yoshiharu Motoo, MD, PhD, FACP, FACG, Professor and Chairman, Department of Medical Oncology, Kanazawa Medical University,1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan

Tam WWS, Wong ELY, Twinn SF. Effect of music on procedure time and sedation during colonoscopy: A meta-analysis. *World J Gastroenterol* 2008; 14(34): 5336-5343 Available from: URL: http://www.wjgnet.com/1007-9327/14/5336.asp DOI: http://dx.doi.org/10.3748/wjg.14.5336

INTRODUCTION

In 2005, colorectal cancer was the fourth most frequent cancer type worldwide for both sexes and colon cancer accounted for about 655 000 deaths per year[1]. Most colon cancers develop from polyps that grow abnormally in the colon. If polyps grow unnoticed and are not removed, they may become cancerous. Indeed, colorectal cancer is among the most preventable and curable cancers and with early detection 75%-90% of them can be prevented[2]. The aim of screening is to find precancerous polyps so they can be removed before they become cancerous. Screening for colon cancer has been shown to be effective in reducing mortality[3].

Colonoscopy is now the recommended method for screening colon cancer[4]. During a colonoscopy procedure, physicians insert a colonoscope into the rectum of a patient from the anus and slowly guide it into the colon for direct visualization and diagnosis[5]. As can be imagined, it is not a comfortable experience. In fact, many people refuse to undergo colonoscopy because of discomfort and anxiety[6], some feel out of control with what would be happening during the procedure and were fearful of looking foolish[7]. Even those who agree to undergo a colonoscopy feel frightened and anxious before the procedure[8]. After it, they express it was an unpleasant and stressful experience[9]. Therefore, most physicians prefer to

perform colonoscopy with conscious sedation[10] although some still start the procedure without sedation, particularly in non-anxious patients[11].

Since the use of sedation is risky-it may contribute to the occurrence of cardiovascular events and is associated with the risk of cardio-respiratory complication[12,13] especially for elderly patients[14]-pand costly, non-pharmacological methods for alleviating patients' discomfort and anxiety have been developed and music therapy is one of them. The use of music therapy to promote relaxation has a long history in medicine[15,16]. The ancient Chinese medical reference, Yellow Emperor's Classics of Internal Medicine, mentioned the use of music for treatment[17], while ancient Indian treatises, like Samaveda, stated the therapeutic utility of music[18]. Earlier studies show the effectiveness of music on patients with acute myocardial infarctions[19] and receiving intensive medical/surgical care[20]. The effects of music on different screening procedures were examined during the early 1990s. For example, Fullhart[21] and Palakanis[6] studied anxiety in patients undergoing flexible sigmoidoscopy and Bampton[22] assessed the role of relaxation music on patient tolerance of gastrointestinal endoscopic procedures. Since then, more and more studies have examined the effect of music during colonoscopy, but no conclusion has yet been reached about its effectiveness. Recently, a meta-analysis[23] focusing on the general endoscopic procedure was published and the authors reported significantly lower anxiety levels and shorter procedure times. The aim of this meta-analysis is to integrate results from different studies in examining the effectiveness of music in reducing procedure time and amount of sedation used during colonoscopy.

MATERIALS AND METHODS

Search strategies

We identified studies to evaluate the effect of listening to music during colonoscopy. The electronic search was conducted on six databases, namely, AMED-Allied and Complementary Medicine (1985 to 2007), ACP journal club (1991 to 2007), CINAHL (1982 to March 2007), Cochrane Central Register of Controlled trials (up to 1st quarter of 2007), EMBASE (1980 to March 2007), and MEDLINE (1966 to March 2007). Only two sets of keywords, i.e. Colonoscopy and Music/Music, were used to include as many articles as possible. We also attempted to identify any potential unpublished studies such as theses and dissertations using the above keywords through the ProQuest Dissertations and Theses database, and the web using Internet search engines, Google and Yahoo, with thesis or dissertation as additional keywords. Reference lists of the collected papers were also checked for any potential articles.

Inclusion criteria

Only randomized controlled studies reported in English were included. At least one of the comparison groups included listening to music during colonoscopy as part of the intervention.

Data extraction

For each eligible study, we extracted information on author/s, year of publication, age distribution, gender proportion, other demographic data, intervention, outcome measures, and results. The data extraction form was modified from the data extraction form of van Tulder[24]. Two investigators (WT and EW) of the study independently extracted the data, which was then summarized by one of them. Agreement was reached on the extracted data before proceeding to data analysis.

Methodological quality

The methodological quality of the included studies was assessed using the Jadad's scale[25]. The quality variables recorded in the criteria list included the procedure of patient allocation, information regarding withdrawals and dropouts, blinding of patients, and outcome assessment. A maximum of five points can be obtained using the scale. However, as blinding of patients is difficult while assessing behavioral interventions[26], only blinding of outcome assessors was considered in the scale. Furthermore, as withdrawals and attrition of patients were unlikely in such a short period, such item in the Jadad's scale was also ignored. Therefore, the maximum points obtainable for each study became four. Besides the Jadad's scale, one additional question was extracted from van Tulder[24] for assessing the comparability of the two comparison groups.

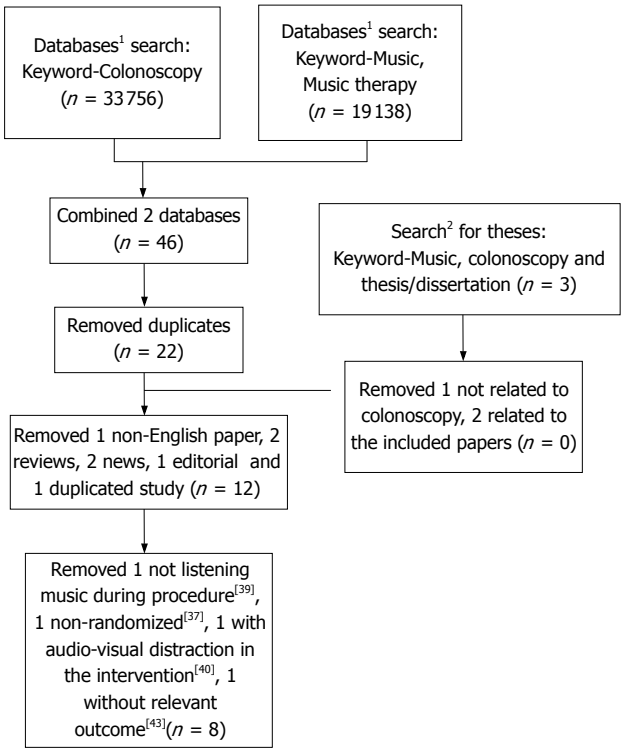
Statistical analysis

Random-effect models were used to combine the outcome effect, i.e. the (standardized) difference between treated and control groups, for each study[27]. Heterogeneity was examined using the Q-test and I^2[27]. All analyses were conducted using RevMan[27]. Publication bias was examined by funnel plot and Egger's regression[28].

RESULTS

Search strategies

The electronic search was conducted in May 2007 and 19 records were found. The title and abstracts of these papers were examined and it was found that one was written in Portuguese[29], one was a systematic review focus on pain[30], one was a discussion paper on the use of sedation[31], one was an editorial[32], and two were news[33,34]. One paper[35] occurred twice in our search as the surname of the first author was recorded differently in two electronic databases (EBASE and MEDLINE), so one was removed. Hence, only 12 studies[35-46] were included and the full articles were obtained for further examination. A study[37] was found to be a non-randomized study



[1]Databases included CINAHL (1982 to 2007 March), Cochrane Central Register of Controlled trials (up to 1st quarter of 2007), EMBASE (1980 to 2007 March) and MEDLINE (1966 to 2006 January); [2]Search in ProQuest Dissertations and Theses database (1861 to 2007) and through search engine yahoo and google (only the first 20 pages of results were checked for detail).

Figure 1 Flowchart of the search history.

and was excluded and another study[43] not examining the procedure time nor the amount of sedation was also excluded. One study[39] required their subjects in the intervention group to listen to music before the colonoscopy procedure on a voluntary basis. The author was contacted to see how many subjects in the intervention group listened to music during the procedure. Although the author implied that most of the subjects continued to listen to music during the procedure, the study was excluded because not all participants in the intervention group listened to music during the procedure. Another study[40] provided audiovisual distraction to patients instead on listening to music alone and the effect may have been enhanced; therefore, it was also excluded from the analysis.

As to the search of potential theses or dissertations, one doctoral dissertation[47] and two master theses[48,49] were identified and their abstracts were obtained. However, one[48] was not related to colonoscopy and the others[47,49] related to the papers from previous search[40,45]. A flowchart is provided to show the search history (Figure 1).

A total of 722 subjects were involved in 8 studies (Table 1), four from Europe[35,36,42,44], two from the US[36,45], and three from Asia[38,41,46]. Two studies[38,45] provided different types of music for their patients to choose from. Four studies broadcasted the music through headphones/earphones[35,38,41,45], three broadcasted as background music[36,42,44], and one did not specify the media method[46]. Seven studies mentioned that all colonoscopy procedures were conducted by an experienced colonoscopist[42,46] or a group of experienced colonoscopists[35,36,38,41] for both comparison groups.

Quality of studies

All of the included studies were randomized controlled studies and only five of them reported the method of randomization[35,36,38,41,42]. No information on attrition was reported in any study probably because most of the procedures were completed in a relatively short period of time. Incomplete procedures were reported in two studies but no significant incomplete rates were detected[35,41]. Although it would be impossible to blind the subjects, outcomes assessor was blinded in two studies[35,38] by requesting the patients in the control group to take the earphones. Six studies[35,36,41,42,45,46] showed that baseline characteristics between control and treatment groups were comparable. One study did not provide the test statistics[44] and one did not report the baseline characteristics[38] (Table 2). Overall, five studies[35,36,38,41,42] got 2 points (half) or above in our modified Jadad's scale.

Procedure time (Figure 2)

The total time taken for the procedure between music and control groups was measured in seven studies[35,36,38,41,44-46]. Six of them[36,38,41,44-46] showed a reduction of time in the music group but only in one case was this significant[44]. Since two studies[36,38] did not provide the means or standard deviations, the corresponding authors of the studies were contacted to obtain this information. Authors from both studies[36,38] generously provided the means and standard deviations of the respective parameters and therefore their results could be included in the analysis. The combined mean difference between the treated and control groups was -2.84 with 95% CI (-5.61 to -0.08). The Q-value and the I^2 were 13.46 ($P = 0.04$) and 55%, suggesting possible heterogeneity among the studies. Publication bias was not detected using Egger's regression method ($P = 0.9133$).

Sedation (Figure 2)

Six studies[36,38,41,42,44,45] examined the use of sedation, i.e. midazolam in mg, and four[38,41,42,45] showed a reduction in the music group. Sedation was given or added based on patients' request[38,41,44] or colonoscopists' decision[36,42]. The means and standard deviations of the 2 studies[36,38] were requested from the authors. The combined mean difference for the six trials was -0.46 with 95% CI (-0.91 to -0.01), showing a marginally significant reduction of the use of sedation in the

Table 1 Characteristics of each of the included studies

Study	Design	Baseline characteristic	Comparison group	Outcome
Andrada *et al* (2004)[35]	RCT	Intervention- 31 males 32 females, mean age: 46 (14.22) Control- 28 males 27 females, mean age: 49 (13.88)	Intervention- Listening music until the end of procedure (classical tracts) Control- Usual colonoscopy screening	Blood pressure, capillary and oxygen saturation, heart rate level of anxiety
Bechtold *et al* (2006)[36]	RCT	Intervention- 41 males and 44 females, age: 58.5 Control- 42 males 39 females, age: 54.1	Intervention- Playing music upon the entrance the patient (same music for all patients) Control- 50 mg of meperidine and 1 or 2 mg of midazolam	Dose consumed, time to time, reach cecum, total procedure insertion difficulty scale, experience scale, pain scale, want music next time
Harikumar *et al* (2006)[38]	RCT	Intervention- 38 (male + female), age 15 - 60 Control- 40 (male + female), age 15-60	Intervention- Listening music (popular film songs based on classical rages, classical music, devotional songs, folk songs, soft instrumental music, and bioacoustics Control- 2 mg intravenous boluses of midazolam on demand; Colonoscopic procedure performed by endoscopists with experience of performing at least 200 full-length colonoscopic procedure	Pain score, discomfort score, procedure time, recovery time, dose consumed
Lee *et al* (2002)[41]	RCT	Intervention 1- 33 male 22 female Inter-quartile age range: 46-68 mean age: 54 Intervention 2- 29 male 26 female, Inter-quartile age range: 39-67 mean age: 47 Intervention 3- 27 males 28 females, Inter-quartile age range: 40-65 mean age: 51	Intervention 1- Patient-controlled sedation Intervention 2- Patient-controlled sedation+ music1 Intervention 3- Music1 Could request intravenous administration of diazemuls (0.1 mg/kg) and meperidine (0.5 mg/kg) Nasal oxygen (2 L/min) to all patients in the study 1Type of music: classical, jazz, popular (Chinese and English) and Chinese opera Examination performed endoscopists having more than 300 similar procedures before	Dose of propofol, pain score, satisfactory score, willingness to repeat the same mode of sedation.
Ovayolu *et al* (2006)[42]	RCT	Intervention- 14 males 16 females, age: 20-39 (n = 10), 40-59 (n = 11), 60 or above (n = 9) Control- 14 males 16 females, age: 20-39 (n = 5), 40-59 (n = 11), 60 or above (n = 14)	Intervention- Listening music (classical Turkish music, a slow and relaxing music) Control- Usual colonoscopy screening; procedure performed by an expert endoscopist with experience of performing at least 200 full-length procedure previously	Dose of sedation consumed, anxiety score, pain score, VAS score, willingness to the procedure score and satisfaction score.
Schimann *et al* (2002)[44]	RCT	Intervention- 25 males 34 females, mean age: 52.3 (13.9), Control- 33 males 27 females, mean age: 55.8 (13.5)	Intervention- Music from radio Control- Conventional procedure	Request of sedation (midazolam), oxygen supplement, procedure time
Smolen *et al* (2002)[45]	RCT	Intervention- 10 male 6 females, mean age: 58.83 (13.64) Control- 7 males 9 females, mean age: 61.06 (9.48)	Intervention- Listening music (classical, jazz, pop rock and easy listening) Control- Undergo standard colonoscopy procedure including explanation of the procedure by the nurse; receive a standard pre-procedure sedation consisting of a slow intravenous injection of 1 mg of midazolam in combination with 50 mg of meperidine	Sedation, anxiety and heart rate
Uedo *et al* (2004)[46]	RCT	Intervention- 7 males 7 females, mean age: 54 (6) Control- 11 males 4 females, mean age: 54 (8)	Intervention- Listen the music (easy listening style) from the beginning and during procedure Control- Usual procedure for undergoing colonoscopy, no anxiolytic medications, no antisecretory agents	Salivart cortisol levels

Table 2 Quality assessment by Jadad's scale and baseline comparison

Study (year of publication)	Is the study randomized?	Is the procedure appropriate & reported?	Is the study blind to the assessors?	Is the blinding method appropriate & reported?	Were the groups similar at baseline?
Andrada *et al* (2004)[35]	C	C	C	C	C
Bechtold *et al* (2006)[36]	C	C	N	NA	C
Harikumar *et al* (2006)[38]	C	C	C	C	P[1]
Lee *et al* (2002)[41]	C	P	C	N	C
Ovayolu *et al* (2006)[42]	C	C	N	NA	C
Schiemann *et al* (2002)[44]	C	N	N	NA	P[2]
Smolen *et al* (2002)[45]	C	N	N	NA	C
Uedo *et al* (2004)[46]	C	N	N	NA	C

Remark: C, correct; N, not correct; P, partly correct; NA, not applicable. [1]No result provided; [2]No test result provided.

music group. The Q-test and I^2 were respectively 34.83 ($P < 0.001$) and 86% suggesting strong heterogeneity. Publication bias was not detected using Egger's regression method ($P = 0.1150$).

DISCUSSION

Colon cancer is the fourth leading cause of death among all cancers[1] but remains one of the most preventable and curable cancers if detected early[2]. Screening for colon cancer has been shown to be an effective method of reducing the risk of mortality, but the compliance rate is still low probably due to the unpleasant feeling of patients during the procedure[9]. Non-pharmacological methods for alleviating patients discomfort and anxiety have been developed and, in the early 1990s, Palakanis[6] demonstrated that listening to music before and during sigmoidoscopy was effective in reducing one's anxiety. Colonoscopy has been the recommended procedure for screening colon cancer[4] and more studies have been conducted in examining the effect of listening to music during this procedure.

Our results show that listening to music during the colonoscopy would effectively reduce the mean procedure time and the amount of sedation used. One possible explanation for the reduction of sedation is that patients in the music group are more relaxed and with less anxiety. Therefore, the physician can complete the procedure in a shorter period of time and use less sedation[45]. The reduction of procedure time implies a reduction of the anxious, frightening, and unpleasant time spent while undergoing the procedure and may be useful in enhancing the compliance rate.

It was reported that conscious sedation with midazolam contributed to the occurrence of cardiovascular events during colonoscopy[12] and was associated with the risk of cardio-respiratory complication[14]. Avoidance of sedation may provide a quicker patient discharge, less need for monitoring, and overall cost savings[50]. Our results also found a significant reduction in anxiety score, but only weak evidence was observed for pain score, blood pressure, and mean recovery time.

Besides the above-mentioned beneficial effects to patients, two advantages of listening to music during colonoscopy are cheapness and ease of implementation[51]. Although cassette players and compact disc players were used in most of the included studies, digital players, like MP3 players, may be a better choice in the future[52]. With advanced technology, a thumb-sized MP3 player can store hundreds of songs at a much lower cost. Therefore, more choices can be given to patients, which is important as personal preference has a strong impact on one's responses to music[53].

No harmful effects from listening to music were reported in any study in the meta-analysis and other references that we read. Only one shortcoming about patients listening to music through headphone/earphone was the isolation of verbal communication between patients and the medical staff during the procedure. However, broadcasting the music as background music might disturb the staff conducting the procedure probably because an imposed choice of musical selection can be annoying to the listener[53].

Recently, a meta-analysis was published on a similar topic[23] but there are several differences between that study and the present one. First of all, colonoscopy was the focus of this paper. Second, this study's search strategy was more comprehensive, meaning that more databases were included and theses/dissertations were also identified. Third, besides the numerically combined results, the characteristics of all included studies were presented and discussed in the text or in the table.

Although our findings confirm the effectiveness of listening to music during the colonoscopy procedure, several areas are worth further investigation. These include the choice of music, the mode of broadcasting music (earphone, background, or both), the possibility of using placebo to the patients in the control group, the possibility of blinding to the colonoscopist/s or medical staff involved in the procedure, the interaction of the medical staff to background music as well as the effect of playing audiovisual materials. Finally, it was suggested that the role of music should be considered whenever applicable[54].

Our results confirm that patient listening to music during colonoscopy is an effective way in reducing

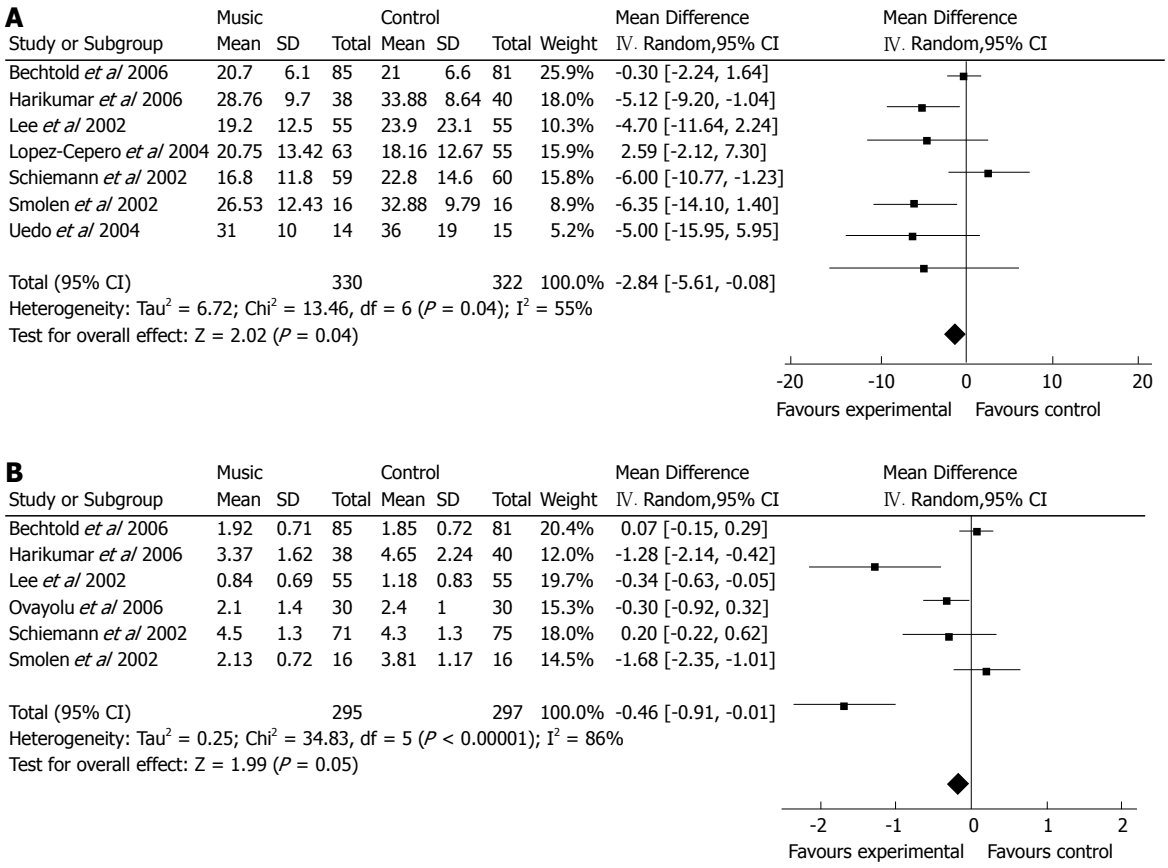


Figure 2 **A**: Forrest plot of the effect of music in procedure time; **B**: Forrest plot of the effect of music in sedation.

procedure time, anxiety, and the amount of sedation. More importantly, no harmful effects were observed for all the studies. Therefore, listening to music during colonoscopy should be recommended.

ACKNOWLEDGMENTS

The authors would like to thank Prof. Mattew Bechtold, Prof. Nair Harikumar, Prof. Ann Hayes and Prof. John Marshall for providing the information in their studies.

COMMENTS

Background

Colonoscopy is the recommended method for screening colon cancer but many people are unwilling to receive it because of fearing the pain, anxiety and other reasons. The use of music in medical research has long history and the beneficial effects of listening music during colonoscopy have been widely reported.

Research frontiers

A meta-analysis was conducted to integrate results from different studies in examining the effectiveness of music in reducing the procedure time and the amount of sedation during colonoscopy.

Innovations and breakthroughs

Our results show that listening to music during colonoscopy may effectively reduce the mean procedure time and the amount of sedation used.

Applications

Listening to music during colonoscopy should be promoted because of its beneficial effect and negligible cost.

Peer review

This is a research on a kind of a complementary and alternative medicine, a "music therapy".

REFERENCES

1 **WHO**. Facts sheets, Cancer, WHO 2006. Available from: http://www.who.int/mediacentre/factsheets/fs297/en/index.html
2 **Winawer SJ**, Fletcher RH, Miller L, Godlee F, Stolar MH, Mulrow CD, Woolf SH, Glick SN, Ganiats TG, Bond JH, Rosen L, Zapka JG, Olsen SJ, Giardiello FM, Sisk JE, Van Antwerp R, Brown-Davis C, Marciniak DA, Mayer RJ. Colorectal cancer screening: clinical guidelines and rationale. *Gastroenterology* 1997; **112**: 594-642
3 **Selby JV**, Friedman GD, Quesenberry CP Jr, Weiss NS. A case-control study of screening sigmoidoscopy and mortality from colorectal cancer. *N Engl J Med* 1992; **326**: 653-657
4 **Davila RE**, Rajan E, Baron TH, Adler DG, Egan JV, Faigel DO, Gan SI, Hirota WK, Leighton JA, Lichtenstein D, Qureshi WA, Shen B, Zuckerman MJ, VanGuilder T, Fanelli RD. ASGE guideline: colorectal cancer screening and surveillance. *Gastrointest Endosc* 2006; **63**: 546-557
5 **Waye JD**, Rex DK, Williams CB. Colonoscopy: Principles and practice. 1st ed. Malden, Mass. Oxford: Blackwell Sci Pub, 2003: 655
6 **Palakanis KC**, DeNobile JW, Sweeney WB, Blankenship CL. Effect of music therapy on state anxiety in patients undergoing flexible sigmoidoscopy. *Dis Colon Rectum* 1994; **37**: 478-481
7 **Salmon P**, Shah R, Berg S, Williams C. Evaluating customer satisfaction with colonoscopy. *Endoscopy* 1994;

26: 342-346
8 **Salmore RG**, Nelson JP. The effect of preprocedure teaching, relaxation instruction, and music on anxiety as measured by blood pressures in an outpatient gastrointestinal endoscopy laboratory. *Gastroenterol Nurs* 2000; **23**: 102-110
9 **Kim LS**, Koch J, Yee J, Halvorsen R, Cello JP, Rockey DC. Comparison of patients' experiences during imaging tests of the colon. *Gastrointest Endosc* 2001; **54**: 67-74
10 **Balsells F**, Wyllie R, Kay M, Steffen R. Use of conscious sedation for lower and upper gastrointestinal endoscopic examinations in children, adolescents, and young adults: a twelve-year review. *Gastrointest Endosc* 1997; **45**: 375-380
11 **Fisher NC**, Bailey S, Gibson JA. A prospective, randomized controlled trial of sedation vs. no sedation in outpatient diagnostic upper gastrointestinal endoscopy. *Endoscopy* 1998; **30**: 21-24
12 **Ristikankare M**, Julkunen R, Laitinen T, Wang SX, Heikkinen M, Janatuinen E, Hartikainen J. Effect of conscious sedation on cardiac autonomic regulation during colonoscopy. *Scand J Gastroenterol* 2000; **35**: 990-996
13 **Bell GD**. Premedication, preparation, and surveillance. *Endoscopy* 2002; **34**: 2-12
14 **Yuno K**, Iishi H, Tatsuta M, Hifumi K, Omori M. Intravenous midazolam as a sedative for colonoscopy: a randomized, double-blind clinical trial. *Aliment Pharmacol Ther* 1996; **10**: 981-984
15 **Rorke MA**. Music therapy in the age of enlightenment. *J Music Ther* 2001; **38**: 66-73
16 **West M**. Music Therapy in Antiquity. Horden P, editor. Music as medicine: the history of music therapy since antiquity. Aldershot; Brookfield, Vt: Ashgate, 2000: 51-68
17 **Ni MS**. The Yellow Emperor's Classic of internal medicine: A new translation of the Neijing Suwen with commentary. Boston: Shambhala Pub, 1995: 14-17
18 **Katz JB**. Music Therapy: Some Possibilities in the Indian Tradition. Horden P, editor. Music as medicine: the history of music therapy since antiquity. Aldershot; Brookfield, Vt: Ashgate, 2000: 84-102
19 **White JM**. Music therapy: an intervention to reduce anxiety in the myocardial infarction patient. *Clin Nurse Spec* 1992; **6**: 58-63
20 **Updike P**. Music therapy results for ICU patients. *Dimens Crit Care Nurs* 1990; **9**: 39-45
21 **Fullhart JW**. Preparatory information and anxiety before sigmoidoscopy: a comparative study. *Gastroenterol Nurs* 1992; **14**: 286-290
22 **Bampton P**, Draper B. Effect of relaxation music on patient tolerance of gastrointestinal endoscopic procedures. *J Clin Gastroenterol* 1997; **25**: 343-345
23 **Rudin D**, Kiss A, Wetz RV, Sottile VM. Music in the endoscopy suite: a meta-analysis of randomized controlled studies. *Endoscopy* 2007; **39**: 507-510
24 **van Tulder M**, Furlan A, Bombardier C, Bouter L. Updated method guidelines for systematic reviews in the cochrane collaboration back review group. *Spine* 2003; **28**: 1290-1299
25 **Jadad AR**, Moore RA, Carroll D, Jenkinson C, Reynolds DJ, Gavaghan DJ, McQuay HJ. Assessing the quality of reports of randomized clinical trials: is blinding necessary? *Control Clin Trials* 1996; **17**: 1-12
26 **Monninkhof E**, van der Valk P, van der Palen J, van Herwaarden C, Partridge MR, Zielhuis G. Self-management education for patients with chronic obstructive pulmonary disease: a systematic review. *Thorax* 2003; **58**: 394-398
27 **Review Manager** (RevMan) [Computer program] Version 5.0. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2008
28 **Egger M**, Davey Smith G, Schneider M, Minder C. Bias in meta-analysis detected by a simple, graphical test. *BMJ* 1997; **315**: 629-634
29 **Arruda Alves PR**, Roncaratti E, Habr Gama A. Reduction in salivary levels of cortisol by music therapy during colonoscopy. *GED-Gastrenterologia Endoscopia Digestiva* 2004; **23**: 83-84
30 **Cepeda MS**, Carr DB, Lau J, Alvarez H. Music for pain relief. *Cochrane Database Syst Rev* 2006; CD004843
31 **Lazzaroni M**, Bianchi Porro G. Preparation, premedication, and surveillance. *Endoscopy* 2005; **37**: 101-109
32 **Lotufo PA**. The noise stops you from hearing good music: the possibilities for a mortality reduction program for cancer of the colon and rectum in Sao Paulo. *Sao Paulo Med J* 2003; **121**: 95-96
33 **Kurth T**, Krevvsky B, Chiu C. Late-breaking news. *Bottom Line Health* 2007; **21**: 1
34 **Anonymous**. Tuned up for colon test. *Nur Standard* 2006; **21**: 8
35 **Lopez-Cepero Andrada JM**, Amaya Vidal A, Castro Aguilar-Tablada T, Garcia Reina I, Silva L, Ruiz Guinaldo A, Larrauri De la Rosa J, Herrero Cibaja I, Ferre Alamo A, Benitez Roldan A. Anxiety during the performance of colonoscopies: modification using music therapy. *Eur J Gastroenterol Hepatol* 2004; **16**: 1381-1386
36 **Bechtold ML**, Perez RA, Puli SR, Marshall JB. Effect of music on patients undergoing outpatient colonoscopy. *World J Gastroenterol* 2006; **12**: 7309-7312
37 **Binek J**, Sagmeister M, Borovicka J, Knierim M, Magdeburg B, Meyenberger C. Perception of gastrointestinal endoscopy by patients and examiners with and without background music. *Digestion* 2003; **68**: 5-8
38 **Harikumar R**, Raj M, Paul A, Harish K, Kumar SK, Sandesh K, Asharaf S, Thomas V. Listening to music decreases need for sedative medication during colonoscopy: a randomized, controlled trial. *Indian J Gastroenterol* 2006; **25**: 3-5
39 **Hayes A**, Buffum M, Lanier E, Rodahl E, Sasso C. A music intervention to reduce anxiety prior to gastrointestinal procedures. *Gastroenterol Nurs* 2003; **26**: 145-149
40 **Lee DW**, Chan AC, Wong SK, Fung TM, Li AC, Chan SK, Mui LM, Ng EK, Chung SC. Can visual distraction decrease the dose of patient-controlled sedation required during colonoscopy? A prospective randomized controlled trial. *Endoscopy* 2004; **36**: 197-201
41 **Lee DW**, Chan KW, Poon CM, Ko CW, Chan KH, Sin KS, Sze TS, Chan AC. Relaxation music decreases the dose of patient-controlled sedation during colonoscopy: a prospective randomized controlled trial. *Gastrointest Endosc* 2002; **55**: 33-36
42 **Ovayolu N**, Ucan O, Pehlivan S, Pehlivan Y, Buyukhatipoglu H, Savas MC, Gulsen MT. Listening to Turkish classical music decreases patients' anxiety, pain, dissatisfaction and the dose of sedative and analgesic drugs during colonoscopy: a prospective randomized controlled trial. *World J Gastroenterol* 2006; **12**: 7532-7536
43 **Salmore RG**, Nelson JP. The effect of preprocedure teaching, relaxation instruction, and music on anxiety as measured by blood pressures in an outpatient gastrointestinal endoscopy laboratory. *Gastroenterol Nurs* 2000; **23**: 102-110
44 **Schiemann U**, Gross M, Reuter R, Kellner H. Improved procedure of colonoscopy under accompanying music therapy. *Eur J Med Res* 2002; **7**: 131-134
45 **Smolen D**, Topp R, Singer L. The effect of self-selected music during colonoscopy on anxiety, heart rate, and blood pressure. *Appl Nurs Res* 2002; **15**: 126-136
46 **Uedo N**, Ishikawa H, Morimoto K, Ishihara R, Narahara H, Akedo I, Ioka T, Kaji I, Fukuda S. Reduction in salivary cortisol level by music therapy during colonoscopic examination. *Hepatogastroenterology* 2004;

51: 451-453
47 **Lee DWH**. The use of patient-controlled and adjunct sedation for colonoscopy. Thesis. Chinese University of Hong Kong 2005
48 **Parker DB**. The effect of Music Therapy for pain and anxiety versus literature on the immediate and future perceptions of cardiac patients. Thesis. The Florida State University, 2004
49 **Smolen D**, Topp R, Singer L. The effect of self-selected music during colonoscopy on anxiety, heart rate, and blood pressure. *Appl Nurs Res* 2002; **15**: 126-136
50 **Takahashi Y**, Tanaka H, Kinjo M, Sakumoto K. Sedation-free colonoscopy. *Dis Colon Rectum* 2005; **48**: 855-859
51 **Kemper KJ**, Danhauer SC. Music as therapy. *South Med J* 2005; **98**: 282-288
52 **Maag M**. Podcasting and MP3 players: emerging education technologies. *Comput Inform Nurs* 2006; **24**: 9-13
53 **Bonny HL**. Music and healing. *Music Ther* 1986; **6**A: 3-12.
54 **Thaut MH**. The future of music in therapy and medicine. *Ann N Y Acad Sci* 2005; **1060**: 303-308

S- Editor Zhong XY **L- Editor** Negro F **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5344

World J Gastroenterol 2008 September 14; 14(34): 5344-5348
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Effect of mutant p27^{kip1} gene on human cholangiocarcinoma cell line, QBC$_{939}$

Jian Luo, Yong-Jun Chen, Wei-Yu Wang, Sheng-Quan Zou

Jian Luo, Yong-Jun Chen, Wei-Yu Wang, Sheng-Quan Zou, Department of General Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China
Author contributions: Luo J, Chen YJ, Wang WY and Zou SQ designed the research; Luo J and Chen YJ performed the research; Wang WY contributed to the new reagents/analytic tools; Wang WY analyzed the data; Luo J wrote the paper.
Supported by The National High Technology Research and Development Program of China (863 Program), No. Z2002AA214061
Correspondence to: Sheng-Quan Zou, Department of General Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China. sqzou@tjh.tjmu.edu.cn
Telephone: +86-27-83662398 Fax: +86-27-83662851
Received: December 16, 2007 Revised: August 8, 2008
Accepted: August 15, 2008
Published online: September 14, 2008

Abstract

AIM: To investigate the effects of exogenously mutated p27^{kip1} (p27) on proliferation and apoptosis of human cholangiocarcinoma cell line, QBC$_{939}$ *in vivo*.

METHODS: Adenoviral vectors were used to transfect mutated p27 cDNA into human QBC$_{939}$ cell line. Expression of *p27* was detected by RT-PCR. Western blot. Cell growth, morphological change, cell cycle, apoptosis and cloning formation were determined by MTT assay and flow cytometry.

RESULTS: The expression of p27 protein and mRNA was increased significantly in QBC$_{939}$ cell line transfected with Ad-p27mt. The transfer of Ad-p27mt could significantly inhibit the growth of QBC$_{939}$ cells, decrease the cloning formation rate and induce apoptosis. *p27* over expression caused cell cycle arrest at G_0/G_1 phase 72 h after infection with Ad-p27mt.

CONCLUSION: *p27* may cause cell cycle arrest at G_0/G_1 phase and subsequently lead to apoptosis. Recombinant adenovirus expressing mutant p27 may be potentially useful in gene therapy for cholangiocarcinoma.

Key words: Adenovirus; Cholangiocarcinoma; Gene therapy; Cell cycle; Apoptosis

Peer reviewer: Gustav Paumgartner, Professor, University of Munich, Klinikum Grosshadern, Marchioninistr. 15, Munich, D-81377, Germany

Luo J, Chen YJ, Wang WY, Zou SQ. Effect of mutant p27^{kip1} gene on human cholangiocarcinoma cell line, QBC$_{939}$. *World J Gastroenterol* 2008; 14(34): 5344-5348 Available from: URL: http://www.wjgnet.com/1007-9327/14/5344.asp DOI: http://dx.doi.org/10.3748/wjg.14.5344

INTRODUCTION

It is well known that cell cycle progression is governed by cyclin-dependent kinases (CDKs). P27^{kip1} (p27), a key inhibitor of CDKs, can directly inhibit the entry of cell cycle from G_1 phase into S phase. A major mechanism underlying the regulation of p27 is proteolysis by the ubiquitin–proteasome pathway. Phosphorylation of p27 on threonine 187 (T187) by Cdk2 creates a binding site for a Skp2-containing E3 ubiquitin-protein ligase, SCF. Ubiquitylation of p27 by SCF results in degradation of p27 by the proteasome.

In this study, a replication-deficient adenovirus vector encoding a mutated p27 at the Thr-187/pyrophosphorylation site was constructed and transfected into the cultured human cholangiocarcinoma cell line QBC$_{939}$, in order to investigate the effects of adenovirus-mediated p27 on proliferation and apoptosis of cholangiocarcinoma cells.

MATERIALS AND METHODS

Materials

Human cholangiocarcinoma cell line QBC$_{939}$ was kindly donated by Professor Wang Shu-Guang of the Hepatobiliary Department of Xinan Hospital, Third Military Medical University. Tetramethyl-azo-zole-cyan (MTT) and iodized-dine (PI) were purchased from Sigma Ltd. Human-source rat anti-p27 monoclonal antibody was purchased from Beijing Zhongshan Ltd. Sense and anti-sense primers of p27 were synthesized by Shanghai Sangon Bioengineering and Technology Service Co. Ltd. Recombinant adenovirus vehicle Ad-p27mt and adenovirus control vehicle Ad-LacZ were kindly donated by Professor Xu Shao-Yong at Digestive Medical Department and Doctor Wang Jia-Ning at

Cardiovascular Department, Yunyang Medical College. CO_2 gas incubator(Binder, Germany), inverted phase contrast microscope (Olympus, Japan), FACsort flow cytometry (USA BD Ltd.) were used in this study. Cells used in experiments were divided into control group (QBC_{939} group), Ad-LacZ group and Ad-p27mt group.

Cell culture and transfection

Human cholangiocarcinoma cell line QBC_{939} was incubated in 10% FCS-containing RPMI 1640 culture medium at 37℃ at an atmosphere containing 50mL/L CO_2, and infected with Ad-LacZ or with Ad-p27mtatant at multiplicity of infection (MOI) of 50 as the density reached to 40%-50%.

Transduction efficacy of recombinant adenovirus

Ad-LacZ was used to infect QBC_{939} cholangiocarcinoma cells when the MOI was set at 25, 50, 100 and 200. X-gal staining was performed after 48 h culture. Blue-staining cells were counted and the percentage was calculated to confirm recombinant adenovirus infection efficacy. Results demonstrated that as MOI ⩾ 50, recombinant adenovirus was able to implement an approximately 100% transduction efficacy rate on the two types of cells.

Cell growth inhibition test (MTT assay)

Cells (4000-6000 cells/well) were inoculated in 96-well plates. The culture fluid was discarded after 48h of grouping, and 150 μL /well (0.5 mg/mL) MTT solution was supplemented at 37℃ for 4 h followed by 150 μL/well DMSO, and shaken for 10 min. Absorbance (A) value was detected with an autokinetic enzyme scaling meter at 492 nm wavelength. Cell growth suppressive rate = (1-A value of experimental group/A value of the same titre QBC_{939} group) × 100%.

Clone formation

The cells infected with Ad-LacZ or with Ad-p27mutant at a MOI of 50 were transferred into a 12-well plate (500 cells/well) in triplicate and cultured for 3, 6, 9, and 12 d, respectively, then fixed with methanol and stained with 0.4% crystal violet. Clones containing at least 50 cells were counted under inverse microscope. Clone formation ratio (%) = cell clone amounts/500 × 100%.

Extraction of total RNA and RT-PCR

Total cellular RNA was extracted from QBC_{939} cells transfected with Ad-p27mt and Ad-LacZ for 48 h using the Trizol method. PCR was performed after reverse transcription. The sequences of P27mt gene are upstream primer: 5'-CCTAGAGGGCAAGTACGAG TG-3', downstream primer: 5'-GAAGAATCGTCGG TTGCAGGTCGCT-3'. Reaction parameters were pre-degenerated at 95℃ for 5 min, degenerated at 94℃ for 30 s, 39 cycles of annealing at 56.3℃ for 35 s, extension at 72℃ for 35 s, a final extension at 72℃ for 10 min. Electrophoresis was performed for the PCR products on a 2% agarose gel.

Western blot

Cellular protein disposed for 72 h was extracted with the same method as described above. The proteins electro-transferred onto nitrocellulose membranes and blocked by confining liquid were bound to p27 monoclonal antibody and secondary antibody, and colored by enhanced chemiluminescence (ECL).

Table 1 In different testing groups QBC_{939} cells

Group	Cell clone count (ratio, cell clone amounts/500)			
	3 d after transfect	6 d after transfect	9 d after transfect	12 d after transfect
Ad-p27mt group	9 (1.8)	14 (2.8)	15 (3.0)	21 (4.2)
Ad-LacZ group (n = 3)	16 (3.2)	27 (5.4)	49 (9.8)	56 (11.2)
QBC_{939} group (n = 3)	18 (3.6)	31 (6.2)	46 (9.2)	62 (12.4)

F = 10.361, P = 0.011 (cell clone ratio, Ad-p27m *vs* AdLacZ group).

Cell cycle and apoptosis counting analysis by flow cytometry

Cells of each group (above 10^6 cells in each group) were harvested at different time points. RNA enzyme was added at 37℃ and reacted for 1 h after cells were fixed in 70% alcohol at 4℃ for 24 h (final concentration 50 μg/mL). After 20-30 min of PI solution (concentration 100 μg/mL) staining, cells were counted by monochromatic fluorescence flow cytometry to observe the apoptosis rate.

Statistical analysis

All data were expressed as mean ± SD. The data were analyzed with SPSS 10.0 software. Variance analysis SNK method was employed in comparison of multi-groups. $P < 0.05$ was considered statistically significant.

RESULTS

Titre of recombinant adenovirus

Ultraviolet spectrophotometry showed that the titre of recombinant adenovirus after multiplication, amplification, and purification was up to 7.95×10^{12} CFU/mL.

Transduction efficacy of recombinant adenovirus

Ad-LacZ was used to infect QBC_{939} cholangiocarcinoma cells. The multiplicity of infection (MOI) was 25, 50, 100 and 200. X-gal staining was performed after 48 h culture. Blue-staining cells were counted and the percentage was calculated to confirm the recombinant adenovirus infection efficacy. Results demonstrated that as MOI ⩾ 50, recombinant adenovirus was able to implement an approximately transduction efficacy rate of 100% in the two types of cells.

Growth suppression of QBC_{939} cells by introduction of mutated p27 gene

Clone formation test: The number and ratio of cellular clones in different groups are shown in Table 1 and Figure 1. The transfer of Ad-p27mt significantly inhibited the growth of QBC_{939} cells, decreased the clone formation, which was significantly different from the Ad-LacZ-infected and uninfected groups (F = 10.361, P = 0.011) with no statistical difference.

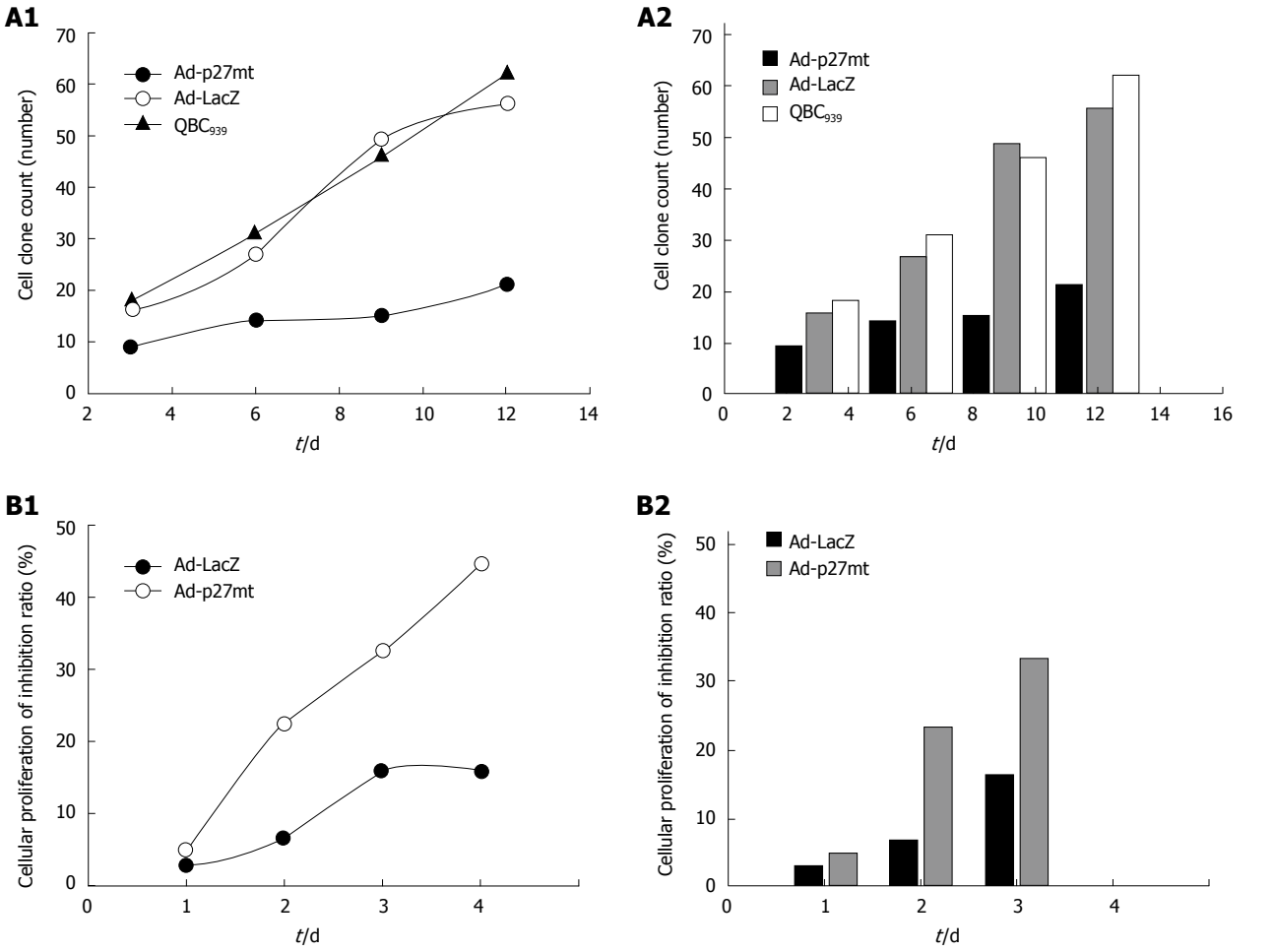


Figure 1 Clone formation test and MTT assay showing effect of Ad-p27mt and Ad-LacZ on growth curve of QBC_{939} cells (**A1**, **B1**) and on the proliferation of QBC_{939} cells (**A2**, **B2**).

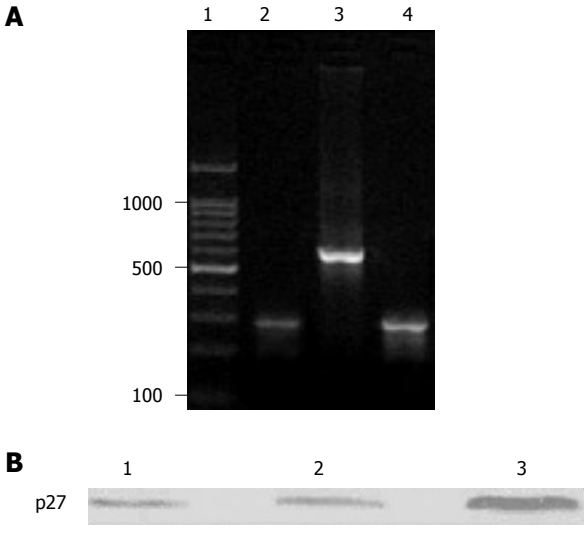


Figure 2 *p27* expression at mRNA (**A**) and protein level (**B**). Lane 1: Marker; Lane 2: QBC_{939}; Lane 3: β-actin; Lane 4: QBC_{939}/Ad-p27mt in Figure 2A; Lane 1: QBC_{939}; Lane 2: QBC_{939}/Ad-LacZ; Lane 3: QBC_{939}/Ad-p27mt in Figure 2B.

MTT assay for cell growth and viability: MTT assays also indicated that the proliferation of QBC_{939} cells was significantly inhibited after Adp27 infection, with its inhibitory effect peaked at 72 h. The transfer of Ad-p27mt could significantly inhibit the growth of QBC_{939} cells and decrease clone formation. After 24, 48 and 72 h of Adp27 infection, the average CD value was remarkably lower in Adp27-infected group than in Ad LacZ-infected and uninfected groups, revealing that introduction of exogenously mutated p27 gene *via* a recombinant adenovirus vector could significantly suppress the growth of QBC_{939} cells in a non time-dependent manner within 72 h.

Expression of p27 in cholangiocarcinoma QBC_{939} cells at mRNA level

Gel electrophoresis for the RT- PCR products displayed that the expression of *p27* was decreased in normal control group, but the expression of QBC_{939} cells was significantly elevated with a distinct 275bp objective gene strap (Figure 2A).

Expression of p27 in cholangiocarcinoma QBC_{939} cells at protein level

The expression of *p27* was significantly increased in Ad-p27mt-tranfected QBC_{939} cells. However, the expression of *p27* could be detected in a small number of Ad-LacZ-tranfected QBC_{939} cells (endogenous) and in the control group (Figure 2B).

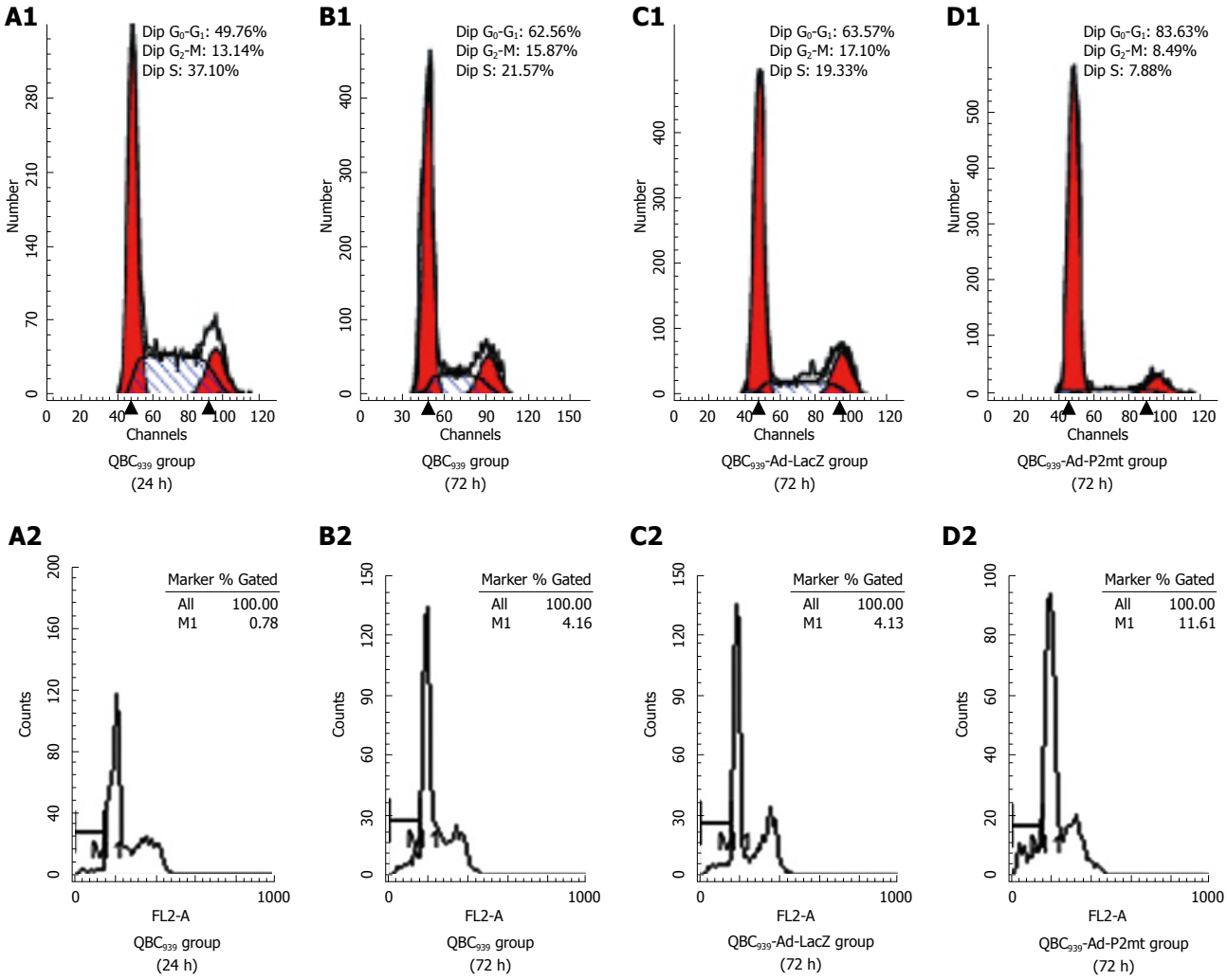


Figure 3 Impact of exogenous *p27* gene on QBC_{939} cell cycle and apoptosis.

Table 2 Influence of gene transfect on QBC_{939} cells at G_0/G_1 phase and apoptosis in different testing groups (mean ± SD)

Group	G_0/G_1 (%)/apoptosis (%)		
	24 h after transfect	48 h after transfect	72 h after transfect
Ad-p27mt group (n = 3)	61.02 ± 1.03/ 0.81 ± 0.052	73.32 ± 2.99/ 5.27 ± 0.030	83.63 ± 2.10/ 11.61 ± 1.23
Ad-LacZgroup (n = 3)	54.91 ± 2.32/ 0.76 ± 0.031	62.56 ± 2.71/ 1.28 ± 0.043	63.57 ± 2.32/ 4.16 ± 0.230
QBC_{939} group (n = 3)	49.76 ± 1.97/ 0.78 ± 0.041	56.95 ± 1.06/ 1.10 ± 0.071	62.56 ± 2.88/ 4.13 ± 0.454

F = 15.954, P = 0.012 (G_0/G_1 cell ratio, Ad-p27m *vs* AdLacZ group); F = 3.236, P > 0.05 (apoptosis rates, Ad-p27m *vs* AdLacZ group).

Impact of exogenous p27 gene on QBC_{939} cell cycle and apoptosis

A high expression level of exogenous p27 protein in QBC_{939} cells evoked a strong cell cycle arrest at G_0/G_1 phase in a time-dependent manner within 72 h. The cell ratio was stabilized at about 83.63% ± 2.10% in a non time-dependent manner after 72 h, which was significantly different from that in the Ad-LacZ-infected and uninfected groups (F = 15.954, P = 0.012; Table 2 and Figure 3). The apoptosis rate was 11.61% ± 1.23% when the cells were infected with Ad-p27mt for 72 h. The sub-G_1 apoptosis was more significant in Ad-p27mt group than in AdLacZ and control groups (Table 2 and Figure 3). All results were obtained from experiments performed in triplicate.

DISCUSSION

Cholangiocarcinoma remains one of the most difficult tumors to treat in clinical practice. Currently, there is no effective chemotherapy for this disease. Surgery offers the only opportunity to cure it. However, the majority of patients fail to qualify for such a treatment. Therefore, new therapeutic modalities are needed. Gene therapy is regard as one of the most important and potential new modalities for this disease.

The CDK inhibitor *p27* plays a major role in controlling the cell cycle, which negatively regulates the transition from the G_1 into the S phase. Moreover, p27^{kip1} is also a tumor suppressor. Loss of p27 function weakens the control of G_1/S checkpoint, thus accelerating cell cycle progression and predisposing cells to malignant transformation[1,2]. Ganoth *et al*[3]

and Troncone *et al*[4] reported that the degradation of *p27* is mainly regulated by post-translational ubiquitin-proteasome-mediated proteolysis of phosphorylation in threonine (Thr) 187. In order to inhibit the degradation of *p27* and restore the function of G_1/S checkpoint, we transfected mutated p27 into cholangiocarcinoma QBC_{939} cell line, which has a mutation of Thr-187/Pro-188 (ACGCCC) to Met-187/Ile-188(ATGATC).

Western blot analysis showed that 72 h after infection with Ad-p27mt, *p27* in the CBC_{939} cells expressed a strong band, whereas Ad-LacZ-infected QBC_{939} cells showed a faint p27 protein product in the uninfected groups, suggesting that tansgenes can be successfully induced and expressed. The elevated level of *p27* expression demonstrated that mutated p27 was resistant to degradation and more stable than wild p27, indicating that phosphorylation of threonine (Thr) 187 can trigger degradation of wild p27. The transfer of Ad-p27mt significantly inhibited the proliferation of QBC_{939} cells, decreased clone formation, strongly induced cell cycle arrest and apoptosis at G_1/S phase within 72 h after infection, which is consistent with the previous findings[5].

It has been well documented that over expression of wild p27 *via* adenoviral gene transfer on p27-deficient tumor cells could strongly inhibit cell cycle arrest and even lead to significantly apoptosis in disparate types of human cancers, such as spongiocytoma, lung cancer, leukaemia. It is a common phenomenon that recombinant adenovirus-mediated p27 can eliminate carcinoma cells through apoptosis. Although there was no significant difference in apoptosis between Adp27mt- and AdLacZ-infected cells, uninfected cells at any time point, our data show that 72 h after infection with Adp27mt, the typical sub-G_1 apoptotic peak could be observed by flow cytometry, which was more apparent than in AdLacZ-infected and uninfected cells. Although the precise mechanism by which *p27* induces apoptosis is unclear, transfer of *p27* is associated with a moderate level of apoptosis as shown by FACS analysis. Since QBC_{939} cells have mutated p53, the mechanism underlying apoptosis induced by transfer of *p27* must be p53-independent. Further investigation is needed on how *p27* regulates and induces apoptosis.

In conclusion, Ad-p27mt at Thr-187 can be used as a novel, potent, tumor-suppressing gene therapy tool in the treatment of cholangiocarcinoma.

ACKNOWLEDGMENTS

The authors thank Doctors Chen YJ and Wang WY for their long, concerted, and fruitful efforts, Professor Wang SG for kindly donation of human cholangiocarcinoma cell line QBC_{939}, Professors Xu SY and Wang JN for their recombinant adenovirus vehicle Ad-p27mt and adenovirus control vehicle Ad-LacZ, Professor Zou SQ for his useful suggestions, and a great debt of gratitude to its organizer, Professor Zou SQ.

COMMENTS

Background

As a cyclin-dependent kinase inhibitor, $p27^{Kip1}$ (*p27*) regulates cell cycle progression by transcriptional, translational and proteolytic mechanisms. G_1/S cell cycle progression requires p27 proteolysis, which is triggered by its phosphorylation of threonine (Thr) 187. Increased *p27* causes proliferating cells to exit from the cell cycle, while decreased *p27* is required for quiescent cells to resume cell division. Low levels of *p27* are associated with excessive cell proliferation in pathological conditions such as inflammation and cancers. High levels of *p27* are observed in such conditions of diminished cell proliferation as in late stages of arterial wound repair in atherosclerosis. Interestingly, in many types of tumors such as gastric, prostate and breast carcinomas, the expression of *p27* gene is down-regulated. Loss of *p27* expression may result in tumor development and/or progression.

Research frontiers

The research involved cell morphologyoncology, cell morphology, molecular biology and gene therapy for cholangiocarcinoma.

Innovations and breakthroughs

It is well know that the degradation of *p27* is mainly regulated by post-translational ubiquitin-proteasome-mediated proteolysis during phosphorylation of threonine (Thr) 187. In order to inhibit the degradation of *p27* and restore the function of G_1/S checkpoint, we transfected mutated p27 into cholangiocarcinoma QBC_{939} cell line, which can mutate from Thr-187/Pro-188 (ACGCCC) to Met-187/Ile-188(ATGATC). The recombinant adenoviral vector cannot replicate in target cells because it lacks the E1 gene, thus only expressing the inserted gene. Because the foreign gene fragment is not incorporated into the genome of target cells, the danger of mutations affecting treatment is reduced. Meanwhile, adenoviral vectors are stable and easy to purify. This technique can effectively affect both proliferating and quiescent cells *ex vivo*. The potential for gene therapy by using the recombinant adenovirus is worthy of extensive attention. The results of our study suggest that adenovirus-mediated p27 gene transfection can be used as a novel gene therapy for cholangiocarcinoma.

Applications

The prognosis of cholangiocarcinoma is extremely poor although aggressive multidisciplinary cancer therapies have been used in clinical practice. Thus, it is imperative to develop new and effective treatment modalities for cholangiocarcinoma, such as gene therapy.

Peer review

The authors showed that transfection of a human cholangiocarcinoma cell line (QBC_{939}) could cause cell cycle arrest and apoptosis, which are of interest in developing new treatment modalities for cholangiocarcinoma. The methods used were well described. The results are of scientific interest.

REFERENCES

1 **Milde-Langosch K**, Hagen M, Bamberger AM, Loning T. Expression and prognostic value of the cell-cycle regulatory proteins, Rb, p16MTS1, p21WAF1, p27KIP1, cyclin E, and cyclin D2, in ovarian cancer. *Int J Gynecol Pathol* 2003; **22**: 168-174

2 **Fukunaga M**. Immunohistochemical characterization of cyclin E and p27KIP1 expression in early hydatidiform moles. *Int J Gynecol Pathol* 2004; **23**: 259-264

3 **Ganoth D**, Bornstein G, Ko TK, Larsen B, Tyers M, Pagano M, Hershko A. The cell-cycle regulatory protein Cks1 is required for SCF(Skp2)-mediated ubiquitinylation of p27. *Nat Cell Biol* 2001; **3**: 321-324

4 **Troncone G**, Martinez JC, Iaccarino A, Zeppa P, Caleo A, Russo M, Migliaccio I, Motti ML, Califano D, Palmieri EA, Palombini L. p27Kip1 is expressed in proliferating cells in its form phosphorylated on threonine 187. *BMC Clin Pathol* 2005; **5**: 3

5 **Katner AL**, Gootam P, Hoang QB, Gnarra JR, Rayford W. A recombinant adenovirus expressing p7(Kip1) induces cell cycle arrest and apoptosis in human 786-0 renal carcinoma cells. *J Urol* 2002; **168**: 766-773

S- Editor Zhong XY **L- Editor** Wang XL **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5349

World J Gastroenterol 2008 September 14; 14(34): 5349-5352
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

A "false positive" octreoscan in ileal Crohn's disease

Alberto Fernandez, Olga Tabuenca, Angeles Peteiro

Alberto Fernandez, Gastroenterology Department, POVISA Hospital, Vigo 36211, Spain
Olga Tabuenca, Nuclear Medicine Department, POVISA Hospital, Vigo 36211, Spain
Angeles Peteiro, Pathology Department, POVISA Hospital, Vigo 36211, Spain
Author contributions: Fernandez A, Tabuenca O and Peteiro A contributed equally to this work and wrote the paper.
Correspondence to: Alberto Fernandez, Servicio de Aparato Digestivo, Hospital POVISA, c/ Salamanca 5, Vigo (Pontevedra) 36211. Spain. afvillaverde@gmail.com
Telephone: +34-98-6413144 Fax: +34-98-6421439
Received: May 24, 2008 Revised: July 28, 2008
Accepted: August 4, 2008
Published online: September 14, 2008

Abstract

We present a case report of a patient with a suspicious ileal carcinoid tumour. Clinical examination as well as computer tomography (CT) scan suggested a tumour. Octeotride scan showed uptake in the same bowel loop reported as pathological in CT. The patient underwent surgery and biopsy which reported Crohn's disease (CD). The interest in the case is due to the fact that this is, to the best of our knowledge, the second report of Crohn's disease as a cause of false positive octeotride scan. Unfortunately, no somatostatin receptors could be found in the sample, so further studies should be performed.

Key words: Crohn's disease; Carcinoid tumour; Octreoscan; Somatostatin receptor scintigraphy; ^{111}In-DTPA- octreotido

Peer reviewer: Gert De Hertogh, Ph.D, Department of Morphology and Molecular Pathology, University Hospitals KU-Leuven, Minderbroedersstraat 12, Leuven 3000, Belgium

Fernandez A, Tabuenca O, Peteiro A. A "false positive" octreoscan in ileal Crohn's disease. *World J Gastroenterol* 2008; 14(34): 5349-5352 Available from: URL: http://www.wjgnet.com/1007-9327/14/5349.asp DOI: http://dx.doi.org/10.3748/wjg.14.5349

INTRODUCTION

Somatostatin receptors (SS-Rs) are membrane glycoproteins spreading over a large number of body tissues and can be found in normal and pathological conditions[1]. Many benign and malignant tumours over express SS-R in their membranes especially in neuroendocrine tumours (NETs) but there are other benign conditions showing an increase number of SS-Rs, such as granulomatous or inflammatory disease[2].

Octeotride is an analogue whose molecule is a shortened version of somatostatin's, from 8 to 14 aminoacides, sharing its active nuclei and allowing the ^{111}Indium-DPTA molecule to bind to the N-terminal (d-phe).

Thus, ^{111}In- DTPA-D- Phe1- octreótido (^{111}In-DTPAOC) is the labelled form of octreotide. There are 5 subtypes of SS-R, and somatostatin shows a high affinity for all of them. However, octeotride has a high affinity for only SS-R 2 subtype and a low affinity for SS-R 3 and SS-R 5 subtypes. Therefore, pathological conditions over expressing these receptors are able to be imaged in ^{111}In- DTPAOC scans.

The sensitivity of ^{111}In- DTPAOC in detecting these pathological conditions is variable being very high in carcinoid tumour (86%)[3]. Uptake in other tissues can lead to false-positive findings when studying suspected NETs. Only one case of Crohn's disease (CD) has been recently reported as a cause of false positive scan[4].

We report a case of a false positive ^{111}In- DTPAOC scintigraphy in a patient with CD mimicking an ileal carcinoid tumour. A discussion about SS-R potential alterations in CD was included.

CASE REPORT

A 40-year-old man was admitted to our outpatient clinic with an 8-mo history of dull abdominal pain and weight loss of 20 kg, but no other gastrointestinal symptoms. In terms of past medical or surgery history, the patient denied of smoking or other diseases. Physical examination only revealed a painful site in the right lower quadrant of the abdomen and a body temperature of 37.3℃. Laboratory test only revealed an increased erythrocyte sedimentation rate of 35 mm/h (normal range: 0-25 mm/h) and C-reactive protein rate of 2.37 mg/dL (normal range: 0-0.5 mg/dL). A computer tomography (CT) scan was performed (Figure 1) and showed thickening of the ileum distal wall and the presence of a 4 cm solid mass with irregular border adjacent to the affected ileum, conditioning retraction, with prominent mesenteric lymph nodes. All these findings suggested a carcinoid tumour.

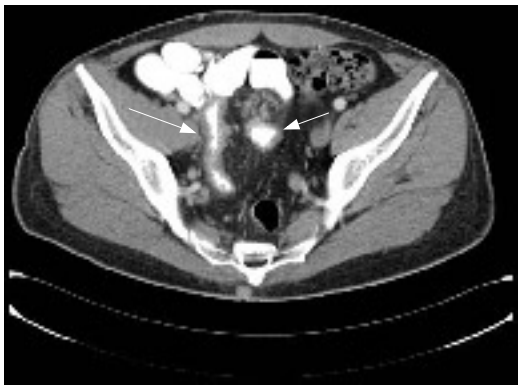


Figure 1 CT-scan showing thickening of the distal ileum (long arrow) with an adjacent solid mass (short arrow), suggesting a carcinoid tumour.

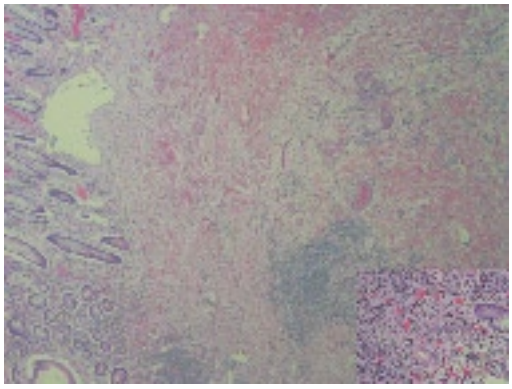


Figure 3 CD showing marked transmural inflammatory changes (involving the walls of veins and arteries): edema, lymphatic dilatation, hyperplasia of the muscularis mucosae, fibrosis ("obliterative muscularization"), and epithelioid granuloma.

Further explorations were performed. Colonoscopy with ileoscopy (at least 15 cm of the distal ileum was explored) showed an irregular ileal mucosa with erythema, irregular nodular areas and marked stiffness, suggesting an infiltrative process. Ten ileal biopsies were taken and showed unspecific inflammatory changes without evidence of malignancy, granulomas or histological features suggestive of carcinoid tumour or CD. A 24-h urine collection for 5-hydroxyindolacetic acid (5-HIAA) showed a normal result of 3.3 mg/24 h (normal range < 10 mg/24 h).

Due to the low sensitivity of the 24-h 5-HIAA test and the high suspicion of carcinoid tumour, an ^{111}In-DTPAOC scan was performed. The patient was injected with 3mCi ^{111}In- DTPAOC. Total body images were obtained at 4 and 24 h, and an abdominal SPECT was also performed at 14 h. The images showed pathological uptake in central and right pelvic fossae at 4 and 24 h (Figure 2). SPECT showed a C-shape uptake in the sagittal plane that matches with the thickened loop on CT. No uptake was observed in the solid mass. The scan was reported as a highly suspicious carcinoid tumour.

With all these results, laparoscopy with curative intention was performed. An inflammatory mass involving the distal ileon was found at surgery, and a distal small bowel resection and right hemicolectomy with lateral anastomosis were performed. Histological analysis

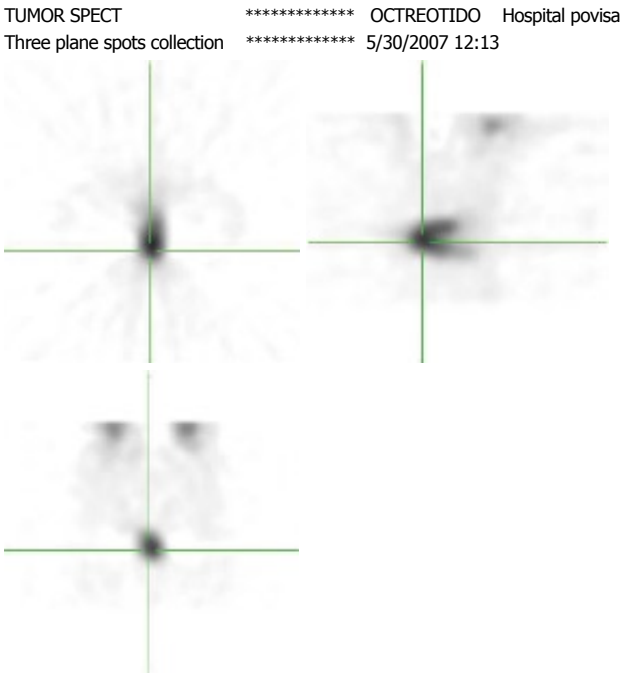


Figure 2 Octeotride-SPECT images showing pathological uptake in the ileal thickened loop at CT-scan.

of macroscopically-affected segments and specimens of non-macroscopically-involved segments was carried out. Thickened serosa and shortened mesentery could be grossly observed in the involved terminal ileum (20 cm), resulting in a corrugated bowel contour in the terminal portion of the small bowel. These changes could probably mimic the impression on CT-scan to an abdominal mass. The mesenteric lymph nodes were enlarged measuring 8 mm in the larger diameter. The detailed macroscopic examination failed to show any tumour mass. Photo of gross specimen neither was nor included. Other gross findings of significance included stricture formation, fissuring, cobblestone appearance (discontinuous involvement with transmural spread) with an intervening normal or edematous mucosa. The main microscopic features were ulceration, fissure, non caseating sarcoid-like granuloma and lymphoid aggregates. The regional lymph nodes showed sinusal dilatation and lymphoid hyperplasia. Chromogranin stain was not performed.

Intestinal tissue sections were routinely fixed in 10% neutral formalin and embedded in paraffin. Immunohistochemical staining for Dako® polyclonal antibodies against human somatostatin protein was performed with the standard avidin-biotin method. The slide-mounted tissue sections were allowed to reach room temperature and incubated for 60 min at a solution of 1:900, using saponic to antigenic recuperation. Somatostatin immunohistochemical expression was undetectable in the veins or arteries of inflamed and non inflamed control intestinal tissue sections. No concordance was reached in immunohistochemistry/scintigraphy somatostatin receptor. No areas of dysplasia or carcinoma were identified.

In the absence of features other than the typical appearance of CD, the pathologic diagnosis of Crohn's ileitis was made (Figure 3).

After surgery, azathioprine was started for prevention of postoperative recurrence. The patient underwent a complete clinical recovery and 6 mo later he was asymptomatic. A surveillance/follow-up colonoscopy did not show any abnormality and inflammatory signs of recurrence.

DISCUSSION

There are many cases of carcinoid tumour misdiagnosed as CD only discovered when treatment is not effective or surgery is performed. It was reported that approximately 2.3% of patients with ileal carcinoid are first diagnosed and treated as CD[5]. However, the reverse is a very uncommon situation.

In our case, the clinic features as well as the image of CT-scan strongly suggested a carcinoid tumour. In order to confirm the suspected disease, ^{111}In- DTPAOC scan was performed and showed a C-shape uptake in the bowel loop that corresponded to that reported as pathologic in CT, but there was no uptake in the solid mass. The patient underwent surgery and the final pathologic report was CD. The mass that did not show uptake was reported as inflammatory.

^{111}In- DTPAOC is a radio-labelled octreotide analogue that binds to SS-R expressed in cell membranes. Many benign and malignant diseases overexpress SS-R and thus, can be imaged with this radioligand[6]. Carcinoid tumour, one of the malignant tumours, shows more uptakes in the bowel loop due to its high density of SS-R. Reported values for the detection of known carcinoid tumour localizations vary from 80% to nearly 100%[1]. Uptake is related to SS-R density and even tumours smaller than 1 cm in diameter can be detected. This is the reason why somatostatin receptor scintigraphy (SRS) plays a central role in locating and assessing the primary gastroenteropancreatic NET[7] and has a marked effect in the clinical management of these malignancies[8,9]. However, 12% of SRS examinations result in false-positive localization of NET, understanding a false-positive as an uptake not related with the tested pathology. Renal parapelvic cysts, accessory spleens, ventral hernias, thyroid or breast disease are the most frequent cases of false positive localizations[10]. SS-R is over expressed in activated peripheric lymphocytes and macrophages in granulomatous and inflammatory diseases, allowing obtaining images[11,12].

In normal bowel, SS-R is expressed in gastrointestinal mucosa, peripheral nervous system and lymphoid tissue. Several tumours, such as carcinoid tumour, express SS-R in peritumoral veins[13]. In intestinal inflammatory disease, a high density of SS-R is detected by autoradiography in intestinal intramural veins but not in normal tissues. SS-R is seen in small muscular veins of submucosa, tunica muscularis and subserosa, and the thickness of veins is labelled. These veins are histologically normal and seldom have a minimal lymphocyte infiltration although label intensity does not seem to depend on them as the surrounding tissues are infiltrated with these cells and do not show labelling[14-16].

Usually no ^{111}In- DTPAOC uptake is found in inflammatory bowel disease. There is only one recent report of ^{111}In- DTPAOC uptake in Crohn's disease in literature although there is no evidence that SS-R is determined[4]. In our case, the samples were studied to assess the existence of SS-R. Immunohistochemical staining of somatostatin receptors was performed and somatostatin immunohistochemical expression was undetectable in the veins or arteries of inflamed and non inflamed control intestines.

Unfortunately, SS-R could not be found in the tissues studied, so we could not explain this abnormal uptake. No concordance was reached in immunohistochemistry/ scintigraphy somatostatin receptor, possibly as a consequence of somatostatin receptor heterogeneity. Further studies should be made to assess the possible causes of uptake in CD.

However, there are several reports of small carcinoid tumours (a few millimetres in diameter) found in patients with CD, suggesting a correlation between the pathogenesis of both disorders[17]. It has been theorized that inflammation creates a favourable environment for the development of carcinoid tumour, although most of the carcinoid tumours reported are found in non-inflamed bowel. Other theories include the hyperstimulation of enteroendocrine cells by inflammation[18] and the role of proinflammatory cytokines such as PTHrP or IL-6[19].

We would like to encourage physicians to be aware of CD as a cause of false positive results when performing ^{111}In- DTPAOC scans.

REFERENCES

1 **Kwekkeboom D**, Krenning EP, de Jong M. Peptide receptor imaging and therapy. *J Nucl Med* 2000; **41**: 1704-1713

2 **van Hagen PM**. Somatostatin receptor expression in clinical immunology. *Metabolism* 1996; **45**: 86-87

3 **Kwekkeboom DJ**, Krenning EP. Somatostatin receptor imaging. *Semin Nucl Med* 2002; **32**: 84-91

4 **Marko J**, Lamba R, Miller F, Buchman A, Spies S, Nikolaidis P. OctreoScan positive Crohn's disease mimicking an ileal carcinoid tumor. *J Clin Gastroenterol* 2008; **42**: 66-68

5 **Hsu EY**, Feldman JM, Lichtenstein GR. Ileal carcinoid tumors stimulating Crohn's disease: incidence among 176 consecutive cases of ileal carcinoid. *Am J Gastroenterol* 1997; **92**: 2062-2065

6 **Warner RR**, O'dorisio TM. Radiolabeled peptides in diagnosis and tumor imaging: clinical overview. *Semin Nucl Med* 2002; **32**: 79-83

7 **Ramage JK**, Davies AH, Ardill J, Bax N, Caplin M, Grossman A, Hawkins R, McNicol AM, Reed N, Sutton R, Thakker R, Aylwin S, Breen D, Britton K, Buchanan K, Corrie P, Gillams A, Lewington V, McCance D, Meeran K, Watkinson A. Guidelines for the management of gastroenteropancreatic neuroendocrine (including carcinoid) tumours. *Gut* 2005; **54** Suppl 4: iv1-iv16

8 **Lebtahi R**, Cadiot G, Sarda L, Daou D, Faraggi M, Petegnief Y, Mignon M, le Guludec D. Clinical impact of somatostatin receptor scintigraphy in the management of patients with neuroendocrine gastroenteropancreatic tumors. *J Nucl Med* 1997; **38**: 853-858

9 **Termanini B**, Gibril F, Reynolds JC, Doppman JL, Chen CC,

Stewart CA, Sutliff VE, Jensen RT. Value of somatostatin receptor scintigraphy: a prospective study in gastrinoma of its effect on clinical management. *Gastroenterology* 1997; **112**: 335-347

10 **Gibril F**, Reynolds JC, Chen CC, Yu F, Goebel SU, Serrano J, Doppman JL, Jensen RT. Specificity of somatostatin receptor scintigraphy: a prospective study and effects of false-positive localizations on management in patients with gastrinomas. *J Nucl Med* 1999; **40**: 539-553

11 **Vanhagen PM**, Krenning EP, Reubi JC, Kwekkeboom DJ, Bakker WH, Mulder AH, Laissue I, Hoogstede HC, Lamberts SW. Somatostatin analogue scintigraphy in granulomatous diseases. *Eur J Nucl Med* 1994; **21**: 497-502

12 **Tabuenca Dopico O**, Gutierrez Mendiguchia C, Rego Iraeta A. [Incidental diagnosis of lymph node tuberculosis in an 111-indium-octreotide scintigraphy during a study of acromegaly] *Rev Esp Med Nucl* 2006; **25**: 193-197

13 **Reubi JC**, Horisberger U, Laissue J. High density of somatostatin receptors in veins surrounding human cancer tissue: role in tumor-host interaction? *Int J Cancer* 1994; **56**: 681-688

14 **Reubi JC**, Mazzucchelli L, Laissue JA. Intestinal vessels express a high density of somatostatin receptors in human inflammatory bowel disease. *Gastroenterology* 1994; **106**: 951-959

15 **Denzler B**, Reubi JC. Expression of somatostatin receptors in peritumoral veins of human tumors. *Cancer* 1999; **85**: 188-198

16 **Reubi JC**, Waser B, Schaer JC, Laissue JA. Somatostatin receptor sst1-sst5 expression in normal and neoplastic human tissues using receptor autoradiography with subtype-selective ligands. *Eur J Nucl Med* 2001; **28**: 836-846

17 **West NE**, Wise PE, Herline AJ, Muldoon RL, Chopp WV, Schwartz DA. Carcinoid tumors are 15 times more common in patients with Crohn's disease. *Inflamm Bowel Dis* 2007; **13**: 1129-1134

18 **Le Marc'hadour F**, Bost F, Peoc'h M, Roux JJ, Pasquier D, Pasquier B. Carcinoid tumour complicating inflammatory bowel disease. A study of two cases with review of the literature. *Pathol Res Pract* 1994; **190**: 1185-1192; discussion 1193-1200

19 **Barhoum M**, Hutchins L, Fonseca VA. Intractable hypercalcemia due to a metastatic carcinoid secreting parathyroid hormone-related peptide and interleukin-6: response to octreotide. *Am J Med Sci* 1999; **318**: 203-205

S- Editor Li DL **L- Editor** Wang XL **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5353

World J Gastroenterol 2008 September 14; 14(34): 5353-5355
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Inflammatory myoglandular polyp causing hematochezia

Shoji Hirasaki, Masato Okuda, Kenichiro Kudo, Seiyuu Suzuki, Atsuko Shirakawa

Shoji Hirasaki, Masato Okuda, Kenichiro Kudo, Seiyuu Suzuki, Department of Internal Medicine, Sumitomo Besshi Hospital, Niihama 7928543, Japan
Atsuko Shirakawa, Department of Pathology, Sumitomo Besshi Hospital, Niihama 7928543, Japan
Author contributions: Hirasaki S, Okuda M, Kudo K and Suzuki S were involved in the care of the patient; Shirakawa A studied the specimen; Hirasaki S performed the endoscopic resection; Hirasaki S wrote the paper.
Correspondence to: Shoji Hirasaki, Department of Internal Medicine, Sumitomo Besshi Hospital, 3-1 Ohji-cho, Niihama 7928543, Japan. shoji_hirasaki@ni.sbh.gr.jp
Telephone: +81-897-377111 Fax: +81-897-377121
Received: April 18, 2008 Revised: August 11, 2008
Accepted: August 18, 2008
Published online: September 14, 2008

Abstract

A case of inflammatory myoglandular polyp (IMGP) causing hematochezia is reported. The patient was a 33-year-old man who visited our hospital for further evaluation of hematochezia. Colonoscopy revealed a red, hard, spherical peduncular polyp with erosion and mucous exudation, about 20 mm in diameter, in the descending colon. Excluding the polyp, there was no lesion in the colorectum. Endoscopic polypectomy was performed. Histological examination of the specimen revealed inflammatory granulation tissue in the lamina propria, proliferation of smooth muscle, and hyperplastic glands with variable cystic changes. This polyp was diagnosed as an IMGP. The symptom of hematochezia was resolved after endoscopic resection. Our case shows that treatment is necessary for IMGP if intestinal bleeding occurs and endoscopists should be aware of the endoscopic characteristics of IMGP.

Key words: Inflammatory myoglandular polyp; Colonoscopy; Endoscopic polypectomy; Gastrointestinal bleeding

Peer reviewer: Dr. James E East, Department of Gastroenterology, St. Mary's Hospital, Clarence Wing, Praed Street, London W2 1NY, United Kingdom

Hirasaki S, Okuda M, Kudo K, Suzuki S, Shirakawa A. Inflammatory myoglandular polyp causing hematochezia. *World J Gastroenterol* 2008; 14(34): 5353-5355 Available from: URL: http://www.wjgnet.com/1007-9327/14/5353.asp DOI: http://dx.doi.org/10.3748/wjg.14.5353

INTRODUCTION

Inflammatory myoglandular polyp (IMGP) is characterized by inflammatory granulation tissue in the lamina propria[1], proliferation of smooth muscle[2], and hyperplastic glands with variable cystic changes[3-7]. Only a small number of cases have been reported and the pathogenesis and natural history remain unclear[8-12]. Herein, we describe a relatively rare case of inflammatory myoglandular polyp causing hematochezia.

CASE REPORT

A 33-year-old man presented with the symptom of hematochezia. He was in good health with no specific family or past medical history. His body temperature was 36.7℃, blood pressure was 148/82 mmHg, and radial pulse rate was 70 beats/min and regular. He had neither anemia nor jaundice. Neurological examination revealed no abnormal findings. Abdominal palpation revealed tenderness in the left lower quadrant. Routine hematological examination and biochemical tests were within normal limits. Colonoscopy revealed a red, hard, spherical peduncular polyp with erosion and mucous exudation, about 20 mm in diameter, in the descending colon (Figure 1). With conventional colonoscopy, the lesion did not show type Ⅲ or Ⅳ pit pattern although magnifying colonoscopy was not performed. We speculated that this polyp was non-neoplastic. It was suspected to be an inflammatory polyp from endoscopic findings although it should be distinguished from a juvenile polyp. An air contrast barium enema also revealed a pedunculated polyp in the descending colon (Figure 2). Excluding the polyp, there was no lesion in the colorectum. We speculated that the polyp in the descending colon was the causative lesion of hematochezia. Endoscopic polypectomy was performed. At polypectomy, polyp erosion was healed (Figure 3). Histological examination of the specimen revealed inflammatory granulation tissue in the lamina propria, proliferation of smooth muscle, and hyperplastic glands with variable cystic changes (Figure 4). The lesion was diagnosed as an IMGP. After endoscopic polypectomy, the symptom of hematochezia was resolved.

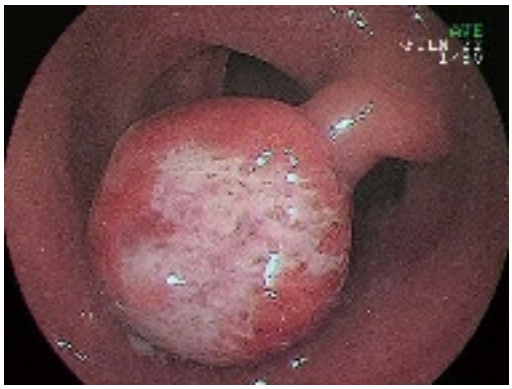


Figure 1 Endoscopy showing a red, hard and spherical peduncular polyp, about 20 mm in diameter, in the descending colon.

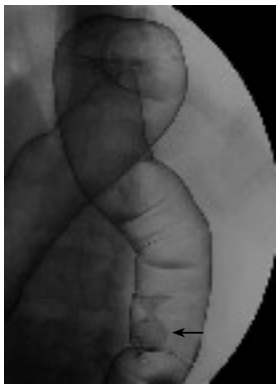


Figure 2 Double contrast radiograph of descending colon showing an about 20 mm peduncular polyp.

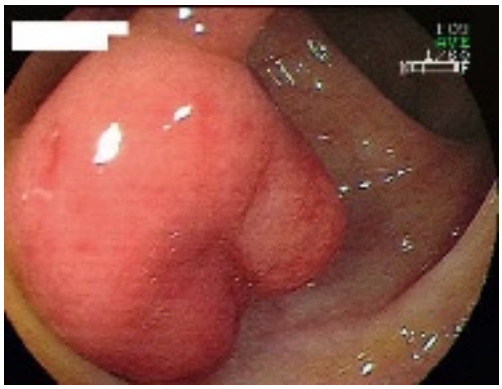
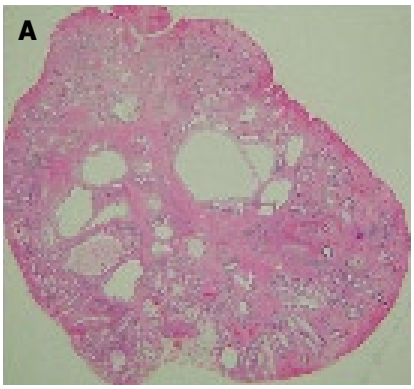


Figure 3 The second colonoscopy showing a healed polyp.



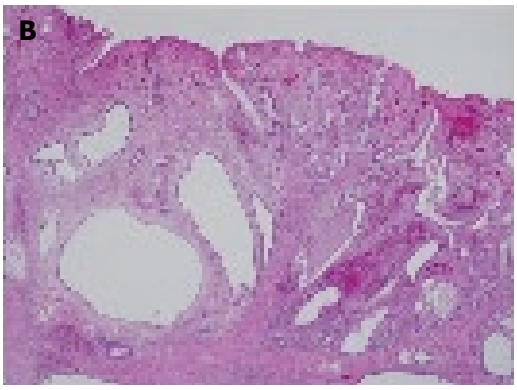


Figure 4 Microscopic findings of the polypectomy specimen. Low-power view of a cross section showing a stalked polyp containing numerous cystically dilated glands (**A**) and inflammatory granulation tissue in the lamina propria mucosae and proliferation of smooth muscle (**B**).

DISCUSSION

IMGP is a non-neoplastic colorectal polyp, first described by Nakamura *et al*[1]. IMGP is solitary, pedunculated and rarely, covered by a fibrin cap, and follows a benign course. Also, IMGP has no association with inflammatory bowel diseases and is located not only in the rectosigmoid, but also in the descending and transverse colon[3]. In the present case, it was located in the descending colon. Although the pathogenesis of IMGP remains unknown, Nakamura[1] proposed that chronic trauma from intestinal peristalsis may contribute to the pathogenesis of IMGP.

Only a small number of IMGP cases have been reported. According to Fujino *et al*[8], a review of the literature revealed 48 cases of IMGP in the large intestine up to 2001. However, recent advances in diagnostic techniques, especially the widespread use of colonoscopy for colorectal tumors, have enabled us to identify small and asymptomatic polyps, and reports on IMGP of the colon have been increasing[9,10]. Fujino *et al*[8] described that the macroscopic appearance is the pedunculated type in 83.3% of cases. In that report, the sites of IMGP in the large intestine were studied and 47 of 48 cases (97.9%) had lesions in the rectum to transverse colon. Thus, IMGPs of the large intestine are predominantly in the distal colon[8,9]. IMGPs in the colon are usually asymptomatic and often detected incidentally on barium enema or endoscopy[8-10]. Another review of the literature revealed that the main clinical feature of colorectal IMGPs is hematochezia[10,11]. Endoscopic characteristic findings include (1) pedunculated or semipedunculated, (2) red and (3) smooth, spherical and hyperemic surface with patchy mucous exudation and erosion[8,9]. In the present case, the endoscopic findings of the polyp were compatible with those of IMGP.

As to therapy, IMGP of the large intestine can best be removed endoscopically, because it is thought to be clinically and histologically benign. Most Japanese cases have been treated with polypectomy or endoscopic mucosal resection (EMR)[5,7-11]. If we could confirm the histological diagnosis of small colonic IMGPs by endoscopic biopsy, endoscopic resection of IMGP might be unnecessary because IMGP follows a benign course.

However, the diagnosis of colorectal IMGPs could seldom be made by endoscopic biopsy and the final diagnosis of colonic IMGP depends on the pathological findings of EMR or endoscopic polypectomy specimens. Endoscopic or surgical treatment is necessary if gastrointestinal bleeding[9] or colonic intussusception occurs. Local excision of the polyp is curative. Kayhan *et al*[12] have reported a case of large IMGP (> 6 cm) that was too large to be removed endoscopically, and was thus treated with surgical resection. We consider that the percentage of patients with colonic IMGP who undergo surgical resection will decrease and endoscopic resection will increase in the future because of recent advances in diagnostic technologies such as improved endoscopic images.

In conclusion, we report a case of IMGP causing hematochezia. IMGP should generally be taken into consideration as a differential diagnosis of peduncular polyp of the colon. IMGP of the large intestine is not fatal and patients remain asymptomatic in their daily lives except for gastrointestinal bleeding or bowel obstruction. Therefore, it is likely that there are many latent patients with IMGP who might be incidentally discovered in the future. Endoscopists should be aware of that IMGP may exhibit the aforementioned endoscopic characteristics and may cause hematochezia. The causes of IMGP are still obscure, and further accumulation of cases may disclose their pathogenesis.

REFERENCES

1 **Nakamura S**, Kino I, Akagi T. Inflammatory myoglandular polyps of the colon and rectum. A clinicopathological study of 32 pedunculated polyps, distinct from other types of polyps. *Am J Surg Pathol* 1992; **16**: 772-779

2 **Griffiths AP**, Hopkinson JM, Dixon MF. Inflammatory myoglandular polyp causing ileo-ileal intussusception. *Histopathology* 1993; **23**: 596-598

3 **Bhathal PS**, Chetty R, Slavin JL. Myoglandular polyps. *Am J Surg Pathol* 1993; **17**: 852-853

4 **Gomez Navarro E**, del Rio Martin JV, Sarasa Corral JL, Melero Calleja E. [Myoglandular inflammatory polyp located in the distal end of the rectum] *Rev Esp Enferm Dig* 1994; **85**: 45-46

5 **Nagata S**, Sumioka M, Sato O, Miyamoto M, Watanabe C, Yamada H, Hirata K, Imagawa M, Haruma K, Kajiyama G. [Five cases of inflammatory myoglandular polyp] *Nippon Shokakibyo Gakkai Zasshi* 1998; **95**: 145-150

6 **Bhardwaj K**, Mohan H, Chopra R, Bhardwaj S, Sachdev A. Inflammatory myoglandular polyp of rectum. *Indian J Gastroenterol* 1998; **17**: 63-64

7 **Harada N**, Chijiiwa Y, Yao T, Koyanagi M, Ono Y, Motomura S. Inflammatory myoglandular polyp. *J Clin Gastroenterol* 1999; **29**: 104-105

8 **Fujino Y**, Orii S, Nakamura S, Sugai T, Saito S, Yamaguchi T, Noro S, Ishii M, Inomata M, Suzuki K. Five cases of colorectal inflammatory myoglandular polyps. *Gastroenterological Endoscopy* 2001; **43**: 1281-1286

9 **Moriyama T**, Matsumoto T, Hizawa K, Tada S, Fuchigami T, Iwai K, Yao T, Iida M. Inflammatory myoglandular colorectal polyps: a case series of nine patients. *Endoscopy* 2003; **35**: 363-365

10 **Tashiro M**, Yoshikawa I, Matsuhashi T, Yamasaki T, Nishikawa S, Taguchi M, Yamasaki M, Kume K, Otsuki M. Images of interest. Gastrointestinal: inflammatory myoglandular polyp of the colon. *J Gastroenterol Hepatol* 2005; **20**: 1123

11 **Becheanu G**, Stamm B. Inflammatory myoglandular polyp--a rare but distinct type of colorectal polyps. *Pathol Res Pract* 2003; **199**: 837-839

12 **Kayhan B**, Kucukel F, Akdogan M, Ozaslan E, Kucukbas TA, Atoglu O. Inflammatory myoglandular polyp: a rare cause of hematochezia. *Turk J Gastroenterol* 2004; **15**: 117-119

S- Editor Zhong XY **L- Editor** Wang XL **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2008 September 14; 14(34): 5356
World Journal of Gastroenterology ISSN 1007-9327

ACKNOWLEDGMENTS

Acknowledgments to Reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

Arno J Dormann, PD, MED
Habil, Medizinische Klinik, Krankenhaus Holweide, Kliniken der Stadt Köln gGmbH, Neufelder St. 32, 51067 Köln, Germany

Yik-Hong Ho, Professor
Department of Surgery, School of Medicine, James Cook University, Townsville 4811, Australia

Dr. Bernardino Rampone
Department of General Surgery and Surgical Oncology, University of Siena, viale Bracci, Siena 53100, Italy

Taku Aoki, MD
Division of Hepato-Biliary-Pancreatic and Transplantation Surgery, Department of Surgery, Graduate School of Medicine, University of Tokyo,7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan

Tadashi Shimoyama, MD
Hirosaki University, 5 Zaifu-cho, Hirosaki 036-8562, Japan

Yoshio Yamaoka, MD, PhD, Associate Professor
Department of Medicine/Gastroenterology, Baylor College of Medicine and VA Medical Center (111D), 2002 Holcombe Blvd, Houston, Texas 77030, United States

Rakesh Aggarwal, Additional Professor
Department of Gastroenterology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow 226014, India

Rosemar Joyce Burnett, PhD
Department of Epidemiology National School of Public Health, University of Limpopo, Medunsa Campus PO Box 173, MEDUNSA, Pretoria 0204, South Africa

Seyed A Taghavi, Associate Professor
Department of Internal Medicine, Nemazee Hospital, No.23, 59th Alley, Ghasrodasht St., Shiraz 71838-95453, Iran

Yik-Hong Ho, Professor
Department of Surgery, School of Medicine, James Cook University, Townsville 4811, Australia

Alastair John Watson, Professor
The Henry Wellcome Laboratory, Nuffield Building, University of Liverpool, Crown St., Liverpool, L69 3GE, United Kingdom

Satoshi Osawa, MD
First Department of Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu, 431-3192, Japan

Elke Cario, MD
Division of Gastroenterology and Hepatology, University Hospital of Essen, Institutsgruppe I, Virchowstr. 171, Essen D-45147, Germany

Yoshio Yamaoka, MD, PhD, Associate Professor
Department of Medicine/Gastroenterology, Baylor College of Medicine and VA Medical Center (111D), 2002 Holcombe Blvd, Houston, Texas 77030, United States

Walter E Longo, Professor
Department of Surgery, Yale University School of Medicine, 205 Cedar Street, New Haven 06510, United States

Conor P Delaney, MD, MCh, PhD, FRCSI, FACS, Professor of Surgery
Case Western Reserve University, Chief, Division of Colorectal Surgery, Vice-Chairman, Department of Surgery, Director, Institute for Surgery and Innovation, University Hospitals, Case Medical Center, 11100 Euclid Avenue Cleveland, OH 44106-5047. United States

Yuan Yuan, Professor
Cancer Institute of China Medical University, 155 North Nanjing Street, Heping District, Shenyang 110001, Liaoning Province,China

Jose JG Marin, Professor
Head of the Departamento Physiology and Pharmacology, University of Salamanca, CIBERehd, Campus Miguel de Unamuno, ED-S09, Salamanca 37007, Spain

Mercedes Susan Mandell, MD, PhD
Department of Anesthesiology, University of Colorado Health Sciences Ctr., 12401 E. 17th Ave, B113 Aurora, CO 80045, United States

Dr. Martin Hennenberg
Dipl-Biol, Medizinische Klinik & Poliklinik I, Uni-Klinik Bonn, Sigmund-Freud Str. 25, 53105 Bonn, Germany

Kurt Lenz, Professor
Department of Internal Medicine, Konventhospital Barmherzige Brueder, A-4020 Linz, Austria

Atsushi Nakajima, Professor
Division of Gastroenterology, Yokohama City University Graduate School of Medicine, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236-0004, Japan

Ana Cristina Simões e Silva, MD, PhD, Professor
Faculdade de Medicina UFMG, Departamento de Pediatria, sala 267, Avenida Professor Alfredo Balena, 190, Bairro Santa Efigênia, Belo Horizonte, Minas Gerais 30130-100, Brazil

Oliviero Riggio, Professor
Dipartimento di Medicina Clinica, Sapienza Università di Roma,Viale dell'Università 37, 00185 Roma, Italy

Robert J Korst, MD
Department of Cardiothoracic Surgery, Weill Medical College of Cornell University, Room M404, 525 East 68th Street, New York 10032, United States

Zsuzsa Szondy, Professor
Department of Biochemistry and Molecular Biol, University of Debrecen, Debrecen H-4012, Hungary

Leonidas G Koniaris, Professor
Alan Livingstone Chair in Surgical Oncology, 3550 Sylvester Comprehensive Cancer Center (310T), 1475 NW 12th Ave., Miami, FL 33136, United States

D Mark Pritchard, PhD, FRCP
Gastroenterology of University of Liverpool, 5th Floor UCD Building, Daulby St, Liverpool L69 3GA, United Kingdom

Dr. Bijan Eghtesad, Associate Professor
Department of General Surgery, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland OH 44195, United States

Katsutoshi Yoshizato, PhD
Academic Advisor , Phoenix Bio Co., Ltd., 3-4-1 Kagamiyama, Higashihiroshima, 739-0046, Japan

Mitsuo Shimada, Professor
Department of Digestive and Pediatric Surgery, Tokushima University, Kuramoto 3-18-15, Tokushima 770-8503, Japan

Peter L Lakatos, MD, PhD, Assistant Professor
1st Department of Medicine, Semmelweis University, Koranyi S 2A, Budapest H1083, Hungary

Lesley A Anderson, PhD
MPHe, BSc (Hons), Academic Fellow in Cancer Prevention, Cancer Epidemiology and Prevention Research Group, Centre for Clinical and Population Sciences, Mulhouse Building Grosvenor Road, Belfast BT12 6BJ, United Kingdom

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2008 September 14; 14(34): 5357
World Journal of Gastroenterology ISSN 1007-9327

Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
January 24-25, Frankfurt, Germany
Falk Workshop: Perspectives in Liver
Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
E-mail: education@asge.org

June 4-7, Helsinki, Finland
The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
ESGAR 2008 19th Annual Meeting and Postgraduate Course
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden
16th International Congress of the European Association for Endoscopic Surgery
E-mail: info@eaes-eur.org

June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
10th World Congress on Gastrointestinal Cancer
Imedex and ESMO
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland
Joint Meeting of the European Pancreatic Club (EPC)
and the International Association of Pancreatology (IAP)
E-mail: office@epc-iap2008.org
www.e-p-c.org
www.pancreatology.org

June 26-28, Bratislava, Slovakia
5th Central European Gastroenterology Meeting
www.ceurgem2008.cz

July 9-12, Paris, France
ILTS 14th Annual International Congress
www.ilts.org

September 10-13, Budapest, Hungary
11th World Congress of the International Society for Diseases of the Esophagus
E-mail: isde@isde.net

September 13-16, New Delhi, India
Asia Pacific Digestive Week
E-mail: apdw@apdw2008.net

APDW 2008
September 13-16, New Delhi, Indian
Organized: Indian Society of Gastroenterology

III FALK GASTRO-CONFERENCE
September 17, Mainz, Germany
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
Falk Symposium 166:
GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
Falk Symposium 167:
Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
Third Annual Meeting
European Society of Coloproctology
www.escp.eu.com

October 8-11, Istanbul, Turkey
18th World Congress of the International Association of Surgeons,
Gastroenterologists and Oncologists
E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria
16th United European Gastroenterology Week
www.negf.org
www.acv.at

October 22-25, Minnesota, USA
Anstralian Gastroenterology Week 2008
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia
71st Annual Colon and Rectal Surgery Conference
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA
59th AASLD Annual Meeting and Postgraduate Course
The Liver Meeting
Information: www.aasld.org

November 6-9, Lucerne, Switzerland
Neurogastroenterology & Motility Joint International Meeting 2008
E-mail: ngm2008@mci-group.com
www.ngm2008.com

November 12, Santiago de Chile, Chile
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt
1st Hepatology and Gastroenterology Post Graduate Course
www.egyptgastrohep.com

December 7-9, Seoul, Korea
6th International Meeting
Hepatocellular Carcinoma: Eastern and Western Experiences
E-mail: sglee@amc.seoul.kr

INFORMATION FOR ALL
FALK FOUNDATION e.V.
E-mail: symposia@falkfoundation.de
www.falkfoundation.de

Advanced Courses - European Institute of Telesurgery EITS - 2008
Strasbourg, France
January 18-19, March 28-29, June 6-7, October 3-4

N.O.T.E.S
April 3-5, November 27-29
Laparoscopic Digestive Surgery

June 27-28, November 7-8
Laparoscopic Colorectal Surgery

July 3-5
Interventional GI Endoscopy Techniques
Contact address for all courses:
E-mail: info@eits.fr

International Gastroenterological Congresses 2009
March 23-26, Glasgow, Scotland
Meeting of the British Society of Gastroenterology (BSG)
E-mail: bsg@mailbox.ulcc.ac.uk

May 17-20, Denver, Colorado, USA
Digestive Disease Week 2009

November 21-25, London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2008 September 14; 14(34): 5358-5360
World Journal of Gastroenterology ISSN 1007-9327

Instructions to authors

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol* ISSN 1007-9327 CN 14-1219/R) is a weekly open access peer-reviewed journal supported by an editorial board consisting of 1208 experts in gastroenterology and hepatology from 60 countries. The aim of the journal is to deliver the most clinically relevant original and commentary articles to readers, and to make the full text publicly available to all clinicians, scientists, patients and biomedical students on an unrestricted platform, so that they can access and learn about the most recent key advances in the field.

In addition to the open access nature, another key characteristic of *WJG* is its reading guidance for each article which includes background, research frontier, related reports, breakthroughs, applications, terminology, and comments of peer reviewers for the general readers.

WJG publishes articles on esophageal, gastrointestinal, hepatobiliary and pancreatic tumors, and other esophageal, gastrointestinal, hepatic-biliary and pancreatic diseases in relation to epidermiology, immunology, microbiology, motility & nerve-gut interaction, endocrinology, nutrition & obesity, endoscopy, imaging and advanced hi-technology.

The main goal of *WJG* is to publish high quality commentary articles contributed by leading experts in gastroenterology and hepatology and original articles that combine the clinical practice and advanced basic research, to provide an interactive platform for clinicians and researchers in internal medicine, surgery, infectious diseases, traditional Chinese medicine, oncology, integrated Chinese and Western medicine, imaging, endoscopy, interventional therapy, pathology and other basic medical specialities, and thus eventually improving the clinical practice and healthcare for patients.

Indexed and abstracted in

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, Index Medicus, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology* and *Hepatology*, CAB Abstracts and Global Health. ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

Published by

The WJG Press

SUBMISSION OF MANUSCRIPTS

Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of The WJG Press, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the International Committee of Medical Journal Editors to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now available, to our knowledge, is http://www. clinicaltrials.gov sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

Authors should retain one copy of the text, tables, photographs and illustrations because rejected manuscripts will not be returned to the author(s) and the editors will not be responsible for loss or damage to photographs and illustrations sustained during mailing.

Online submissions

Manuscripts should be submitted through the Online Submission System at: http://wjg.wjgnet.com/wjg. Authors are highly recommended to consult the ONLINE INSTRUCTIONS TO AUTHORS (http://www.wjgnet.com/wjg/help/instructions.jsp) before attempting to submit online. For assistance, authors encountering problems with the Online Submission System may send an email describing the problem to submission@wjgnet.com, or by telephone: +86-10-85381892. If you submit your manuscript online, do not make a postal contribution. Repeated online submission for the same manuscript is strictly prohibited.

MANUSCRIPT PREPARATION

All contributions should be written in English. All articles must be submitted using word-processing software. All submissions must be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Style should conform to our house format. Required information for each of the manuscript sections is as follows:

Title page

Full manuscript title, running title, all author(s) name(s), affiliations, institution(s) and/or department(s) where the work was carried out; author contributions; disclosure of any financial support for the research; and the name, full address, telephone and fax numbers and email address of the corresponding author should be included. Titles should be concise and informative (remove all unnecessary words), emphasize what is new, and avoid abbreviations. A short running title of less than 40 letters should be provided. List the author(s)' name(s) as follows: initial and/or first name, middle name or initial(s), and full family name.

Author controbutions: The format of this section should be like this: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed research; Wang CL, Zou CC, Hong F and Wu XM performed research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed data; and Wang CL, Liang L and Fu JF wrote the paper.

Peer reviewers: All articles received are subject to peer review. Normally, three experts are invited for each article. Decision for acceptance is made only when at least two experts recommend an article for publication. Reviewers for accepted manuscripts are acknowledged in each manuscript, and reviewers of articles which were not accepted will be acknowledged at the end of each issue. To ensure the quality of the articles published in *WJG*, reviewers of accepted manuscripts will be announced by publishing the name, title/position and institution of the reviewer in the footnote accompanying the printed article. For example, reviewers: Professor Jing-Yuan Fang, Shanghai Institute of Digestive Disease, Shanghai, Affiliated Renji Hospital, Medical Faculty, Shanghai Jiaotong University, Shanghai, China; Professor Xin-Wei Han, Department of Radiology, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan Province, China; and Professor Anren Kuang, Department of Nuclear Medicine, Huaxi Hospital, Sichuan University, Chengdu, Sichuan Province, China.

Abstract

An informative, structured abstract of no more than 350 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections: AIM: Only the purpose should be included. METHODS: The materials, techniques, instruments and equipment, and the experimental procedures should be included. RESULTS: The observed and experimental results, including data, effects, outcome, *etc.* should be included. Authors should present *P* value where necessary, and also include any significant data. CONCLUSION: Accurate view and the value of the results should be included.

The format for structured abstracts can be found at: http://www.wjgnet.com/wjg/help/11.doc.

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles, rapid communication

and case reports, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the body text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, should be found at: http://www.wjgnet.com/wjg/help/instructions.jsp.

Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: …*etc.* It is our principle to publish high resolution-figures for the printed and E-versions.

Tables

Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of P values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscripts and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

Coding system

The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability[1,2]". If references are cited directly in the text, they should be put together within the text, for example, "From references[19,22-24], we know that..."

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

PMID requirement

PMID roots in the abstract serial number indexed by PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed). The author should supply the PMID for journal citation. For those references that have not been indexed by PubMed, a printed copy of the first page of the full reference should be submitted.

The accuracy of the information for journal citations is very important. Using the reference testing system, the authors and editor should check the authors name, title, journal title, publication date, volume number, start page, and end page. We will interlink all references with PubMed in an ASP file so that the readers can immediately access the abstract of the citations online.

DOI requirement

A CrossRef DOI® (Digital Object Identifier) name is a unique string created to identify a piece of scholarly content in the online environment. The author should supply the DOIs for journal citation(doi:10.3748/wjg.13.6458). This link (http://www.crossref.org/SimpleTextQuery/) allows you to retrieve Digital Object Identifiers (DOIs) for journal articles, books, and chapters by simply cutting and pasting the reference list into the box. You may use the form with any reference style, although the tool works most reliably if references are formatted in a standard style such as shown in this example: Assimakopoulos SF, Scopa CD, Vagianos CE. Pathophysiology of increased intestinal permeability in obstructive jaundice. *World J Gastroenterol* 2007; 13(48): 6458-6464

The accuracy of the information of journal citations is very important. We will interlink all references with DOI in ASP file so that readers can access the abstracts of cited articles online immediately.

Style for journal references

Authors: the name of the first author should be typed in bold-faced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

Style for book references

Authors: the name of the first author should be typed in bold-faced letters. The surname of all authors should be typed with the initial letter capitalized, followed by their abbreviated middle and first initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR) Book title. Publication number. Publication place: Publication press, Year: start page and end page.

Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Inappropriate references

Authors should always cite references that are relevant to their article, and avoid any inappropriate references. Inappropriate references include those linked with a hyphen when the difference between the two numbers is greater than five. For example, [1-6], [2-14] and [1, 3, 4-10, 22] are all considered inappropriate references. Authors should not cite their own unrelated published articles.

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/wjg/help/15.doc.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H pylori*, *E coli*, *etc.*

SUBMISSION OF THE REVISED MANUSCRIPTS AFTER ACCEPTED

Please revise your article according to the revision policies of *WJG*. The revised version including manuscript and high-resolution image figures (if any) should be copied on a floppy or compact disk. The author should send the revised manuscript, along with printed high-resolution color or black and white photos, copyright transfer letter, and responses to the reviewers by courier (such as EMS/DHL).

Editorial Office

World Journal of Gastroenterology
Editorial Department: Room 903
Ocean International Center, Building D
No. 62 Dongsihuan Zhonglu
Chaoyang District, Beijing 100025, China
E-mail: wjg@wjgnet.com
http://www.wjgnet.com
Telephone: +86-10-59080039
Fax: +86-10-85381893

Language evaluation

The language of a manuscript will be graded before it is sent for revision. (1) Grade A: priority publishing; (2) Grade B: minor language polishing; (3) Grade C: a great deal of language polishing needed; (4) Grade D: rejected. Revised articles should reach Grade A or B.

Copyright assignment form

Please download a Copyright assignment form from http://www.wjgnet.com/wjg/help/9.doc.

Responses to reviewers

Please revise your article according to the comments/suggestions provided by the reviewers. The format for responses to the reviewers' comments can be found at: http://www.wjgnet.com/wjg/help/10.doc.

Proof of financial support

For paper supported by a foundation, authors should provide a copy of the document and serial number of the foundation.

Links to documents related to the manuscript

WJG will be initiating a platform to promote dynamic interactions between the editors, peer reviewers, readers and authors. After a manuscript is published online, links to the PDF version of the submitted manuscript, the peer-reviewers' report and the revised manuscript will be put on-line. Readers can make comments on the peer reviewer's report, authors' responses to peer reviewers, and the revised manuscript. We hope that authors will benefit from this feedback and be able to revise the manuscript accordingly in a timely manner.

Science news releases

Authors of accepted manuscripts are suggested to write a science news item to promote their articles. The news will be released rapidly at EurekAlert/AAAS (http://www.eurekalert.org). The title for news items should be less than 90 characters; the summary should be less than 75 words; and main body less than 500 words. Science news items should be lawful, ethical, and strictly based on your original content with an attractive title and interesting pictures.

Publication fee

Authors of accepted articles must pay a publication fee.
EDITORIAL, TOPIC HIGHLIGHTS, BOOK REVIEWS and LETTERS TO THE EDITOR are published free of charge.

ISSN 1007-9327
CN 14-1219/R

World Journal of Gastroenterology®

Indexed and Abstracted in:

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, *Index Medicus*, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology and Hepatology*, CAB Abstracts and Global Health.
ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

Volume 14 Number 35
September 21, 2008
World J Gastroenterol
2008 September 21; 14(35): 5361-5488

Online Submissions
wjg.wjgnet.com
www.wjgnet.com
Printed on Acid-free Paper
世界胃肠病学杂志

A Weekly Journal of Gastroenterology and Hepatology

World Journal of Gastroenterology®

Editorial Board

2007-2009

Published by The WJG Press and Baishideng
Room 903, Ocean International Center, Building D
No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Fax: +86-10-8538-1893 E-mail: wjg@wjgnet.com http://www.wjgnet.com

The World Journal of Gastroenterology Editorial Board consists of 1208 members, representing a team of worldwide experts in gastroenterology and hepatology. They are from 60 countries, including Albania (1), Argentina (4), Australia (39), Austria (10), Belarus (1), Belgium (15), Brazil (2), Bulgaria (1), Canada (28), Chile (1), China (60), Croatia (2), Cuba (1), Czech (2), Denmark (7), Egypt (4), Estonia (1), Finland (4), France (44), Germany (108), Greece (9), Hungary (2), Iceland (1), India (12), Iran (3), Ireland (4), Israel (8), Italy (96), Japan (176), Lebanon (3), Lithuania (1), Macedonia (1), Malaysia (3), Mexico (6), Monaco (1), Morocco (1), The Netherlands (26), New Zealand (1), Nigeria (1), Norway (3), Pakistan (2), Peru (1), Poland (6), Portugal (1), Russia (3), Saudi Arabia (2), Serbia (1), Singapore (4), Slovakia (2), Slovenia (1), South Africa (2), South Korea (14), Spain (38), Sweden (15), Switzerland (13), Turkey (8), United Arab Emirates (1), United Kingdom (83), United States (316) and Uruguay (2).

Andrew V Biankin, *Sydney*
Filip Braet, *Sydney*
Andrew D Clouston, *Sydney*
Graham Cooksley, *Queensland*
Darrell HG Crawford, *Brisbane*
Adrian G Cummins, *Woodville South*
Guy D Eslick, *Sydney*
Michael A Fink, *Melbourne*
Robert JL Fraser, *Daw Park*
Peter Raymond Gibson, *Victoria*
Jacob George, *Westmead*
Mark D Gorrell, *Sydney*
Yik-Hong Ho, *Townsville*
Gerald J Holtmann, *Adelaide*
Michael Horowitz, *Adelaide*
John E Kellow, *Sydney*
Rupert Leong, *Concord*
Geoffrey W McCaughan, *Sydney*
Finlay A Macrae, *Victoria*
Daniel Markovich, *Brisbane*
Phillip S Oates, *Perth*
Jacqui Richmond, *Victoria*
Stephen M Riordan, *Sydney*
Ian C Roberts-Thomson, *Adelaide*
Devanshi Seth, *Camperdown*
Arthur Shulkes, *Melbourne*
Ross C Smith, *Sydney*
Kevin J Spring, *Brisbane*
Huy A Tran, *New South Wales*
Debbie Trinder, *Fremantle*
Martin J Veysey, *Gosford*
Daniel L Worthley, *Bedford*

Austria

Peter Ferenci, *Vienna*
Valentin Fuhrmann, *Vienna*
Alfred Gangl, *Vienna*
Christoph Gasche, *Vienna*
Kurt Lenz, *Linz*
Markus Peck-Radosavljevic, *Vienna*
Rudolf E Stauber, *Auenbruggerplatz*
Herbert Tilg, *Innsbruck*
Michael Trauner, *Graz*
Harald Vogelsang, *Vienna*
Guenter Weiss, *Innsbruck*

Belarus

Yury K Marakhouski, *Minsk*

Belgium

Rudi Beyaert, *Gent*
Bart Rik De Geest, *Leuven*
Inge I Depoortere, *Leuven*
Olivier Detry, *Liège*
Benedicte Y De Winter, *Antwerp*
Karel Geboes, *Leuven*
Thierry Gustot, *Brussels*
Yves J Horsmans, *Brussels*
Geert G Leroux-Roels, *Ghent*
Louis Libbrecht, *Leuven*
Etienne M Sokal, *Brussels*
Marc Peeters, *De Pintelaan*
Gert A Van Assche, *Leuven*
Yvan Vandenplas, *Brussels*
Eddie Wisse, *Keerbergen*

Brazil

Heitor Rosa, *Goiania*
Ana Cristina Simões e Silva, *Belo Horizonte*

Bulgaria

Zahariy Krastev, *Sofia*

Canada

Fernando Alvarez, *Québec*
David Armstrong, *Ontario*
Jeffrey P Baker, *Toronto*
Olivier Barbier, *Québec*
Nancy Baxter, *Toronto*
Matthew Bjerknes, *Toronto*
Frank J Burczynski, *Manitoba*
Michael F Byrne, *Vancouver*
Wang-Xue Chen, *Ottawa*
Chantal Guillemette, *Québec*
Samuel S Lee, *Calgary*
Gary A Levy, *Toronto*
Andrew L Mason, *Alberta*
John K Marshall, *Ontario*
Donna-Marie McCafferty, *Calgary*
Thomas I Michalak, *St. John's*
Gerald Y Minuk, *Manitoba*
Paul Moayyedi, *Hamilton*
Kostas Pantopoulos, *Québec*
William G Paterson, *Kingston*
Eldon Shaffer, *Calgary*
Morris Sherman, *Toronto*
Martin Storr, *Calgary*
Elena F Verdu, *Ontario*
John L Wallace, *Calgary*
Eric M Yoshida, *Vancouver*

Chile

Silvana Zanlungo, *Santiago*

China

Henry LY Chan, *Hongkong*
Xiao-Ping Chen, *Wuhan*
Zong-Jie Cui, *Beijing*
Da-Jun Deng, *Beijing*
Er-Dan Dong, *Beijing*
Sheung-Tat Fan, *Hong Kong*
Jin Gu, *Beijing*
Xin-Yuan Guan, *Pokfulam*
De-Wu Han, *Taiyuan*
Ming-Liang He, *Hong Kong*
Wayne HC Hu, *Hong Kong*
Chee-Kin Hui, *Hong Kong*
Ching-Lung Lai, *Hong Kong*
Kam Chuen Lai, *Hong Kong*
James YW Lau, *Hong Kong*
Yuk-Tong Lee, *Hong Kong*
Suet-Yi Leung, *Hong Kong*
Wai-Keung Leung, *Hong Kong*
John M Luk, *Pokfulam*
Chung-Mau Lo, *Hong Kong*
Jing-Yun Ma, *Beijing*
Ronnie Tung Ping Poon, *Hong Kong*
Lun-Xiu Qin, *Shanghai*
Yu-Gang Song, *Guangzhou*
Qin Su, *Beijing*
Wai-Man Wong, *Hong Kong*
Hong Xiao, *Shanghai*
Dong-Liang Yang, *Wuhan*
Winnie Yeo, *Hong Kong*
Yuan Yuan, *Shenyang*
Man-Fung Yuen, *Hong Kong*
Jian-Zhong Zhang, *Beijing*
Xin-Xin Zhang, *Shanghai*
Bo-Jian Zheng, *Hong Kong*
Shu Zheng, *Hangzhou*

Croatia

Tamara Cacev, *Zagreb*
Marko Duvnjak, *Zagreb*

Cuba

Damian C Rodriguez, *Havana*

Czech

Milan Jirsa, *Praha*
Pavel Trunečka, *Prague*

Denmark

Peter Bytzer, *Copenhagen*
Asbjørn M Drewes, *Aalborg*
Hans Gregersen, *Aalborg*
Jens H Henriksen, *Hvidovre*
Claus P Hovendal, *Odense*
Fin S Larsen, *Copenhagen*
Søren Møller, *Hvidovre*

Egypt

Abdel-Rahman El-Zayadi, *Giza*
Amr M Helmy, *Cairo*
Ayman Yosry, *Cairo*

Estonia

Riina Salupere, *Tartu*

Finland

Irma E Jarvela, *Helsinki*
Katri M Kaukinen, *Tampere*
Minna Nyström, *Helsinki*
Pentti Sipponen, *Espoo*

France

Bettaieb Ali, *Dijon*
Corlu Anne, *Rennes*
Denis Ardid, *Clermont-Ferrand*
Charles P Balabaud, *Bordeaux*
Soumeya Bekri, *Rouen*
Jacques Belghiti, *Clichy*
Jacques Bernuau, *Clichy Cedex*
Pierre Brissot, *Rennes*
Patrice P Cacoub, *Paris*
Franck Carbonnel, *Besancon*
Laurent Castera, *Pessac*
Bruno Clément, *Rennes*
Benoit Coffin, *Colombes*
Jacques Cosnes, *Paris*
Thomas Decaens, *Cedex*

Francoise L Fabiani, *Angers*
Gérard Feldmann, *Paris*
Jean Fioramonti, *Toulouse*
Jean-Noël Freund, *Strasbourg*
Jean-Paul Galmiche, *Nantes*
Catherine Guettier, *Villejuif*
Chantal Housset, *Paris*
Juan L Iovanna, *Marseille*
Rene Lambert, *Lyon*
Patrick Marcellin, *Paris*
Philippe Mathurin, *Lille*
Tamara Matysiak–Budnik, *Paris*
Francis Mégraud, *Bordeaux*
Richard Moreau, *Clichy*
Thierry Piche, *Nice*
Raoul Poupon, *Paris*
Jean Rosenbaum, *Bordeaux*
Dominique Marie Roulot, *Bobigny*
Thierry Poynard, *Paris*
Jean-Philippe Salier, *Rouen*
Didier Samuel, *Villejuif*
Jean-Yves Scoazec, *Lyon*
Khalid A Tazi, *Clichy*
Emmanuel Tiret, *Paris*
Baumert F Thomas, *Strasbourg*
Marie-Catherine Vozenin-brotons, *Villejuif*
Jean-Pierre H Zarski, *Grenoble*
Jessica Zucman-Rossi, *Paris*

Germany

Hans-Dieter Allescher, *G-Partenkirchen*
Martin Anlauf, *Kiel*
Rudolf Arnold, *Marburg*
Max G Bachem, *Ulm*
Thomas F Baumert, *Freiburg*
Daniel C Baumgart, *Berlin*
Hubert Blum, *Freiburg*
Thomas Bock, *Tuebingen*
Katja Breitkopf, *Mannheim*
Dunja Bruder, *Braunschweig*
Markus W Büchler, *Heidelberg*
Christa Buechler, *Regensburg*
Reinhard Buettner, *Bonn*
Elke Cario, *Essen*
Uta Dahmen, *Essen*
Christoph F Dietrich, *Bad Mergentheim*
Arno J Dormann, *Koeln*
Rainer J Duchmann, *Berlin*
Volker F Eckardt, *Wiesbaden*
Paul Enck, *Tuebingen*
Fred Fändrich, *Kiel*
Ulrich R Fölsch, *Kiel*
Helmut Friess, *Heidelberg*
Peter R Galle, *Mainz*
Nikolaus Gassler, *Aachen*
Andreas Geier, *Aachen*
Markus Gerhard, *Munich*
Wolfram H Gerlich, *Giessen*
Dieter Glebe, *Giessen*
Burkhard Göke, *Munich*
Florian Graepler, *Tuebingen*
Axel M Gressner, *Aachen*
Veit Gülberg, *Munich*
Rainer Haas, *Munich*
Eckhart G Hahn, *Erlangen*
Stephan Hellmig, *Kiel*
Martin Hennenberg, *Bonn*
Johannes Herkel, *Hamburg*
Klaus R Herrlinger, *Stuttgart*
Eva Herrmann, *Homburg/Saar*
Eberhard Hildt, *Berlin*
Joerg C Hoffmann, *Berlin*
Ferdinand Hofstaedter, *Regensburg*
Werner Hohenberger, *Erlangen*
Jörg C Kalff, *Bonn*
Ralf Jakobs, *Ludwigshafen*
Jutta Keller, *Hamburg*
Andrej Khandoga, *Munich*
Sibylle Koletzko, *München*
Stefan Kubicka, *Hannover*
Joachim Labenz, *Siegen*
Frank Lammert, *Bonn*
Thomas Langmann, *Regensburg*
Christian Liedtke, *Aachen*
Matthias Löhr, *Mannheim*
Christian Maaser, *Muenster*
Ahmed Madisch, *Dresden*
Peter Malfertheiner, *Magdeburg*
Michael P Manns, *Hannover*
Helmut Messmann, *Augsburg*
Stephan Miehlke, *Dresden*
Sabine Mihm, *Göttingen*
Silvio Nadalin, *Essen*
Markus F Neurath, *Mainz*
Johann Ockenga, *Berlin*
Florian Obermeier, *Regensburg*
Gustav Paumgartner, *Munich*
Ulrich KS Peitz, *Magdeburg*
Markus Reiser, *Bochum*
Emil C Reisinger, *Rostock*
Steffen Rickes, *Magdeburg*
Tilman Sauerbruch, *Bonn*
Dieter Saur, *Munich*
Hans Scherubl, *Berlin*
Joerg Schirra, *Munich*
Roland M Schmid, *München*
Volker Schmitz, *Bonn*
Andreas G Schreyer, *Regensburg*
Tobias Schroeder, *Essen*
Henning Schulze-Bergkamen, *Mainz*
Hans Seifert, *Oldenburg*
Norbert Senninger, *Muenster*
Manfred V Singer, *Mannheim*
Gisela Sparmann, *Rostock*
Christian J Steib, *München*
Jurgen M Stein, *Frankfurt*
Ulrike S Stein, *Berlin*
Manfred Stolte, *Bayreuth*
Christian P Strassburg, *Hannover*
Wolfgang R Stremmel, *Heidelberg*
Harald F Teutsch, *Ulm*
Robert Thimme, *Freiburg*
Hans L Tillmann, *Leipzig*
Tung-Yu Tsui, *Regensburg*
Axel Ulsenheimer, *Munich*
Patrick Veit-Haibach, *Essen*
Claudia Veltkamp, *Heidelberg*
Siegfried Wagner, *Deggendorf*
Henning Walczak, *Heidelberg*
Heiner Wedemeyer, *Hannover*
Fritz von Weizsacker, *Berlin*
Jens Werner, *Heidelberg*
Bertram Wiedenmann, *Berlin*
Reiner Wiest, *Regensburg*
Stefan Wirth, *Wuppertal*
Stefan JP Zeuzem, *Homburg*

Greece

Alexandra A Alexopoulou, *Athens*
George N Dalekos, *Larissa*
Christos Dervenis, *Athens*
Melanie Maria Deutsch, *Athens*
Tsianos Epameinondas, *Ioannina*
Elias A Kouroumalis, *Heraklion*
George Papatheodoridis, *Athens*
Spiros Sgouros, *Athens*

Hungary

Peter L Lakatos, *Budapest*
Zsuzsa Szondy, *Debrecen*

Iceland

Hallgrimur Gudjonsson, *Reykjavik*

India

Philip Abraham, *Mumbai*
Rakesh Aggarwal, *Lucknow*
Kunissery A Balasubramanian, *Vellore*
Deepak Kumar Bhasin, *Chandigarh*
Sujit K Bhattacharya, *Kolkata*
Yogesh K Chawla, *Chandigarh*
Radha K Dhiman, *Chandigarh*
Sri Prakash Misra, *Allahabad*
Ramesh Roop Rai, *Jaipur*
Nageshwar D Reddy, *Hyderabad*
Rakesh Kumar Tandon, *New Delhi*

Iran

Seyed-Moayed Alavian, *Tehran*
Reza Malekzadeh, *Tehran*
Seyed A Taghavi, *Shiraz*

Ireland

Billy Bourke, *Dublin*
Ronan A Cahill, *Cork*
Anthony P Moran, *Galway*

Israel

Simon Bar-Meir, *Hashomer*
Abraham R Eliakim, *Haifa*
Zvi Fireman, *Hadera*
Yaron Ilan, *Jerusalem*
Avidan U Neumann, *Ramat-Gan*
Yaron Niv, *Pardesia*
Ran Oren, *Tel Aviv*
Ami D Sperber, *Beer-Sheva*

Italy

Giovanni Addolorato, *Roma*
Luigi E Adinolfi, *Naples*
Domenico Alvaro, *Rome*
Mario Angelico, *Rome*
Vito Annese, *San Giovanni Rotond*
Filippo Ansaldi, *Genoa*
Adolfo F Attili, *Roma*
Giovanni Barbara, *Bologna*
Claudio Bassi, *Verona*
Gabrio Bassotti, *Perugia*
Pier M Battezzati, *Milan*
Stefano Bellentani, *Carpi*
Antomio Benedetti, *Ancona*
Mauro Bernardi, *Bologna*
Livia Biancone, *Rome*
Luigi Bonavina, *Milano*
Flavia Bortolotti, *Padova*
Giuseppe Brisinda, *Rome*
Elisabetta Buscarini, *Crema*
Giovanni Cammarota, *Roma*

Antonino Cavallari, *Bologna*
Giuseppe Chiarioni, *Valeggio*
Michele Cicala, *Rome*
Massimo Colombo, *Milan*
Amedeo Columbano, *Cagliari*
Massimo Conio, *Sanremo*
Dario Conte, *Milano*
Gino R Corazza, *Pavia*
Francesco Costa, *Pisa*
Antonio Craxi, *Palermo*
Silvio Danese, *Milan*
Roberto de Franchis, *Milano*
Roberto De Giorgio, *Bologna*
Maria Stella De Mitri, *Bologna*
Giovanni D De Palma, *Naples*
Fabio Farinati, *Padua*
Giammarco Fava, *Ancona*
Francesco Feo, *Sassari*
Fiorucci Stefano, *Perugia*
Andrea Galli, *Firenze*
Valeria Ghisett, *Turin*
Gianluigi Giannelli, *Bari*
Edoardo G Giannini, *Genoa*
Paolo Gionchetti, *Bologna*
Fabio Grizzi, *Milan*
Salvatore Gruttadauria, *Palermo*
Mario Guslandi, *Milano*
Pietro Invernizzi, *Milan*
Ezio Laconi, *Cagliari*
Giacomo Laffi, *Firenze*
Giovanni Maconi, *Milan*
Lucia Malaguarnera, *Catania*
Emanuele D Mangoni, *Napoli*
Paolo Manzoni, *Torino*
Giulio Marchesini, *Bologna*
Fabio Marra, *Florence*
Marco Marzioni, *Ancona*
Giuseppe Mazzella, *Bologna*
Mario U Mondelli, *Pavia*
Giuseppe Montalto, *Palermo*
Giovanni Monteleone, *Rome*
Giovanni Musso, *Torino*
Gerardo Nardone, *Napoli*
Valerio Nobili, *Rome*
Fabio Pace, *Milano*
Luisi Pagliaro, *Palermo*
Francesco Pallone, *Rome*
Fabrizio R Parente, *Milan*
Maurizio Parola, *Torino*
Francesco Perri, *San Giovanni Rotondo*
Raffaele Pezzilli, *Bologna*
Alberto Pilotto, *San Giovanni Rotondo*
Alberto Piperno, *Monza*
Mario Pirisi, *Novara*
Anna C Piscaglia, *Roma*
Paolo Del Poggio, *Treviglio*
Gabriele B Porro, *Milano*
Piero Portincasa, *Bari*
Cosimo Prantera, *Roma*
Bernardino Rampone, *Siena*
Oliviero Riggio, *Rome*
Claudio Romano, *Messina*
Marco Romano, *Napoli*
Gerardo Rosati, *Potenza*
Mario Del Tacca, *Pisa*
Gloria Taliani, *Rome*
Pier A Testoni, *Milan*
Enrico Roda, *Bologna*
Domenico Sansonno, *Bari*
Vincenzo Savarino, *Genova*
Vincenzo Stanghellini, *Bologna*
Giovanni Tarantino, *Naples*
Roberto Testa, *Genoa*
Dino Vaira, *Bologna*
Anna Linda Zignego, *Florence*

Japan

Kyoichi Adachi, *Izumo*
Yasushi Adachi, *Sapporo*
Taiji Akamatsu, *Matsumoto*
Sk Md Fazle Akbar, *Ehime*
Takafumi Ando, *Nagoya*
Akira Andoh, *Otsu*
Taku Aoki, *Tokyo*
Masahiro Arai, *Tokyo*
Tetsuo Arakawa, *Osaka*
Yasuji Arase, *Tokyo*
Masahiro Asaka, *Sapporo*
Hitoshi Asakura, *Tokyo*
Takeshi Azuma, *Fukui*
Yoichi Chida, *Fukuoka*
Takahiro Fujimori, *Tochigi*
Jiro Fujimoto, *Hyogo*
Kazuma Fujimoto, *Saga*
Mitsuhiro Fujishiro, *Tokyo*
Yoshihide Fujiyama, *Otsu*
Hiroyuki Fukui, *Tochigi*
Hiroyuki Hanai, *Hamamatsu*
Naohiko Harada, *Fukuoka*
Makoto Hashizume, *Fukuoka*
Tetsuo Hayakawa, *Nagoya*
Toru Hiyama, *Higashihiroshima*
Kazuhide Higuchi, *Osaka*
Keisuke Hino, *Ube*
Keiji Hirata, *Kitakyushu*
Yuji Iimuro, *Nishinomiya*
Kenji Ikeda, *Tokyo*
Toru Ikegami, *Fukuoka*
Kenichi Ikejima, *Bunkyo-ku*
Fumio Imazeki, *Chiba*
Yutaka Inagaki, *Kanagawa*
Yasuhiro Inokuchi, *Yokohama*
Haruhiro Inoue, *Yokohama*
Masayasu Inoue, *Osaka*
Hiromi Ishibashi, *Nagasaki*
Shunji Ishihara, *Izumo*
Toru Ishikawa, *Niigata*
Kei Ito, *Sendai*
Masayoshi Ito, *Tokyo*
Hiroaki Itoh, *Akita*
Ryuichi Iwakiri, *Saga*
Yoshiaki Iwasaki, *Okayama*
Terumi Kamisawa, *Tokyo*
Hiroshi Kaneko, *Aichi-Gun*
Shuichi Kaneko, *Kanazawa*
Takashi Kanematsu, *Nagasaki*
Mitsuo Katano, *Fukuoka*
Junji Kato, *Sapporo*
Mototsugu Kato, *Sapporo*
Shinzo Kato, *Tokyo*
Norifumi Kawada, *Osaka*
Sunao Kawano, *Osaka*
Mitsuhiro Kida, *Kanagawa*
Yoshikazu Kinoshita, *Izumo*
Tsuneo Kitamura, *Chiba*
Seigo Kitano, *Oita*
Kazuhiko Koike, *Tokyo*
Norihiro Kokudo, *Tokyo*
Satoshi Kondo, *Sapporo*
Shoji Kubo, *Osaka*
Shigeki Kuriyama, *Kagawa*[2]
Katsunori Iijima, *Sendai*
Masato Kusunoki, *Tsu Mie*
Shin Maeda, *Tokyo*
Shigeru Marubashi, *Suita*
Masatoshi Makuuchi, *Tokyo*
Osamu Matsui, *Kanazawa*
Yasuhiro Matsumura, *Chiba*
Yasushi Matsuzaki, *Tsukuba*
Kiyoshi Migita, *Omura*
Kenji Miki, *Tokyo*
Tetsuya Mine, *Kanagawa*
Hiroto Miwa, *Hyogo*
Masashi Mizokami, *Nagoya*
Yoshiaki Mizuguchi, *Tokyo*
Motowo Mizuno, *Hiroshima*
Morito Monden, *Suita*
Hisataka S Moriwaki, *Gifu*
Yasuaki Motomura, *Iizuka*
Yoshiharu Motoo, *Kanazawa*
Naofumi Mukaida, *Kanazawa*
Kazunari Murakami, *Oita*
Kunihiko Murase, *Tusima*
Hiroaki Nagano, *Suita*
Masahito Nagaki, *Gifu*
Masaki Nagaya, *Kawasaki*
Yujl Naito, *Kyoto*
Atsushi Nakajima, *Yokohama*
Hisato Nakajima, *Tokyo*
Hiroki Nakamura, *Yamaguchi*
Shotaro Nakamura, *Fukuoka*
Mikio Nishioka, *Niihama*
Shuji Nomoto, *Nagoya*
Susumu Ohmada, *Maebashi*
Hirohide Ohnishi, *Akita*
Masayuki Ohta, *Oita*
Tetsuo Ohta, *Kanazawa*
Kazuichi Okazaki, *Osaka*
Katsuhisa Omagari, *Nagasaki*
Saburo Onishi, *Nankoku*
Morikazu Onji, *Ehime*
Satoshi Osawa, *Hamamatsu*
Masanobu Oshima, *Kanazawa*
Hiromitsu Saisho, *Chiba*
Hidetsugu Saito, *Tokyo*
Yutaka Saito, *Tokyo*
Isao Sakaida, *Yamaguchi*
Michiie Sakamoto, *Tokyo*
Yasushi Sano, *Chiba*
Hiroki Sasaki, *Tokyo*
Iwao Sasaki, *Sendai*
Motoko Sasaki, *Kanazawa*
Chifumi Sato, *Tokyo*
Shuichi Seki, *Osaka*
Hiroshi Shimada, *Yokohama*
Mitsuo Shimada, *Tokushima*
Tomohiko Shimatan, *Hiroshima*
Hiroaki Shimizu, *Chiba*
Ichiro Shimizu, *Tokushima*
Yukihiro Shimizu, *Kyoto*
Shinji Shimoda, *Fukuoka*
Tooru Shimosegawa, *Sendai*
Tadashi Shimoyama, *Hirosaki*
Ken Shirabe, *Iizuka City*
Yoshio Shirai, *Niigata*
Katsuya Shiraki, *Mie*
Yasushi Shiratori, *Okayama*
Masayuki Sho, *Nara*
Yasuhiko Sugawara, *Tokyo*
Hidekazu Suzuki, *Tokyo*
Minoru Tada, *Tokyo*
Tadatoshi Takayama, *Tokyo*
Tadashi Takeda, *Osaka*
Koji Takeuchi, *Kyoto*
Kiichi Tamada, *Tochigi*
Akira Tanaka, *Kyoto*
Eiji Tanaka, *Matsumoto*
Noriaki Tanaka, *Okayama*
Shinji Tanaka, *Hiroshima*
Hideki Taniguchi, *Yokohama*
Kyuichi Tanikawa, *Kurume*
Akira Terano, *Shimotsugagun*
Hitoshi Togash, *Yamagata*
Shinji Togo, *Yokohama*
Kazunari Tominaga, *Osaka*
Takuji Torimura, *Fukuoka*

Minoru Toyota, *Sapporo*
Akihito Tsubota, *Chiba*
Takato Ueno, *Kurume*
Naomi Uemura, *Tokyo*
Shinichi Wada, *Tochigi*
Hiroyuki Watanabe, *Kanazawa*
Toshio Watanabe, *Osaka*
Yuji Watanabe, *Ehime*
Toshiaki Watanabe, *Tokyo*
Chun-Yang Wen, *Nagasaki*
Satoshi Yamagiwa, *Niigata*
Koji Yamaguchi, *Fukuoka*
Takayuki Yamamoto, *Yokkaichi*
Takashi Yao, *Fukuoka*
Masashi Yoneda, *Tochigi*
Hiroshi Yoshida, *Tokyo*
Masashi Yoshida, *Tokyo*
Norimasa Yoshida, *Kyoto*
Hitoshi Yoshiji, *Nara*
Kentaro Yoshika, *Toyoake*
Yasunobu Yoshikai, *Fukuoka*
Masahide Yoshikawa, *Kashihara*
Katsutoshi Yoshizato, *Higashihiroshima*

Lebanon

Bassam N Abboud, *Beirut*
Ala I Sharara, *Beirut*
Joseph D Boujaoude, *Beirut*

Lithuania

Limas Kupcinskas, *Kaunas*

Macedonia

Vladimir C Serafimoski, *Skopje*

Malaysia

Andrew Seng Boon Chua, *Ipoh*
Khean-Lee Goh, Kuala *Lumpur*
Jayaram Menon, *Sabah*

Mexico

Diego Garcia-Compean, *Monterrey*
Eduardo R Marin-Lopez, *Jesús García*
Nahum Méndez-Sánchez, *Mexico*
Saúl Villa-Treviño, *México*

Monaco

Patrick Rampal, *Monaco*

Morocco

Abdellah Essaid, *Rabat*

The Netherlands

Ulrich Beuers, *Amsterdam*
Gerd Bouma, *Amsterdam*
Lee Bouwman, *Leiden*
J Bart A Crusius, *Amsterdam*
NKH de Boer, *Amsterdam*
Koert P de Jong, *Groningen*
Henrike Hamer, *Maastricht*
Frank Hoentjen, *Haarlem*
Janine K Kruit, *Groningen*
Ernst J Kuipers, *Rotterdam*
CBHW Lamers, *Leiden*
Ton Lisman, *Utrecht*
Yi Liu, *Amsterdam*
Jeroen Maljaars, *Maastricht*
Servaas Morré, *Amsterdam*
Chris JJ Mulder, *Amsterdam*
Michael Müller, *Wageningen*
Amado S Peña, *Amsterdam*
Robert J Porte, *Groningen*
Ingrid B Renes, *Rotterdam*
Andreas Smout, *Utrecht*
Paul E Sijens, *Groningen*
Reinhold W Stockbrugger, *Maastricht*
Luc JW van der Laan, *Rotterdam*
Karel van Erpecum, *Utrecht*
Gerard P VanBerge-Henegouwen,*Utrecht*

New Zealand

Ian D Wallace, *Auckland*

Nigeria

Samuel B Olaleye, *Ibadan*

Norway

Trond Berg, *Oslo*
Tom H Karlsen, *Oslo*
Helge L Waldum, *Trondheim*

Pakistan

Muhammad S Khokhar, *Lahore*
Syed MW Jafri, *Karachi*

Peru

Hector H Garcia, *Lima*

Poland

Tomasz Brzozowski, *Cracow*
Robert Flisiak, *Bialystok*
Hanna Gregorek, *Warsaw*
Dariusz M Lebensztejn, *Bialystok*
Wojciech G Polak, *Wroclaw*
Marek Hartleb, *Katowice*

Portugal

Miguel C De Moura, *Lisbon*

Russia

Vladimir T Ivashkin, *Moscow*
Leonid Lazebnik, *Moscow*
Vasiliy I Reshetnyak, *Moscow*

Saudi Arabia

Ibrahim A Al Mofleh, *Riyadh*
Ahmed Helmy, *Riyadh*

Serbia

Dusan M Jovanovic, *Sremska Kamenica*

Singapore

Bow Ho, *Singapore*
Khek-Yu Ho, *Singapore*
Fock Kwong Ming, *Singapore*
Francis Seow-Choen, *Singapore*

Slovakia

Silvia Pastorekova, *Bratislava*
Anton Vavrecka, *Bratislava*

Slovenia

Sasa Markovic, *Ljubljana*

South Africa

Rosemar Joyce Burnett, *Pretoria*
Michael C Kew, *Parktown*

South Korea

Byung Ihn Choi, *Seoul*
Ho Soon Choi, *Seoul*
Marie Yeo, *Suwon*
Sun Pyo Hong, *Gyeonggi-do*
Jae J Kim, *Seoul*
Jin-Hong Kim, *Suwon*
Myung-Hwan Kim, *Seoul*
Chang Hong Lee, *Seoul*
Jong Kyun Lee, *Seoul*
Eun-Yi Moon, *Seoul*
Jae-Gahb Park, *Seoul*
Dong Wan Seo, *Seoul*
Dong Jin Suh, *Seoul*
Byung Chul Yoo, *Seoul*

Spain

Juan G Abraldes, *Barcelona*
Agustin Albillos, *Madrid*
Raul J Andrade, *Málaga*
Luis Aparisi, *Valencia*
Fernando Azpiroz, *Barcelona*
Ramon Bataller, *Barcelona*
Josep M Bordas, *Barcelona*
Xavier Calvet, *Sabadell*
Jordi Camps, *Catalunya*
Andres Cardenas, *Barcelona*
Vicente Carreño, *Madrid*
Jose Castellote, *Barcelona*
Antoni Castells, *Barcelona*
Vicente Felipo, *Valencia*
Juan C Garcia-Pagán, *Barcelona*
Jaime B Genover, *Barcelona*
Javier P Gisbert, *Madrid*
Jaime Guardia, *Barcelona*
Isabel Fabregat, *Barcelona*
Mercedes Fernandez, *Barcelona*
Angel Lanas, *Zaragoza*
Juan-Ramón Larrubia, *Guadalajara*
Laura Lladóa, *Barcelona*
María IT López, *Jaén*
Juan R Malagelada, *Barcelona*
José M Mato, *Derio*
Juan F Medina, *Pamplona*
Miguel A Muñoz-Navas, *Pamplona*
Julian Panes, *Barcelona*
Miguel M Perez, *Valencia*
Miguel Perez-Mateo, *Alicante*

Josep M Pique, *Barcelona*
Jesús M Prieto, *Pamplona*
Sabino Riestra, *Pola De Siero*
Luis Rodrigo, *Oviedo*
Manuel Romero-Gómez, *Sevilla*
Joan Roselló-Catafau, *Barcelona*

Sweden

Einar S Björnsson, *Gothenburg*
Curt Einarsson, *Huddinge*
Per M Hellström, *Stockholm*
Ulf Hindorf, *Lund*
Elisabeth Hultgren-Hörnquist, *Örebro*
Anders E Lehmann, *Mölndal*
Hanns-Ulrich Marschall, *Stockholm*
Lars C Olbe, *Molndal*
Lars A Pahlman, *Uppsala*
Matti Sallberg, *Stockholm*
Magnus Simrén, *Göteborg*
Xiao-Feng Sun, *Linköping*
Ervin Tóth, *Malmö*
Weimin Ye, *Stockholm*
Christer S von Holstein, *Lund*

Switzerland

Chrish Beglinger, *Basel*
Pierre A Clavien, *Zurich*
Jean-Francois Dufour, *Bern*
Franco Fortunato, *Zürich*
Jean L Frossard, *Geneva*
Gerd A Kullak-Ublick, *Zurich*
Pierre Michetti, *Lausanne*
Francesco Negro, *Genève*
Bruno Stieger, *Zurich*
Radu Tutuian, *Zurich*
Stephan R Vavricka, *Zurich*
Gerhard Rogler, *Zurich*
Arthur Zimmermann, *Berne*

Turkey

Yusuf Bayraktar, *Ankara*
Figen Gurakan, *Ankara*
Aydin Karabacakoglu, *Konya*
Serdar Karakose, *Konya*
Hizir Kurtel, *Istanbul*
Osman C Ozdogan, *Istanbul*
Özlem Yilmaz, *Izmir*
Cihan Yurdaydin, *Ankara*

United Arab Emirates

Sherif M Karam, *Al-Ain*

United Kingdom

David H Adams, *Birmingham*
Simon Afford, *Birmingham*
Navneet K Ahluwalia, *Stockport*
Ahmed Alzaraa, *Manchester*
Lesley A Anderson, *Belfast*
Charalambos G Antoniades, *London*
Anthony TR Axon, *Leeds*
Qasim Aziz, *Manchester*
Nicholas M Barnes, *Birmingham*
Jim D Bell, *London*
Mairi Brittan, *London*
Alastair D Burt, *Newcastle*
Simon S Campbell, *Manchester*
Simon R Carding, *Leeds*
Paul J Ciclitira, *London*
Eithne Costello, *Liverpool*
Tatjana Crnogorac-Jurcevic, *London*
Harry Dalton, *Truro*
Amar P Dhillon, *London*
William Dickey, *Londonderry*
James E East, *London*
Emad M El-Omar, *Aberdeen*
Ahmed M Elsharkawy, *Newcastle Upon Tyne*
Annette Fristscher-Ravens, *London*
Elizabeth Furrie, *Dundee*
Daniel R Gaya, *Edinburgh*
Subrata Ghosh, *London*
William Greenhalf, *Liverpool*
Indra N Guha, *Southampton*
Peter C Hayes, *Edinburgh*
Gwo-Tzer Ho, *Edinburgh*
Anthony R Hobson, *Salford*
Lesley A Houghton, *Manchester*
Stefan G Hübscher, *Birmingham*
Robin Hughes, *London*
Pali Hungin, *Stockton*
David P Hurlstone, *Sheffield*
Rajiv Jalan, *London*
Janusz AZ Jankowski, *Oxford*
Brian T Johnston, *Belfast*
David EJ Jones, *Newcastle*
Roger Jones, *London*
Michael A Kamm, *Harrow*
Peter Karayiannis, *London*
Laurens Kruidenier, *Harlow*
Patricia F Lalor, *Birmingham*
Chee Hooi Lim, *Midlands*
Hong-Xiang Liu, *Cambridge*
Yun Ma, *London*
Kenneth E L McColl, *Glasgow*
Stuart AC McDonald, *London*
Dermot P Mcgovern, *Oxford*
Giorgina Mieli-Vergani, *London*
Nikolai V Naoumov, *London*
John P Neoptolemos, *Liverpool*
James Neuberger, *Birmingham*
Philip Noel Newsome, *Birmingham*
Mark S Pearce, *Newcastle Upon Tyne*
Stephen P Pereira, *London*
D Mark Pritchard, *Liverpool*
Sakhawat Rahman, *London*
Stephen E Roberts, *Swansea*
Marco Senzolo, *Padova*
Soraya Shirazi-Beechey, *Liverpool*
Robert Sutton, *Liverpool*
Simon D Taylor-Robinson, *London*
Paris P Tekkis, *London*
Ulrich Thalheimer, *London*
David G Thompson, *Salford*
Nick P Thompson, *Newcastle*
David Tosh, *Bath*
Frank I Tovey, *London*
Chris Tselepis, *Birmingham*
Diego Vergani, *London*
Geoffrey Warhurst, *Salford*
Alastair John Watson, *Liverpool*
Peter J Whorwell, *Manchester*
Roger Williams, *London*
Karen L Wright, *Bath*
Min Zhao, *Foresterhill*

United States

Manal F Abdelmalek, *Durham*
Gary A Abrams, *Birmingham*
Maria T Abreu, *New York*
Reid B Adams, *Virginia*
Golo Ahlenstiel, *Bethesda*
BS Anand, *Houston*
Frank A Anania, *Atlanta*
M Ananthanarayanan, *New York*
Gavin E Arteel, *Louisville*
Jasmohan S Bajaj, *Milwaukee*
Subhas Banerjee, *Palo Alto*
Peter A Banks, *Boston*
Jamie S Barkin, *Miami Beach*
Kim E Barrett, *San Diego*
Marc D Basson, *Detroit*
Anthony J Bauer, *Pittsburgh*
Wallace F Berman, *Durham*
Timothy R Billiar, *Pittsburgh*
Edmund J Bini, *New York*
David G Binion, *Milwaukee*
Jennifer D Black, *Buffalo*
Herbert L Bonkovsky, *Charlotte*
Carla W Brady, *Durham*
Andrea D Branch, *New York*
Robert S Bresalier, *Houston*
Alan L Buchman, *Chicago*
Ronald W Busuttil, *Los Angeles*
Alan Cahill, *Philadelphia*
John M Carethers, *San Diego*
David L Carr-Locke, *Boston*
Maurice A Cerulli, *New York*
Ravi S Chari, *Nashville*
Jiande Chen, *Galveston*
Xian-Ming Chen, *Omaha*
Xin Chen, *San Francisco*
Ramsey Chi-man Cheung, *Palo Alto*
William D Chey, *Ann Arbor*
John Y Chiang, *Rootstown*
Parimal Chowdhury, *Arkansas*
Raymond T Chung, *Boston*
James M Church, *Cleveland*
Ram Chuttani, *Boston*
Mark G Clemens, *Charlotte*
Ana J Coito, *Los Angeles*
Vincent Coghlan, *Beaverton*
David Cronin II, *New Haven*
John Cuppoletti, *Cincinnati*
Mark J Czaja, *New York*
Peter V Danenberg, *Los Angeles*
Kiron M Das, New *Brunswick*
Conor P Delaney, *Cleveland*
Jose L del Pozo, *Rochester*
Sharon DeMorrow, *Temple*
Deborah L Diamond, *Seattle*
Douglas A Drossman, *Chapel Hill*
Katerina Dvorak, *Tucson*
Bijan Eghtesad, *Cleveland*
Hala El-Zimaity, *Houston*
Michelle Embree-Ku, *Providence*
Sukru Emre, New *Haven*
Douglas G Farmer, *Los Angeles*
Alessio Fasano, *Baltimore*
Mark A Feitelson, *Philadelphia*
Ariel E Feldstein, *Cleveland*
Alessandro Fichera, *Chicago*
Robert L Fine, *New York*
Magali Fontaine, *Stanford*
Chris E Forsmark, *Gainesville*
Glenn T Furuta, *Aurora*
Chandrashekhar R Gandhi, *Pittsburgh*
Susan L Gearhart, *Baltimore*
Xupeng Ge, *Boston*
Xin Geng, *New Brunswick*
M Eric Gershwin, *Suite*
Jean-Francois Geschwind, *Baltimore*
Ignacio Gil-Bazo, *New York*
Shannon S Glaser, *Temple*
Ajay Goel, *Dallas*
Richard M Green, *Chicago*
Julia B Greer, *Pittsburgh*

James H Grendell, *New York*
David R Gretch, *Seattle*
Stefano Guandalini, *Chicago*
Anna S Gukovskaya, *Los Angeles*
Sanjeev Gupta, *Bronx*
David J Hackam, *Pittsburgh*
Stephen B Hanauer, *Chicago*
Gavin Harewood, *Rochester*
Margaret M Heitkemper, *Washington*
Alan W Hemming, *Gainesville*
Samuel B Ho, *San Diego*
Peter R Holt, *New York*
Colin W Howden, *Chicago*
Hongjin Huang, *Alameda*
Jamal A Ibdah, *Columbia*
Atif Iqbal, *Omaha*
Hajime Isomoto, *Rochester*
Hartmut Jaeschke, *Tucson*
Dennis M Jensen, *Los Angeles*
Cheng Ji, *Los Angeles*
Leonard R Johnson, *Memphis*
Michael P Jones, *Chicago*
Peter J Kahrilas, *Chicago*
Anthony N Kalloo, *Baltimore*
Marshall M Kaplan, *Boston*
Neil Kaplowitz, *Los Angeles*
Serhan Karvar, *Los Angeles*
Rashmi Kaul, *Tulsa*
Jonathan D Kaunitz, *Los Angeles*
Ali Keshavarzian, *Chicago*
Miran Kim, *Providence*
Joseph B Kirsner, *Chicago*
Leonidas G Koniaris, *Miami*
Burton I Korelitz, *New York*
Robert J Korst, *New York*
Richard A Kozarek, *Seattle*
Alyssa M Krasinskas, *Pittsburgh*
Michael Kremer, *Chapel Hill*
Shiu-Ming Kuo, *Buffalo*
Paul Y Kwo, *Indianapolis*
Daryl Tan Yeung Lau, *Galvesto*
Stephen J Lanspa, *Omaha*
Joel E Lavine, *San Diego*
Bret Lashner, *Cleveland*
Dirk J van Leeuwen, *Lebanon*
Glen A Lehman, *Indianapolis*
Alex B Lentsch, *Cincinnati*
Andreas Leodolter, *La Jolla*
Gene LeSage, *Houston*
Josh Levitsky, *Chicago*
Cynthia Levy, *Gainesville*
Ming Li, *New Orleans*
Zhiping Li, *Baltimore*
Zhe-Xiong Lian, *Davis*
Lenard M Lichtenberger, *Houston*
Gary R Lichtenstein, *Philadelphia*
Otto Schiueh-Tzang Lin, *Seattle*
Martin Lipkin, *New York*
Chen Liu, *Gainesville*
Edward V Loftus, *Rocheste*
Robin G Lorenz, *Birmingham*
Michael R Lucey, *Madison*
James D Luketich, *Pittsburgh*
Guangbin Luo, *Cheveland*
Henry T Lynch, *Omaha*
Patrick M Lynch, *Houston*
John S Macdonald, *New York*
Bruce V MacFadyen, *Augusta*
Willis C Maddrey, *Dallas*
Ashok Malani, *Los Angeles*
Mercedes Susan Mandell, *Aurora*
Peter J Mannon, *Bethesda*
Charles M Mansbach, *Tennessee*
John F Di Mari, *Texas*
John M Mariadason, *Bronx*
Jorge A Marrero, *Ann Arbor*
Paul Martin, *New York*
Paulo Ney Aguiar Martins, *Boston*
Wendy M Mars, *Pittsburgh*
Laura E Matarese, *Pittsburgh*
Richard W McCallum, *Kansas*
Beth A McCormick, *Charlestown*
Lynne V McFarland, *Washington*
Kevin McGrath, *Pittsburgh*
Harihara Mehendale, *Monroe*
Ali Mencin, *New York*
Fanyin Meng, *Ohio*
Stephan Menne, *New York*
Didier Merlin, *Atlanta*
Howard Mertz, *Nashville*
George W Meyer, *Sacramento*
George Michalopoulos, *Pittsburgh*
James M Millis, *Chicago*
Fabrizio Michelassi, *New York*
Albert D Min, *New York*
Pramod K Mistry, *New Haven*
Emiko Mizoguchi, *Boston*
Smruti R Mohanty, *Chicago*
Satdarshan S Monga, *Pittsburgh*
Timothy H Moran, *Baltimore*
Peter L Moses, *Burlington*
Steven F Moss, *Providence*
Andrew J Muir, *Durham*
Milton G Mutchnick, *Detroit*
Masaki Nagaya, *Boston*
Victor Navarro, *Philadelphia*
Laura E Nagy, *Cleveland*
Hiroshi Nakagawa, *Philadelphia*
Douglas B Nelson, *Minneapolis*
Justin H Nguyen, *Florida*
Patrick G Northup, *Charlottesville*
Christopher O'Brien, *Miami*
Robert D Odze, *Boston*
Brant K Oelschlager, *Washington*
Curtis T Okamoto, *Los Angeles*
Stephen JD O'Keefe, *Pittsburgh*
Dimitry Oleynikov, *Omaha*
Stephen J Pandol, *Los Angeles*
Georgios Papachristou, *Pittsburgh*
Pankaj J Pasricha, *Galveston*
Zhiheng Pei, *New York*
Michael A Pezzone, *Pittsburgh*
CS Pitchumoni, *New Brunswiuc*
Paul J Pockros, *La Jolla*
Jay Pravda, *Gainesville*
Massimo Raimondo, *Jacksonville*
GS Raju, *Galveston*
Raymund R Razonable, *Minnesota*
Murray B Resnick, *Providence*
Adrian Reuben, *Charleston*
Douglas K Rex, *Indianapolis*
Victor E Reyes, *Galveston*
Basil Rigas, *New York*
Yehuda Ringel, *Chapel Hill*
Richard A Rippe, *Chapel Hill*
Maribel Rodriguez-Torres, *Santurce*
Marcos Rojkind, *Washington*
Philip Rosenthal, *San Francisco*
Barry Rosser, *Jacksonville Florida*
Hemant K Roy, *Evanston*
Sammy Saab, *Los Angeles*
Shawn D Safford, *Norfolk*
Dushyant V Sahani, *Boston*
Bruce E Sands, *Boston*
James M Scheiman, *Ann Arbor*
Eugene R Schiff, *Miami*
Nicholas J Shaheen, *Chapel Hill*
Vanessa M Shami, *Charlottesville*
Prateek Sharma, *Kansas City*
Harvey L Sharp, *Minneapolis*
Stuart Sherman, *Indianapolis*
Shivendra Shukla, *Columbia*
Alphonse E Sirica, *Virginia*
Shanthi V Sitaraman, *Atlanta*
Stuart J Spechler, *Dallas*
Shanthi Srinivasan, *Atlanta*
Michael Steer, *Boston*
Peter D Stevens, *New York*
Charmaine A Stewart, *Rochester*
Christian D Stone, *Saint Louis*
Gary D Stoner, *Columbus*
R Todd Stravitz, *Richmond*
Liping Su, *Chicago*
Christina Surawicz, *Seattle*
Robert W Summers, *Iowa City*
Wing-Kin Syn, *Durham*
Gyongyi Szabo, *Worcester*
Yvette Taché, *Los Angeles*
Seng-Lai Tan, *Seattle*
Andrzej S Tarnawski, *Orange*
K-M Tchou-Wong, *New York*
Jonathan P Terdiman, *San Francisco*
Neil D Theise, *New York*
Christopher C Thompson, *Boston*
Swan N Thung, *New York*
Michael Torbenson, *Baltimore*
Natalie J Torok, *Sacramento*
RA Travagli, *Baton Rouge*
George Triadafilopoulos, *Stanford*
Chung-Jyi Tsai, *Lexington*
Janet Elizabeth Tuttle-Newhall, *Durham*
Andrew Ukleja, *Florida*
Michael F Vaezi, *Nashville*
Hugo E Vargas, *Scottsdale*
Arnold Wald, *Wisconsin*
Scott A Waldman, *Philadelphia*
Jian-Ying Wang, *Baltimore*
Timothy C Wang, *New York*
Irving Waxman, *Chicago*
Steven A Weinman, *Galveston*
Steven D Wexner, *Weston*
Keith T Wilson, *Baltimore*
Jacqueline L Wolf, *Boston*
Jackie Wood, *Ohio*
George Y Wu, *Farmington*
Jian Wu, *Sacramento*
Samuel Wyllie, *Houston*
Wen Xie, *Pittsburgh*
Vijay Yajnik, *Boston*
Vincent W Yang, *Atlanta*
Francis Y Yao, *San Francisco*
Hal F Yee, *San Francisco*
Xiao-Ming Yin, *Pittsburgh*
Min You, *Tampa*
Zobair M Younossi, *Virginia*
Liqing Yu, *Winston-Salem*
David Yule, *Rochester*
Ruben Zamora, *Pittsburgh*
Michael E Zenilman, *New York*
Zhi Zhong, *Chapel Hill*
Michael A Zimmerman, *Colorado*
Stephen D Zucker, *Cincinnati*

Uruguay

Henry Cohen, *Montevideo*

[1]Passed away on October 20, 2007
[2]Passed away on June 11, 2007

National Journal Award
2005

World Journal of Gastroenterology®

Weekly Established in October 1995

Volume 14 Number 35
September 21, 2008

Contents

World Journal of Gastroenterology
Volume 14 **Number** 35 **September** 21, 2008

Contents

NAME OF JOURNAL
World Journal of Gastroenterology

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

EDITING
Editorial Board of *World Journal of Gastroenterology,* Room 903, Ocean International Center, Building D, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

PUBLISHING
The WJG Press and Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Ocean International Center, Building D, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

PRINTING
Beijing Kexin Printing House

OVERSEAS DISTRIBUTOR
Beijing Bureau for Distribution of Newspapers and Journals
(Code No. 82-261)
China International Book Trading Corporation PO Box 399, Beijing, China (Code No. M4481)

PUBLICATION DATE
September 21, 2008

EDITOR-IN-CHIEF
Lian-Sheng Ma, *Beijing*

SUBSCRIPTION
RMB 50 Yuan for each issue, RMB 2400 Yuan for one year

CSSN
ISSN 1007-9327
CN 14-1219/R

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5361

World J Gastroenterol 2008 September 21; 14(35): 5361-5370
World Journal of Gastroenterology ISSN 1007-9327

EDITORIAL

Acute mesenteric ischemia after cardio-pulmonary bypass surgery

Bassam Abboud, Ronald Daher, Joe Boujaoude

Bassam Abboud, Ronald Daher, Department of General Surgery, Hotel Dieu de France Hospital, Faculty of Medicine, Saint-Joseph University, Beirut 16-6830, Lebanon
Joe Boujaoude, Department of Gastroenterology, Hotel Dieu de France Hospital, Faculty of Medicine, Saint-Joseph University, Beirut 16-6830, Lebanon
Author contributions: Abboud B designed research; Daher R, Abboud B and Boujaoude J performed research, Daher R and Abboud B wrote the paper.
Correspondence to: Bassam Abboud, MD, Department of General Surgery, Hotel Dieu de France Hospital, Alfred Naccache Street, Beirut 16-6830, Lebanon. dbabboud@yahoo.fr
Telephone: +961-1-615300 Fax: +961-1-615295
Received: May 28, 2008 Revised: July 14, 2008
Accepted: July 21, 2008
Published online: September 21, 2008

Abstract

Acute mesenteric ischemia (AMI) is a highly-lethal surgical emergency. Several pathophysiologic events (arterial obstruction, venous thrombosis and diffuse vasospasm) lead to a sudden decrease in mesenteric blood flow. Ischemia/reperfusion syndrome of the intestine is responsible for systemic abnormalities, leading to multi-organ failure and death. Early diagnosis is difficult because the clinical presentation is subtle, and the biological and radiological diagnostic tools lack sensitivity and specificity. Therapeutic options vary from conservative resuscitation, medical treatment, endovascular techniques and surgical resection and revascularization. A high index of suspicion is required for diagnosis, and prompt treatment is the only hope of reducing the mortality rate. Studies are in progress to provide more accurate diagnostic tools for early diagnosis. AMI can complicate the post-operative course of patients following cardio-pulmonary bypass (CPB). Several factors contribute to the systemic hypo-perfusion state, which is the most frequent pathophysiologic event. In this particular setting, the clinical presentation of AMI can be misleading, while the laboratory and radiological diagnostic tests often produce inconclusive results. The management strategies are controversial, but early treatment is critical for saving lives. Based on the experience of our team, we consider prompt exploratory laparotomy, irrespective of the results of the diagnostic tests, is the only way to provide objective assessment and adequate treatment, leading to dramatic reduction in the mortality rate.

Key words: Acute mesenteric ischemia; Non-occlusive; Cardio-pulmonary bypass; Laparotomy; Prognosis; Mortality

Peer reviewer: James David Luketich, MD, Professor and Chief, Division of Thoracic and Foregut Surgery University of Pittsburgh Medical Center Pittsburgh, PA 15213, United States

Abboud B, Daher R, Boujaoude J. Acute mesenteric ischemia after cardio-pulmonary bypass surgery. *World J Gastroenterol* 2008; 14(35): 5361-5370 Available from: URL: http://www.wjgnet.com/1007-9327/14/5361.asp DOI: http://dx.doi.org/10.3748/wjg.14.5361

INTRODUCTION

Acute mesenteric ischemia (AMI) is a life-threatening surgical emergency in which the outcome is closely dependent on the elapsed time to diagnosis and treatment. The diagnosis is typically difficult and delayed due to non-specific results of biological and radiological tests. Since prompt treatment is the key to a better outcome, AMI remains a challenging condition because of controversial algorithms and numerous therapeutic options.

When AMI occurs after a cardio-pulmonary bypass (CPB) procedure, the condition has a more subtle clinical presentation, is more difficult to diagnose and treat, leading to a higher mortality rate.

This report is an updated review of AMI with respect to the pathophysiologic events, diagnostic tests, therapeutic options, mortality rate and promising new areas of research. A separate section is dedicated to AMI in CPB patients and focuses on the main differences in the diagnosis, management and outcome.

AMI IN STANDARD CONTEXT

Definitions

AMI is caused by a sudden decrease in the blood flow to the bowel and abdominal viscera. Important features of AMI include: bacterial translocation, systemic

inflammatory response syndrome and reperfusion injury, which exacerbate the ischemic damage of the intestinal microcirculation and negatively impact the outcome. Although rare, the incidence of AMI is increasing, in parallel with the aging population[1].

Pathophysiology

AMI is the result of four distinct pathophysiologic mechanisms: arterial embolus, arterial thrombosis, splanchnic vasoconstriction, known as non-occlusive mesenteric ischemia (NOMI) and venous thrombosis.

Arterial embolus is the most common cause, responsible for almost half of all cases[2,3]. The source of the embolus is usually the heart, and the affected vessel is the superior mesenteric artery in 50% of cases. In general, the obstruction occurs at the mid to distal bifurcation points of the blood vessel[1,4].

Arterial thrombus is the underlying cause in approximately 30% of patients[2,3], with rupture of an atherosclerotic plaque in the mesenteric arteries. The site of the occlusion tends to occur at the origin of the blood vessels. The patients can tolerate major visceral artery obstruction because of the slow progressive nature of atherosclerosis, with the development of collaterals. Nearly 75% of patients have pre-existing chronic mesenteric ischemia[5], and acute bowel ischemia or infarction only ensues if the last remaining visceral artery or an important collateral artery occludes. The extent of bowel ischemia or infarction is typically greater than that seen with embolism.

In NOMI, diffuse vasospasm of the mesenteric and other visceral arteries occurs as a result of a sustained hypoperfusion state[6,7]. No vascular occlusion is usually demonstrated because pulsatile blood flow is present in larger arteries[8]. There are several predisposing factors, which are often interrelated, such as heart failure, arterial hypotension, elevated sympathetic activity, hypovolemia, sepsis, use of vasopressors and pre-existing atherosclerotic lesions[7]. Catecholamines and medications such as digitalis[9], by interfering with the auto-regulation of mesenteric circulation, can also cause vasospasm[10].

Mesenteric venous thrombosis accounts for approximately 10% of all AMI cases[2] and involves the superior mesenteric vein in over 90% of patients. Mesenteric venous thrombosis is usually secondary to an underlying coagulopathy, while in 10% the cause is idiopathic[11-15]. Patients should be screened for genetic thrombophilias. Compromised venous return leads to interstitial swelling in the bowel wall, with subsequent arterial flow disturbances and eventual necrosis. The etiologic factors responsible for venous thrombosis include portal hypertension, intra-abdominal sepsis, cirrhosis, pancreatitis, malignancy and trauma.

Other rare causes of mesenteric ischemia are aortic dissection, lupus, vasculitis, median ligament syndrome, ergot administration and post laparoscopic cholecystectomy[16]. In young patients, arterial occlusion due to inherited coagulopathy is exceedingly rare and only isolated cases have been reported[17].

The clinical features of AMI originate from factors such as the site of involvement, systemic inflammatory response triggered by damage to the microcirculation, and reperfusion injury.

At the cellular level, ischemia causes mitochondrial dysfunction, loss of ion transfer regulation, and intracellular acidosis. Alterations in membrane permeability, and the release of free radicals and degradative enzymes leads to cell death and tissue necrosis[18]. In the ischemic tissue, numerous cells including neutrophils, endothelium, monocytes and platelets are activated. Proinflammatory substances are produced such as tumor necrosis factor, interleukines, platelet-activating factor and leukotrienes. Subsequently, the injury is due to leukocyte adhesion, platelet aggregation[19] and nitric oxide production impairment[20]. The activated neutrophils release superoxide substances such as superoxide O_2, peroxide H_2O_2 and hydroxyl radicals OH[21], along with neutrophil enzymes, which result in further damage to the surrounding tissues.

Ischemic/reperfusion double-hit injury consists of an initial hypoxic episode followed by the subsequent reperfusion injury due to reestablishment of forward flow[10]. Superoxide molecules, neutrophil enzymes and pro-inflammatory substances are carried in the bloodstream, causing distant organ damage. Moreover, reperfusion causes swelling of the corresponding organs since capillary permeability is considerably increased during ischemia[22]. Finally, damage to the intestinal micro-vessels and the disruption of the intestinal mucosal barrier results in leakage of water and bacteria, with resulting endotoxemia[23] and bacteremia[24,25]. Ultimately, multi-organ failure ensues and involves the liver[18], heart[26], kidneys[27] and lungs[28]. Acute pulmonary edema resulting from mesenteric ischemia/reperfusion is caused by an increase in pulmonary microvascular permeability to fluids and proteins, as well as smooth muscle dysfunction[29,30].

Clinical presentation

Abdominal pain is the primary symptom. The pain is characteristically out of proportion to the clinical findings. It is described as colicky and is most severe in the paraumbilical region. Other symptoms are present inconstantly and include nausea (93%), vomiting (80%) and diarrhea (48%)[31]. Physical examination is unremarkable unless peritonitis has developed. During the late stages, abdominal distension and guarding, as well as systemic complications may be encountered.

Laboratory tests

Soon after onset but prior to the development of mesenteric infarction, the sensitivity of laboratory tests in detecting mesenteric ischemia is poor[32]. Even at the time when ischemia is confirmed at laparotomy, elevation of serum lactate, amylase, creatine kinase and C-reactive protein (CRP), as well as leucocytes may be absent[31]. At present, no laboratory test is available for accurately establishing or eliminating the diagnosis[33,34]. One study

reported that hemoconcentration and hyperamylasemia were independent predictive factors of massive ischemic infarction[35].

Imaging studies

It is important to remember that when intestinal ischemia is clinically suspected, diagnostic imaging studies should be performed if peritoneal signs are absent.

Plain abdominal radiographs are of little help in the diagnosis of mesenteric ischemia. The presence of dilated loops is non-specific[36,37], and thickened bowel loops, "ground-glass" appearance suggesting ascites, or "thumbprinting" caused by submucosal edema or hemorrhage are seen in less than 40% patients. Twenty-five percent patients with bowel infarction have negative plain radiographs of the abdomen[38].

Barium enema has no place in the diagnosis of AMI since it may increase intra-luminal pressure and reduce perfusion to the bowel wall, causing translocation of bacteria and potentially, perforation. In addition, the presence of barium may compromise subsequent diagnostic tests, such as computed tomography (CT) and angiography[32].

Magnetic resonance imaging has shown promising results in detecting mesenteric ischemia but remains a slow-processing technique that seems to be inadequate in an emergent situation such as AMI[39,40].

Mesenteric duplex sonography is a highly user-dependent modality that can only confirm diminished blood flow in the trunks of the mesenteric blood vessels. Mesenteric duplex scanning identifies stenosis of the superior mesenteric and celiac arteries by the mean of elevated peak systolic velocities. A velocity > 275 cm/s is indicative of >70% stenosis with a sensitivity of 92% and a negative predictive value of 99%[41]. Doppler sonography is useful in diagnosing chronic mesenteric arterial occlusive disease but has limited role in AMI[42-45]. Other applications for duplex sonography are detection of reversible celiac flux alteration such as in median ligament syndrome, and follow-up of mesenteric bypass grafts and stents[41]. The new technique of contrast-enhanced ultrasonography is a promisingly non-invasive tool for the diagnosis of bowel ischemia[46].

Angiography is the gold standard diagnostic test in acute mesenteric artery occlusion[47], providing both anatomical visualization of the vessels and therapeutic options[48]. The sensitivity and specificity are 74% to 100% and 100%, respectively[49]. When used in the absence of peritonitis signs, angiography has been shown to improve the survival rate[50,51]. Mesenteric angiography can usually identify the underlying pathophysiologic event, by differentiating between embolic and thrombotic occlusion[52]. NOMI characteristically shows narrowing and multiple irregularities of the major SMA tributaries recognized as the "string of sausages" sign. Mesenteric venous thrombosis is characterized by a generalized slowing of arterial flow (up to 20 s) in conjunction with a lack of opacification of the corresponding mesenteric or portal venous outflow tracts. However, angiography is an invasive, time consuming and potentially nephrotoxic procedure. Its routine use is controversial in emergency situations[53] and therefore, it is employed only in selected patients.

Since CT is a fast, widely available non-invasive modality, it is considered as the initial imaging test[54]. It is useful in detecting intestinal signs suggestive of ischemia, as well as vascular abnormalities such as occlusion and stenosis. It is also useful in assessing other causes of acute abdominal pain. Still, the CT findings of mesenteric ischemia and infarction are not pathognomonic, and a direct correlation between CT findings and the final diagnosis is not accurate[55]. Overall, the sensitivity and specificity of contrast-enhanced CT for mesenteric ischemia are 64% and 92%, respectively[31,56,57]. Because of these drawbacks, the American Gastrointestinal Association[49] concluded that CT is of limited use in the diagnosis of AMI and that unremarkable CT findings in the context of a high suspicion of mesenteric ischemia should prompt an angiography without delay. An exception to this rule is when superior mesenteric vein thrombosis is suspected; a situation where CT scan remains the test of choice with sensitivity rate in the range of 90%[14,50].

Recently, the multi-detector row CT has emerged as a widely established non-invasive technique that not only delineates the blood vessels, but also shows an anatomical three dimensional relationship with the surrounding tissues, and allows evaluation of tissue perfusion[58]. The sensitivity and specificity rates are 92%-96% and 94%-100%, respectively[31,59,60], with positive and negative predictive values of 90% and 98%, respectively[61]. Moreover, when a cardiac source of mesenteric emboli is suspected, scanning of the heart provides a method for concomitant detection of the source of the embolus[62].

Therapeutic approaches

Therapeutic decisions are taken based on four main considerations: the presence or absence of peritonitis, the presence or absence of irreversible ischemia or infarcted segments of the intestine, the general condition of the patient, and the pathophysiologic phenomenon responsible for the event.

Once a diagnosis of AMI is made, treatment should be initiated without delay. This should include active resuscitation and treatment of the underlying condition, along with efforts directed toward reducing the associated vasospasm. Broad-spectrum antibiotics and intravenous heparin at therapeutic doses should be initiated as early as possible. If the diagnosis was established through angiography, intra-arterial infusion of papaverine, a phosphodiesterase inhibitor, is recommended for NOMI and for occlusive arterial AMI, since arterial vasospasm persists even after successful treatment of the precipitating event[1,3]. When angiography is not performed, intravenous glucagon may help reduce the vasospasm[52].

In the setting of a hemodynamically stable patient, with no signs of peritonitis, conservative medical

management may be attempted. For embolus- and thrombus-induced events, thrombolytic agents such as streptokinase, urokinase or recombinant tissue plasminogen activator are effective treatments[63-66]. Thrombolytic therapy seems to be most successful in distal clots, when used within 12 h after the onset of symptoms[66]. Ultimately, primary endovascular techniques and surgical resection may prevent mesenteric infarction when performed promptly in hemodynamically stable patients with arterial mesenteric ischemia[67]. For NOMI, especially when diagnosed by angiography, selective intra-arterial infusion of papaverine at the usual dose of 30 to 60 mg/h, is an adequate treatment[1,52,53]. It reduces the mortality rate from 70% to 50%-55%[68]. Early treatment with continuous intravenous high dose prostaglandin E(1)[69] or a prostacyclin analogue[70] have shown promising results in the treatment of NOMI. As for venous mesenteric ischemia, the standard treatment is anticoagulation, while venous thrombectomy has not improved the outcome and is controversial[71,72]. Heparin should be initiated as soon as the diagnosis is established, and is associated with reduction in the recurrence rate and mortality[13,14]. Another appropriate therapeutic modality is thrombolysis[73-75].

At any time during evaluation, should signs of peritonitis develop, the patient should undergo exploratory laparotomy without delay. First, the intestine is assessed for viability. Visual evaluation of the bowel relies on arterial pulsations and intestinal peristalsis and colour, although these findings are not specific[52]. Another technique is the use of sodium fluorescein, which is injected intravenously; it is detected with a Wood's lamp in the presence of hypoxic damage. Both retrospective analysis and randomized trials have shown that this technique is more reliable than clinical evaluation of mesenteric viability[76-78]. When compared with histological results, intraoperative laser Doppler flowmetry has been shown to be 100% accurate in assessing bowel viability[79]. Doppler ultrasound can be used intraoperatively but does not provide a quick assessment of the entire length of the intestine and thus does not carry any advantage over clinical judgment[80].

In patients with arterial occlusive AMI, when sufficient bowel is potentially viable, revascularization prior to resection of the infarcted bowel may improve the survival[2]. Although surgical revascularization is the standard procedure[81], embolectomy, thrombectomy, endarterectomy[38], as well as endovascular techniques such as antegrade percutaneous stenting[82], and open retrograde stenting[83-85] provide attractive alternatives with good short-term outcome. A high stent restenosis rate is the drawback of these techniques, requiring close follow-up of the patients[85,86]. Contraindications to revascularization include obvious infarction of the bowel supplied by the affected artery, patient's instability precluding further resection, and mesenteric vein thrombosis[53,87].

Surgical bowel resection must include all of the clearly non viable and infarcted portions of the bowel. Primary anastomosis can be performed if perfusion is adequate. A second-look laparotomy is scheduled within 12 to 24 h, if large portions or multiple segments of intestine of questionable viability were left behind[88], provided that complete resection should not result in a short bowel syndrome. Although widely approved[1,3], some authors question its routine use, and limit second-look laparotomy to individual cases[89].

Alternatively, second-look laparoscopy has emerged as a minimally-invasive, technically simple procedure that can provide diagnostic and therapeutic advantages[90] despite the fact that the evaluation is limited to the serosa and that mucosal lesions can be missed[91]. As a result, the value of second-look laparotomy in preventing morbidity is uncertain[90,92-94].

Outcome

Despite advances in the identification of mortality risk factors and greater therapeutic options, the overall mortality associated with AMI is as high today as it was several decades ago[31,95], ranging from 60% to 90%[49,96-99]. When specific etiologies are considered separately, arterial thrombosis has the highest mortality rate of 70% to 100%[3,98,100] in part because of the extensive ischemia-infarction of the bowel, and the need for more complex surgical revascularization. The mortality associated with NOMI is also within this range[98], whereas arterial embolism and venous thrombosis have much better prognosis, with mortality rates of 0% to 50%[15,98,101] and 20%[5], respectively. The peri-operative factors predicting mortality after mesenteric ischemia have been extensively studied[7,99]. Of the various factors examined, age > 70 (where diagnosis is more frequently overlooked), and prolonged duration of symptoms were independent predictors of mortality[97,102-104]. It cannot be overemphasized that a high index of suspicion, prompt diagnosis and aggressive early treatment are the only surgeon-dependent factors that have a positive influence on the outcome.

Perspectives for the future

A number of biochemical and genetic studies are in progress, designed to elucidate the pathophysiologic changes in an ischemic/reperfused intestine. Several molecules have been identified which attenuate intestinal injury and reduce the production of proinflammatory cytokines[105-110]. Intra-luminal infusion of hyperoxygenated solution during ischemia may improve the functional and structural status of the enterocyte mitochondria associated with ischemia/reperfusion syndrome[111]. Most therapies have to be initiated prior to the onset of ischemia, making their clinical application difficult to foresee.

Since the prognosis is closely related to delay in the treatment, and since the diagnostic tools currently available are not very accurate, great effort is being focused on identifying more accurate methods of early diagnosis. Serum assays such as D-dimers[112-114], alcohol dehydrogenase[115], glutathione S-transferase[116] and cobalt-albumin binding[117], and measurement of pH and potassium in peritoneal irrigation fluid[118], as well

as liver tissue oxygenation index[119] are good examples of current research. Seidel *et al* showed that mesenteric electrical activity may detect mesenteric ischemia with a high degree of sensitivity and specificity[120]. However, promising studies in animals remain to be validated under clinical conditions.

Experimental studies in which laparoscopy was combined with ultraviolet light and IV injection of fluoresceine showed that this technique may be useful in detecting mesenteric ischemia and viability of the intestine at an early stage[121,122]. Moreover, trans-serosal pulse oximetry may help determine bowel viability and resection extension prior to laparotomy[123].

AMI AFTER CPB

Incidence and frequency

Abdominal complications after CPB for cardiac surgery are seen in < 1% of patients[124-132] but carry a high mortality of 14.1%[131]. AMI represents 10%-67% of these complications[129,133,134], and is the most lethal, with a case-fatality rate of 70% to 100%[129,131,132,135,136].

Pathophysiology

AMI occurring after CPB, is due to NOMI in the vast majority of cases[137-139]. The various contributory factors are: low cardiac output (frequent in this category of patients), use of vasopressors and underlying atherosclerotic disease. It is well established that CPB is responsible for mesenteric endothelial dysfunction and microcirculation disturbances even under stable hemodynamic conditions. An increase in the contractile response to alpha1-adrenergic agonist and an early release of pro-inflammatory substances has been observed after CPB[140-142]. Nevertheless, the effect of pulseless extracorporeal circulation on bowel hypoperfusion is still under debate[143,144], and off-pump coronary artery bypass does not prevent subsequent mesenteric ischemia[134,145,146]. The physiologic changes in intestinal perfusion during cardiac surgery remain to be elucidated. It has been observed that there is significant mesenteric hypoperfusion followed by hyperemic response[147] after off-pump cardiac surgery along with an increase in the resistive and pulsatility indexes[148]. Moreover, studies focusing on the identification of predisposing factors for mesenteric ischemia after CPB[135,124-126], have produced different, and even opposite results. Rare cases of embolic acute arterial infarction can be prevented when a calcified aorta is detected on pre-operative CT scan[149].

Clinical presentation

In the context of CPB, patients are usually sedated and mechanically ventilated for a few days. Consequently, symptoms are not reported and the physical examination is equivocal due to masked, late-appearing, or missing clinical signs[125,139,150]. This accounts for the delay in diagnosis, and the disease may progress to a late, even irreversible stage by the time clinical signs become obvious (i.e. cyanosis). Finally, extracorporeal circulation induces a systemic inflammatory response with vasodilatation, such that hemodynamic instability can no longer be interpreted as a clue to an underlying mesenteric ischemia. A high index of suspicion in detecting subtle clinical evidence of AMI is the key to reducing the delay in diagnosis.

Laboratory tests

Pulseless perfusion during extracorporeal circulation causes systemic hypoperfusion, as illustrated by major derangements in the biochemical tests. These abnormalities are difficult to distinguish from those related to an underlying AMI. Moreover, laboratory test abnormalities are observed inconstantly in AMI. Even if unexplained metabolic acidosis with elevation of lactate level is considered as an early sign[151], several studies have shown that serum lactate may remain normal in the presence of extensive mesenteric infarction[139]. As indicated by Edwards *et al*[130], neither routine clinical investigations nor biological tests (such as leucocytosis, and elevation in serum creatinine, creatine kinase, hepatic or pancreatic enzymes) are discriminatory for mesenteric ischemia when the diagnosis is clinically suspected.

Imaging

When AMI is suspected after CPB, imaging studies should be such that they provide a rapid and accurate diagnosis, while being safe and avoid further morbidity in an already fragile patient. Traditional radiologic studies are not accurate for the diagnosis of AMI (as stated above) and are not recommended. Abdominal ultrasound is a non-invasive technique but remains highly operator-dependent. Its accuracy decreases significantly in emergent situations, especially in the presence of ileus and dilated bowel loops, which have a negative impact on image quality. Multi-detector abdominal CT scan theoretically provides good diagnostic results in AMI, but its specificity and sensitivity after CPB are reduced dramatically; accurate diagnosis correlates with laparotomy findings in < 50% of patients[139]. This is in part due to the limitation in the use of intravenous contrast because of borderline renal function and frequent presence of diabetes in these patients. This consideration applies also to angiography that remains the gold standard diagnostic tool in peripheral splanchnic disease[152], despite its invasive nature and time-consumption.

Therapeutic options

An early diagnosis and prompt treatment based on a high index of suspicion are the only hope for reducing the mortality and improving the outcome[153,154]. The initial treatment consists of hemodynamic support, but if these measures fail, prompt intervention is mandatory. Some experts believe that surgery within the first 6 h has a positive impact on the prognosis[151]. Since most mesenteric ischemic episodes after CPB are due to NOMI, some authors argue in favour of selective angiography as the initial test, as it provides the potential for both diagnosis and therapy. Intra-arterial infusion

of papaverine[155] or tolazoline with heparin[125] are both effective treatments.

Perspectives for the future

AMI occurring in the post CPB period remains a challenging surgical emergency, characterized by extremely high mortality and a controversial management approach. As stated above, clinical assessment as well as laboratory and radiological tests are typically unreliable in establishing the diagnosis.

In view of these considerations, we have adopted a uniform treatment strategy. When a diagnosis of AMI after CPB was suspected, exploratory laparotomy was carried out, irrespective of the results of the diagnostic tests. We performed a retrospective analysis on 1634 consecutive patients undergoing CPB for coronary artery bypass alone or combined with valvular surgery, between January 1st, 2000 and July 31st, 2007. A total of thirteen patients were suspected to have mesenteric ischemia, based on clinical and/or laboratory and/or radiological findings. All patients underwent exploratory laparotomy and were divided into two groups (Group 1 and Group 2) depending upon whether or not ischemic bowel was present. There was no difference in the clinical findings, laboratory tests and radiological results between the two groups. The mean delay in laparotomy was 13.7 h and 51.4 h in Group 1 and Group 2, respectively; the difference was statistically significant. Mortality rates in Group 1 and Group 2 were 42.8% and 50%, respectively. Based on these findings, we concluded that in the context of post-CPB AMI, diagnostic tests do not provide any information of practical value, but instead consume valuable time. By performing early exploratory laparotomy, we were able to reduce the mortality rate considerably. Since all of our patients had NOMI, no revascularization was required and resection of the irreversibly ischemic and infarcted segments of the bowel helped in preventing the vicious circle leading to multi-organ failure and death.

CONCLUSION

Although AMI has been known for several decades, it remains a highly lethal emergency, characterized by numerous controversies. The pathophysiologic process has not been completely resolved, the clinical presentation is often subtle and misleading, and despite the introduction of new technologies the diagnostic tools are often inaccurate. A high index of suspicion and prompt treatment are the only means to reduce mortality.

AMI in cardiac patients undergoing CPB is an extremely challenging surgical emergency. The role of clinical evaluation becomes even more relevant since the laboratory and radiological tests are no longer effective. In this context, prompt laparotomy is the only method of providing objective assessment and targeted treatment. Using this approach we achieved considerable improvement in the mortality rate. Although promising, this practice needs to be confirmed in a larger series of patients.

REFERENCES

1 **Stoney RJ**, Cunningham CG. Acute mesenteric ischemia. *Surgery* 1993; **114**: 489-490
2 **Safioleas MC**, Moulakakis KG, Papavassiliou VG, Kontzoglou K, Kostakis A. Acute mesenteric ischaemia, a highly lethal disease with a devastating outcome. *Vasa* 2006; **35**: 106-111
3 **Bradbury AW**, Brittenden J, McBride K, Ruckley CV. Mesenteric ischaemia: a multidisciplinary approach. *Br J Surg* 1995; **82**: 1446-1459
4 **Batellier J**, Kieny R. Superior mesenteric artery embolism: eighty-two cases. *Ann Vasc Surg* 1990; **4**: 112-116
5 **Falkensammer J**, Oldenburg WA. Surgical and medical management of mesenteric ischemia. *Curr Treat Options Cardiovasc Med* 2006; **8**: 137-143
6 **Imanaka K**, Kyo S, Abe K. Severe hepatic artery spasm and nonocclusive mesenteric ischemia after cardiac surgery. *Ann Thorac Surg* 2006; **82**: 1127
7 **Acosta S**, Ogren M, Sternby NH, Bergqvist D, Bjorck M. Fatal nonocclusive mesenteric ischaemia: population-based incidence and risk factors. *J Intern Med* 2006; **259**: 305-313
8 **Howard TJ**, Plaskon LA, Wiebke EA, Wilcox MG, Madura JA. Nonocclusive mesenteric ischemia remains a diagnostic dilemma. *Am J Surg* 1996; **171**: 405-408
9 **Weil J**, Sen Gupta R, Herfarth H. Nonocclusive mesenteric ischemia induced by digitalis. *Int J Colorectal Dis* 2004; **19**: 277-280
10 **Chang RW**, Chang JB, Longo WE. Update in management of mesenteric ischemia. *World J Gastroenterol* 2006; **12**: 3243-3247
11 **Dobrinja C**, Trevisan G, Petronio B, Liguori G. Idiopathic mesenteric venous thrombosis: report of a case. *J Mal Vasc* 2008; **33**: 96-100
12 **Grendell JH**, Ockner RK. Mesenteric venous thrombosis. *Gastroenterology* 1982; **82**: 358-372
13 **Abdu RA**, Zakhour BJ, Dallis DJ. Mesenteric venous thrombosis--1911 to 1984. *Surgery* 1987; **101**: 383-388
14 **Rhee RY**, Gloviczki P, Mendonca CT, Petterson TM, Serry RD, Sarr MG, Johnson CM, Bower TC, Hallett JW Jr, Cherry KJ Jr. Mesenteric venous thrombosis: still a lethal disease in the 1990s. *J Vasc Surg* 1994; **20**: 688-697
15 **Imperato M**, Moujahid M, Mennecier D, Bechade D, Duverger V, Farret O, Algayres JL, Baranger B. [Acute superior mesenteric vein thrombosis. A retrospective study of 9 patients] *Ann Chir* 2006; **131**: 595-600
16 **Leduc LJ**, Mitchell A. Intestinal ischemia after laparoscopic cholecystectomy. *JSLS* 2006; **10**: 236-238
17 **Scally N**, O'Donnell ME, McClintock C, Allen M, Kirk SJ, Dolan S. Mesenteric artery occlusion secondary to activated protein C resistance: a life-threatening combination. *Angiology* 2007; **58**: 768-771
18 **Cerqueira NF**, Hussni CA, Yoshida WB. Pathophysiology of mesenteric ischemia/reperfusion: a review. *Acta Cir Bras* 2005; **20**: 336-343
19 **Harward TR**, Brooks DL, Flynn TC, Seeger JM. Multiple organ dysfunction after mesenteric artery revascularization. *J Vasc Surg* 1993; **18**: 459-467; discussion 467-469
20 **Ma XL**, Johnson G 3rd, Lefer AM. Mechanisms of inhibition of nitric oxide production in a murine model of splanchnic artery occlusion shock. *Arch Int Pharmacodyn Ther* 1991; **311**: 89-103
21 **McCord JM**, Roy RS. The pathophysiology of superoxide: roles in inflammation and ischemia. *Can J Physiol Pharmacol* 1982; **60**: 1346-1352
22 **Granger DN**, McCord JM, Parks DA, Hollwarth ME. Xanthine oxidase inhibitors attenuate ischemia-induced vascular permeability changes in the cat intestine. *Gastroenterology* 1986; **90**: 80-84
23 **Turnage RH**, Guice KS, Oldham KT. Endotoxemia and remote organ injury following intestinal reperfusion. *J Surg Res* 1994; **56**: 571-578

24 **Crissinger KD**, Granger DN. Mucosal injury induced by ischemia and reperfusion in the piglet intestine: influences of age and feeding. *Gastroenterology* 1989; **97**: 920-926
25 **Koike K**, Moore EE, Moore FA, Read RA, Carl VS, Banerjee A. Gut ischemia/reperfusion produces lung injury independent of endotoxin. *Crit Care Med* 1994; **22**: 1438-1444
26 **Horton JW**, White DJ. Lipid peroxidation contributes to cardiac deficits after ischemia and reperfusion of the small bowel. *Am J Physiol* 1993; **264**: H1686-H1692
27 **Rothenbach P**, Turnage RH, Iglesias J, Riva A, Bartula L, Myers SI. Downstream effects of splanchnic ischemia-reperfusion injury on renal function and eicosanoid release. *J Appl Physiol* 1997; **83**: 530-536
28 **Vinardi S**, Pierro A, Parkinson EJ, Vejchapipat P, Stefanutti G, Spitz L, Eaton S. Hypothermia throughout intestinal ischaemia-reperfusion injury attenuates lung neutrophil infiltration. *J Pediatr Surg* 2003; **38**: 88-91; discussion 88-91
29 **Iglesias JL**, LaNoue JL, Rogers TE, Inman L, Turnage RH. Physiologic basis of pulmonary edema during intestinal reperfusion. *J Surg Res* 1998; **80**: 156-163
30 **Koksoy C**, Kuzu MA, Ergun H, Demirpence E, Zulfikaroglu B. Intestinal ischemia and reperfusion impairs vasomotor functions of pulmonary vascular bed. *Ann Surg* 2000; **231**: 105-111
31 **Kassahun WT**, Schulz T, Richter O, Hauss J. Unchanged high mortality rates from acute occlusive intestinal ischemia: six year review. *Langenbecks Arch Surg* 2008; **393**: 163-171
32 **Kurland B**, Brandt LJ, Delany HM. Diagnostic tests for intestinal ischemia. *Surg Clin North Am* 1992; **72**: 85-105
33 **Woo K**, Major K, Kohanzadeh S, Allins AD. Laparotomy for visceral ischemia and gangrene. *Am Surg* 2007; **73**: 1006-1008
34 **Corke C**, Glenister K. Monitoring intestinal ischaemia. *Crit Care Resusc* 2001; **3**: 176-180
35 **Acosta-Merida MA**, Marchena-Gomez J, Cruz-Benavides F, Hernandez-Navarro J, Roque-Castellano C, Rodriguez-Mendez A, Alonso-Alvarado A, Hernandez-Romero J. [Predictive factors of massive intestinal necrosis in acute mesenteric ischemia] *Cir Esp* 2007; **81**: 144-149
36 **Chien-Hua L**, Jyh-Cherng Y, Huan-Fa H, Hurng-Sheng W, Shih-Yi C, Chu-Hsin C. Pneumatosis intestinalis and hepatic-portal-mesenteric venous gas in intestinal ischemia. *Rev Esp Enferm Dig* 2007; **99**: 96-99
37 **Wolf EL**, Sprayregen S, Bakal CW. Radiology in intestinal ischemia. Plain film, contrast, and other imaging studies. *Surg Clin North Am* 1992; **72**: 107-124
38 **Karwowski J**, Arko F. Surgical management of mesenteric ischemia. *Tech Vasc Interv Radiol* 2004; **7**: 151-154
39 **Ha HK**, Rha SE, Kim AY, Auh YH. CT and MR diagnoses of intestinal ischemia. *Semin Ultrasound CT MR* 2000; **21**: 40-55
40 **Rha SE**, Ha HK, Lee SH, Kim JH, Kim JK, Kim JH, Kim PN, Lee MG, Auh YH. CT and MR imaging findings of bowel ischemia from various primary causes. *Radiographics* 2000; **20**: 29-42
41 **Mitchell EL**, Moneta GL. Mesenteric duplex scanning. *Perspect Vasc Surg Endovasc Ther* 2006; **18**: 175-183
42 **Bowersox JC**, Zwolak RM, Walsh DB, Schneider JR, Musson A, LaBombard FE, Cronenwett JL. Duplex ultrasonography in the diagnosis of celiac and mesenteric artery occlusive disease. *J Vasc Surg* 1991; **14**: 780-786; discussion 786-788
43 **Danse EM**, Van Beers BE, Goffette P, Dardenne AN, Laterre PF, Pringot J. Acute intestinal ischemia due to occlusion of the superior mesenteric artery: detection with Doppler sonography. *J Ultrasound Med* 1996; **15**: 323-326
44 **Hamada T**, Yamauchi M, Tanaka M, Hashimoto Y, Nakai K, Suenaga K. Prospective evaluation of contrast-enhanced ultrasonography with advanced dynamic flow for the diagnosis of intestinal ischaemia. *Br J Radiol* 2007; **80**: 603-608
45 **Zwolak RM**. Can duplex ultrasound replace arteriography in screening for mesenteric ischemia? *Semin Vasc Surg* 1999; **12**: 252-260
46 **Hata J**, Kamada T, Haruma K, Kusunoki H. Evaluation of bowel ischemia with contrast-enhanced US: initial experience. *Radiology* 2005; **236**: 712-715
47 **Clavien PA**. Diagnosis and management of mesenteric infarction. *Br J Surg* 1990; **77**: 601-603
48 **Sardo P**, Cepich MS, Giulini SM. [Diagnostic and therapeutic role of angiography in acute vascular insufficiency of the superior mesenteric artery] *Radiol Med* (Torino) 1988; **75**: 319-325
49 **Brandt LJ**, Boley SJ. AGA technical review on intestinal ischemia. American Gastrointestinal Association. *Gastroenterology* 2000; **118**: 954-968
50 **Desai SR**, Cox MR, Martin CJ. Superior mesenteric vein thrombosis: computed tomography diagnosis. *Aust N Z J Surg* 1998; **68**: 811-812
51 **Grace PA**, Da Costa M, Qureshi A, Sheehan S, Burke P, Bouchier-Hayes D. An aggressive approach to acute superior mesenteric arterial ischemia. *Eur J Vasc Surg* 1993; **7**: 731-732
52 **Oldenburg WA**, Lau LL, Rodenberg TJ, Edmonds HJ, Burger CD. Acute mesenteric ischemia: a clinical review. *Arch Intern Med* 2004; **164**: 1054-1062
53 **Bradbury AW**, Brittenden J, McBride K, Ruckley CV. Mesenteric ischaemia: a multidisciplinary approach. *Br J Surg* 1995; **82**: 1446-1459
54 **Fock CM**, Kullnig P, Ranner G, Beaufort-Spontin F, Schmidt F. Mesenteric arterial embolism--the value of emergency CT in diagnostic procedure. *Eur J Radiol* 1994; **18**: 12-14
55 **Romano S**, Lassandro F, Scaglione M, Romano L, Rotondo A, Grassi R. Ischemia and infarction of the small bowel and colon: spectrum of imaging findings. *Abdom Imaging* 2006; **31**: 277-292
56 **Kim AY**, Ha HK. Evaluation of suspected mesenteric ischemia: efficacy of radiologic studies. *Radiol Clin North Am* 2003; **41**: 327-342
57 **Taourel PG**, Deneuville M, Pradel JA, Regent D, Bruel JM. Acute mesenteric ischemia: diagnosis with contrast-enhanced CT. *Radiology* 1996; **199**: 632-636
58 **Wildermuth S**, Leschka S, Alkadhi H, Marincek B. Multislice CT in the pre- and postinterventional evaluation of mesenteric perfusion. *Eur Radiol* 2005; **15**: 1203-1210
59 **Zandrino F**, Musante F, Gallesio I, Benzi L. Assessment of patients with acute mesenteric ischemia: multislice computed tomography signs and clinical performance in a group of patients with surgical correlation. *Minerva Gastroenterol Dietol* 2006; **52**: 317-325
60 **Kirkpatrick ID**, Kroeker MA, Greenberg HM. Biphasic CT with mesenteric CT angiography in the evaluation of acute mesenteric ischemia: initial experience. *Radiology* 2003; **229**: 91-98
61 **Wiesner W**. [Is multidetector computerized tomography currently the primary diagnostic method of choice in diagnostic imaging of acute intestinal ischemia?] *Praxis* (Bern 1994) 2003; **92**: 1315-1317
62 **Hellinger JC**. Evaluating mesenteric ischemia with multidetector-row CT angiography. *Tech Vasc Interv Radiol* 2004; **7**: 160-166
63 **Jamieson AC**, Thomas RJ, Cade JF. Lysis of a superior mesenteric artery embolus following local infusion of streptokinase and heparin. *Aust N Z J Surg* 1979; **49**: 355-356
64 **Boyer L**, Delorme JM, Alexandre M, Boissier A, Gimbergues P, Glanddier G, Viallet JF. Local fibrinolysis for superior mesenteric artery thromboembolism. *Cardiovasc Intervent Radiol* 1994; **17**: 214-216
65 **Antico E**, Paci E, Crosta F, Maniscalco L, De Bernardinis S, Candelari R. [Acute embolic superior mesenteric artery ischemia by embolism: a case treated with fibrinolysis and endoarterial aspiration of embolic material] *Radiol Med* (Torino) 2001; **102**: 293-295
66 **Schoots IG**, Levi MM, Reekers JA, Lameris JS, van Gulik TM. Thrombolytic therapy for acute superior mesenteric artery occlusion. *J Vasc Interv Radiol* 2005; **16**: 317-329
67 **Demirpolat G**, Oran I, Tamsel S, Parildar M, Memis A.

Acute mesenteric ischemia: endovascular therapy. *Abdom Imaging* 2007; **32**: 299-303
68 **Bassiouny HS**. Nonocclusive mesenteric ischemia. *Surg Clin North Am* 1997; **77**: 319-326
69 **Mitsuyoshi A**, Obama K, Shinkura N, Ito T, Zaima M. Survival in nonocclusive mesenteric ischemia: early diagnosis by multidetector row computed tomography and early treatment with continuous intravenous high-dose prostaglandin E(1). *Ann Surg* 2007; **246**: 229-235
70 **Kang H**, Manasia A, Rajamani S, Rajaram SS, Hannon E, Lu Y, Oropello JM, Benjamin E. Intravenous iloprost increases mesenteric blood flow in experimental acute nonocclusive mesenteric ischemia. *Crit Care Med* 2002; **30**: 2528-2534
71 **Martinez JP**, Hogan GJ. Mesenteric ischemia. *Emerg Med Clin North Am* 2004; **22**: 909-928
72 **Rhee RY**, Gloviczki P. Mesenteric venous thrombosis. *Surg Clin North Am* 1997; **77**: 327-338
73 **Poplausky MR**, Kaufman JA, Geller SC, Waltman AC. Mesenteric venous thrombosis treated with urokinase via the superior mesenteric artery. *Gastroenterology* 1996; **110**: 1633-1635
74 **Train JS**, Ross H, Weiss JD, Feingold ML, Khoury-Yacoub A, Khoury PT. Mesenteric venous thrombosis: successful treatment by intraarterial lytic therapy. *J Vasc Interv Radiol* 1998; **9**: 461-464
75 **Rivitz SM**, Geller SC, Hahn C, Waltman AC. Treatment of acute mesenteric venous thrombosis with transjugular intramesenteric urokinase infusion. *J Vasc Interv Radiol* 1995; **6**: 219-223; discussion 224-228
76 **Bulkley GB**, Zuidema GD, Hamilton SR, O'Mara CS, Klacsmann PG, Horn SD. Intraoperative determination of small intestinal viability following ischemic injury: a prospective, controlled trial of two adjuvant methods (Doppler and fluorescein) compared with standard clinical judgment. *Ann Surg* 1981; **193**: 628-637
77 **Bergman RT**, Gloviczki P, Welch TJ, Naessens JM, Bower TC, Hallett JW Jr, Pairolero PC, Cherry KJ Jr. The role of intravenous fluorescein in the detection of colon ischemia during aortic reconstruction. *Ann Vasc Surg* 1992; **6**: 74-79
78 **Mann A**, Fazio VW, Lucas FV. A comparative study of the use of fluorescein and the Doppler device in the determination of intestinal viability. *Surg Gynecol Obstet* 1982; **154**: 53-55
79 **Redaelli CA**, Schilling MK, Buchler MW. Intraoperative laser Doppler flowmetry: a predictor of ischemic injury in acute mesenteric infarction. *Dig Surg* 1998; **15**: 55-59
80 **Ballard JL**, Stone WM, Hallett JW, Pairolero PC, Cherry KJ. A critical analysis of adjuvant techniques used to assess bowel viability in acute mesenteric ischemia. *Am Surg* 1993; **59**: 309-311
81 **Shaw RS**, Maynard EP 3rd. Acute and chronic thrombosis of the mesenteric arteries associated with malabsorption; a report of two cases successfully treated by thromboendarterectomy. *N Engl J Med* 1958; **258**: 874-878
82 **Gartenschlaeger S**, Bender S, Maeurer J, Schroeder RJ. Successful percutaneous transluminal angioplasty and stenting in acute mesenteric ischemia. *Cardiovasc Intervent Radiol* 2008; **31**: 398-400
83 **Moyes LH**, McCarter DH, Vass DG, Orr DJ. Intraoperative retrograde mesenteric angioplasty for acute occlusive mesenteric ischaemia: a case series. *Eur J Vasc Endovasc Surg* 2008; **36**: 203-206
84 **Sonesson B**, Hinchliffe RJ, Dias NV, Resch TA, Malina M, Ivancev K. Hybrid recanalization of superior mesenteric artery occlusion in acute mesenteric ischemia. *J Endovasc Ther* 2008; **15**: 129-132
85 **Wyers MC**, Powell RJ, Nolan BW, Cronenwett JL. Retrograde mesenteric stenting during laparotomy for acute occlusive mesenteric ischemia. *J Vasc Surg* 2007; **45**: 269-275
86 **Resch T**, Lindh M, Dias N, Sonesson B, Uher P, Malina M, Ivancev K. Endovascular recanalisation in occlusive mesenteric ischemia--feasibility and early results. *Eur J Vasc Endovasc Surg* 2005; **29**: 199-203
87 **Kaleya RN**, Sammartano RJ, Boley SJ. Aggressive approach to acute mesenteric ischemia. *Surg Clin North Am* 1992; **72**: 157-182
88 **Levy PJ**, Krausz MM, Manny J. The role of second-look procedure in improving survival time for patients with mesenteric venous thrombosis. *Surg Gynecol Obstet* 1990; **170**: 287-291
89 **Kaminsky O**, Yampolski I, Aranovich D, Gnessin E, Greif F. Does a second-look operation improve survival in patients with peritonitis due to acute mesenteric ischemia? A five-year retrospective experience. *World J Surg* 2005; **29**: 645-648
90 **Yanar H**, Taviloglu K, Ertekin C, Ozcinar B, Yanar F, Guloglu R, Kurtoglu M. Planned second-look laparoscopy in the management of acute mesenteric ischemia. *World J Gastroenterol* 2007; **13**: 3350-3353
91 **Sauerland S**, Agresta F, Bergamaschi R, Borzellino G, Budzynski A, Champault G, Fingerhut A, Isla A, Johansson M, Lundorff P, Navez B, Saad S, Neugebauer EA. Laparoscopy for abdominal emergencies: evidence-based guidelines of the European Association for Endoscopic Surgery. *Surg Endosc* 2006; **20**: 14-29
92 **Gagne DJ**, Malay MB, Hogle NJ, Fowler DL. Bedside diagnostic minilaparoscopy in the intensive care patient. *Surgery* 2002; **131**: 491-496
93 **Anadol AZ**, Ersoy E, Taneri F, Tekin EH. Laparoscopic "second-look" in the management of mesenteric ischemia. *Surg Laparosc Endosc Percutan Tech* 2004; **14**: 191-193
94 **Seshadri PA**, Poulin EC, Mamazza J, Schlachta CM. Simplified laparoscopic approach to "second-look" laparotomy: a review. *Surg Laparosc Endosc Percutan Tech* 1999; **9**: 286-289
95 **Mamode N**, Pickford I, Leiberman P. Failure to improve outcome in acute mesenteric ischaemia: seven-year review. *Eur J Surg* 1999; **165**: 203-208
96 **Klempnauer J**, Grothues F, Bektas H, Pichlmayr R. Long-term results after surgery for acute mesenteric ischemia. *Surgery* 1997; **121**: 239-243
97 **Acosta-Merida MA**, Marchena-Gomez J, Hemmersbach-Miller M, Roque-Castellano C, Hernandez-Romero JM. Identification of risk factors for perioperative mortality in acute mesenteric ischemia. *World J Surg* 2006; **30**: 1579-1585
98 **Schoots IG**, Koffeman GI, Legemate DA, Levi M, van Gulik TM. Systematic review of survival after acute mesenteric ischaemia according to disease aetiology. *Br J Surg* 2004; **91**: 17-27
99 **Merle C**, Lepouse C, De Garine A, Frayssinet N, Leymarie F, Leon A, Jolly D. Surgery for mesenteric infarction: prognostic factors associated with early death within 72 hours. *J Cardiothorac Vasc Anesth* 2004; **18**: 734-741
100 **Mansour MA**. Management of acute mesenteric ischemia. *Arch Surg* 1999; **134**: 328-330; discussion 331
101 **Kumar S**, Sarr MG, Kamath PS. Mesenteric venous thrombosis. *N Engl J Med* 2001; **345**: 1683-1688
102 **Kougias P**, Lau D, El Sayed HF, Zhou W, Huynh TT, Lin PH. Determinants of mortality and treatment outcome following surgical interventions for acute mesenteric ischemia. *J Vasc Surg* 2007; **46**: 467-474
103 **Wadman M**, Syk I, Elmstahl S. Survival after operations for ischaemic bowel disease. *Eur J Surg* 2000; **166**: 872-877
104 **Ritz JP**, Germer CT, Buhr HJ. Prognostic factors for mesenteric infarction: multivariate analysis of 187 patients with regard to patient age. *Ann Vasc Surg* 2005; **19**: 328-334
105 **Zhang W**, Zhu W, Zhang J, Li N, Li J. Protective effects of glucagon-like peptide 2 on intestinal ischemia-reperfusion rats. *Microsurgery* 2008; **28**: 285-290
106 **Mori S**, Sawada T, Okada T, Kubota K. Erythropoietin and its derivative protect the intestine from severe ischemia/reperfusion injury in the rat. *Surgery* 2008; **143**: 556-565
107 **Higa OH**, Parra ER, Ab'Saber AM, Farhat C, Higa R, Capelozzi VL. Protective effects of ascorbic acid pretreatment in a rat model of intestinal ischemia-

reperfusion injury: a histomorphometric study. *Clinics* 2007; **62**: 315-320
108 **Spanos CP**, Papaconstantinou P, Spanos P, Karamouzis M, Lekkas G, Papaconstantinou C. The effect of L-arginine and aprotinin on intestinal ischemia-reperfusion injury. *J Gastrointest Surg* 2007; **11**: 247-255
109 **Kaneko H**, Tamura A, Ishii T, Maeda T, Katagiri T, Ishii J, Kubota Y, Suzuki T, Tsuchiya M, Otsuka Y, Yamazaki K, Watanabe M, Tatsuo T. Bacterial translocation in small intestinal ischemia-reperfusion injury and efficacy of Anti-CINC antibody treatment. *Eur Surg Res* 2007; **39**: 153-159
110 **Horie Y**, Yamagishi Y, Kato S, Kajihara M, Kimura H, Ishii H. Low-dose ethanol attenuates gut ischemia/reperfusion-induced liver injury in rats via nitric oxide production. *J Gastroenterol Hepatol* 2003; **18**: 211-217
111 **Gao C**, Xu L, Chai W, Sun X, Zhang H, Zhang G. Amelioration of intestinal ischemia-reperfusion injury with intraluminal hyperoxygenated solution: studies on structural and functional changes of enterocyte mitochondria. *J Surg Res* 2005; **129**: 298-305
112 **Kulacoglu H**, Kocaerkek Z, Moran M, Kulah B, Atay C, Kulacoglu S, Ozmen M, Coskun F. Diagnostic value of blood D-dimer level in acute mesenteric ischaemia in the rat: an experimental study. *Asian J Surg* 2005; **28**: 131-135
113 **Kurt Y**, Akin ML, Demirbas S, Uluutku AH, Gulderen M, Avsar K, Celenk T. D-dimer in the early diagnosis of acute mesenteric ischemia secondary to arterial occlusion in rats. *Eur Surg Res* 2005; **37**: 216-219
114 **Altinyollar H**, Boyabatli M, Berberoglu U. D-dimer as a marker for early diagnosis of acute mesenteric ischemia. *Thromb Res* 2006; **117**: 463-467
115 **Gumaste UR**, Joshi MM, Mourya DT, Barde PV, Shrivastav GK, Ghole VS. Alcohol dehydrogenase: a potential new marker for diagnosis of intestinal ischemia using rat as a model. *World J Gastroenterol* 2005; **11**: 912-916
116 **Khurana S**, Corbally MT, Manning F, Armenise T, Kierce B, Kilty C. Glutathione S-transferase: a potential new marker of intestinal ischemia. *J Pediatr Surg* 2002; **37**: 1543-1548
117 **Polk JD**, Rael LT, Craun ML, Mains CW, Davis-Merritt D, Bar-Or D. Clinical utility of the cobalt-albumin binding assay in the diagnosis of intestinal ischemia. *J Trauma* 2008; **64**: 42-45
118 **Gönüllü D**, Yankol Y, Işiman F, Akyildiz Iğdem A, Yücel O, Köksoy FN. [pH value and potassium level of diagnostic peritoneal lavage fluid in the early diagnosis of acute mesenteric ischemia secondary to arterial occlusion in rats] *Ulus Travma Acil Cerrahi Derg* 2007; **13**: 261-267
119 **Vanderhaegen J**, Dehing L, Naulaers G, Devlieger H, Al-Olayet Y, Penninckx F, Miserez M. Use of the liver tissue oxygenation index as a noninvasive parameter of intestinal ischemia in rabbits. *World J Surg* 2007; **31**: 2359-2362
120 **Seidel SA**, Hegde SS, Bradshaw LA, Ladipo JK, Richards WO. Intestinal tachyarrhythmias during small bowel ischemia. *Am J Physiol* 1999; **277**: G993-G999
121 **Paral J**, Ferko A, Plodr M, Raupach J, Hadzi-Nikolov D, Dolezal D, Chovanec V. Laparoscopic diagnostics of acute bowel ischemia using ultraviolet light and fluorescein dye: an experimental study. *Surg Laparosc Endosc Percutan Tech* 2007; **17**: 291-295
122 **McGinty JJ Jr**, Hogle N, Fowler DL. Laparoscopic evaluation of intestinal ischemia using fluorescein and ultraviolet light in a porcine model. *Surg Endosc* 2003; **17**: 1140-1143
123 **Erikoglu M**, Kaynak A, Beyatli EA, Toy H. Intraoperative determination of intestinal viability: a comparison with transserosal pulse oximetry and histopathological examination. *J Surg Res* 2005; **128**: 66-69
124 **Allen KB**, Salam AA, Lumsden AB. Acute mesenteric ischemia after cardiopulmonary bypass. *J Vasc Surg* 1992; **16**: 391-395; discussion 395-396
125 **Huwer H**, Winning J, Straub U, Isringhaus H, Kalweit G. Clinically diagnosed nonocclusive mesenteric ischemia after cardiopulmonary bypass: retrospective study. *Vascular* 2004; **12**: 114-120
126 **Venkateswaran RV**, Charman SC, Goddard M, Large SR. Lethal mesenteric ischaemia after cardiopulmonary bypass: a common complication? *Eur J Cardiothorac Surg* 2002; **22**: 534-538
127 **Yilmaz AT**, Arslan M, Demirkile U, Ozal E, Kuralay E, Bingol H, Oz BS, Tatar H, Ozturk OY. Gastrointestinal complications after cardiac surgery. *Eur J Cardiothorac Surg* 1996; **10**: 763-767
128 **Gennaro M**, Ascer E, Matano R, Jacobowitz IJ, Cunningham JN Jr, Uceda P. Acute mesenteric ischemia after cardiopulmonary bypass. *Am J Surg* 1993; **166**: 231-236
129 **Mangi AA**, Christison-Lagay ER, Torchiana DF, Warshaw AL, Berger DL. Gastrointestinal complications in patients undergoing heart operation: an analysis of 8709 consecutive cardiac surgical patients. *Ann Surg* 2005; **241**: 895-901; discussion 901-904
130 **Edwards M**, Sidebotham D, Smith M, Leemput JV, Anderson B. Diagnosis and outcome from suspected mesenteric ischaemia following cardiac surgery. *Anaesth Intensive Care* 2005; **33**: 210-217
131 **Bolcal C**, Iyem H, Sargin M, Mataraci I, Sahin MA, Temizkan V, Yildirim V, Demirkilic U, Tatar H. Gastrointestinal complications after cardiopulmonary bypass: sixteen years of experience. *Can J Gastroenterol* 2005; **19**: 613-617
132 **Andersson B**, Nilsson J, Brandt J, Hoglund P, Andersson R. Gastrointestinal complications after cardiac surgery. *Br J Surg* 2005; **92**: 326-333
133 **D'Ancona G**, Baillot R, Poirier B, Dagenais F, de Ibarra JI, Bauset R, Mathieu P, Doyle D. Determinants of gastrointestinal complications in cardiac surgery. *Tex Heart Inst J* 2003; **30**: 280-285
134 **Poirier B**, Baillot R, Bauset R, Dagenais F, Mathieu P, Simard S, Dionne B, Caouette M, Hould FS, Doyle D, Poirier P. [Abdominal complications associated with cardiac surgery. Review of a contemporary surgical experience and of a series done without extracorporeal circulation] *Can J Surg* 2003; **46**: 176-182
135 **Schutz A**, Eichinger W, Breuer M, Gansera B, Kemkes BM. Acute mesenteric ischemia after open heart surgery. *Angiology* 1998; **49**: 267-273
136 **Fitzgerald T**, Kim D, Karakozis S, Alam H, Provido H, Kirkpatrick J. Visceral ischemia after cardiopulmonary bypass. *Am Surg* 2000; **66**: 623-626
137 **Pinson CW**, Alberty RE. General surgical complications after cardiopulmonary bypass surgery. *Am J Surg* 1983; **146**: 133-137
138 **Wilson C**, Gupta R, Gilmour DG, Imrie CW. Acute superior mesenteric ischaemia. *Br J Surg* 1987; **74**: 279-281
139 **Hasan S**, Ratnatunga C, Lewis CT, Pillai R. Gut ischaemia following cardiac surgery. *Interact Cardiovasc Thorac Surg* 2004; **3**: 475-478
140 **Doguet F**, Litzler PY, Tamion F, Richard V, Hellot MF, Thuillez C, Tabley A, Bouchart F, Bessou JP. Changes in mesenteric vascular reactivity and inflammatory response after cardiopulmonary bypass in a rat model. *Ann Thorac Surg* 2004; **77**: 2130-2137; author reply 2137
141 **Rossi M**, Sganga G, Mazzone M, Valenza V, Guarneri S, Portale G, Carbone L, Gatta L, Pioli C, Sanguinetti M, Montalto M, Glieca F, Fadda G, Schiavello R, Silveri NG. Cardiopulmonary bypass in man: role of the intestine in a self-limiting inflammatory response with demonstrable bacterial translocation. *Ann Thorac Surg* 2004; **77**: 612-618
142 **Sack FU**, Dollner R, Reidenbach B, Schledt A, Taylor S, Gebhard MM, Hagl S. Extracorporeal circulation induced microvascular perfusion injury of the small bowel. *Eur Surg Res* 2002; **34**: 418-424
143 **Velissaris T**, Tang A, Murray M, El-Minshawy A, Hett D, Ohri S. A prospective randomized study to evaluate splanchnic hypoxia during beating-heart and conventional

coronary revascularization. *Eur J Cardiothorac Surg* 2003; **23**: 917-924; discussion 924

144 **Rotker J**, Knichwitz G, Erren M, Twelker L, Hammel D, Scheld HH. Mesenteric blood flow during cardiopulmonary bypass in pigs. *Thorac Cardiovasc Surg* 2000; **48**: 351-355

145 **Akpinar B**, Guden M, Sagbas E, Sanisoglu I, Aytekin V, Bayindir O. Off-pump coronary artery bypass grafting with use of the octopus 2 stabilization system. *Heart Surg Forum* 2000; **3**: 282-286

146 **Musleh GS**, Patel NC, Grayson AD, Pullan DM, Keenan DJ, Fabri BM, Hasan R. Off-pump coronary artery bypass surgery does not reduce gastrointestinal complications. *Eur J Cardiothorac Surg* 2003; **23**: 170-174

147 **Fiore G**, Brienza N, Cicala P, Tunzi P, Marraudino N, Schinosa Lde L, Fiore T. Superior mesenteric artery blood flow modifications during off-pump coronary surgery. *Ann Thorac Surg* 2006; **82**: 62-67

148 **Straub U**, Winning J, Greilach P, Isringhaus H, Kalweit G, Huwer H. Alterations of mesenteric blood flow after cardiopulmonary bypass: a Doppler sonographic study. *J Cardiothorac Vasc Anesth* 2004; **18**: 731-733

149 **Lee R**, Matsutani N, Polimenakos AC, Levers LC, Lee M, Johnson RG. Preoperative noncontrast chest computed tomography identifies potential aortic emboli. *Ann Thorac Surg* 2007; **84**: 38-41; discussion 42

150 **Leitman IM**, Paull DE, Barie PS, Isom OW, Shires GT. Intra-abdominal complications of cardiopulmonary bypass operations. *Surg Gynecol Obstet* 1987; **165**: 251-254

151 **Ghosh S**, Roberts N, Firmin RK, Jameson J, Spyt TJ. Risk factors for intestinal ischaemia in cardiac surgical patients. *Eur J Cardiothorac Surg* 2002; **21**: 411-416

152 **Trompeter M**, Brazda T, Remy CT, Vestring T, Reimer P. Non-occlusive mesenteric ischemia: etiology, diagnosis, and interventional therapy. *Eur Radiol* 2002; **12**: 1179-1187

153 **Yoshida K**, Matsumoto M, Sugita T, Nishizawa J, Matsuyama K, Tokuda Y, Matsuo T. Gastrointestinal complications in patients undergoing coronary artery bypass grafting. *Ann Thorac Cardiovasc Surg* 2005; **11**: 25-28

154 **Waspe SR**, Agrawal NK, Spyt TJ. Acute abdomen after cardiac surgery: three cases, one fatal. *J R Soc Med* 2001; **94**: 30-31

155 **Eker A**, Malzac B, Teboul J, Jourdan J. Mesenteric ischemia after coronary artery bypass grafting: should local continuous intra-arterial perfusion with papaverine be regarded as a treatment? *Eur J Cardiothorac Surg* 1999; **15**: 218-220

S- Editor Li DL **L- Editor** Anand BS **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5371

World J Gastroenterol 2008 September 21; 14(35): 5371-5376
World Journal of Gastroenterology ISSN 1007-9327

REVIEW

Satiety testing: Ready for the clinic?

Michael P Jones

Michael P Jones, Division of Gastroenterology, Northwestern University, Chicago, IL 60611, United States
Correspondence to: Michael P Jones, MD, Division of Gastroenterology, Suite 1400, 676 North St Clair Street, Chicago, IL 60611, United States. mpjones@nmh.org
Telephone: +1-312-6954054 Fax: +1-312-6953999
Received: February 8, 2008 Revised: June 2, 2008
Accepted: June 9, 2008
Published online: September 21, 2008

Abstract

Drink tests are advocated as an inexpensive, nonivasive technique to assess gastric function in patients with a variety of upper digestive symptoms. Many patients with dyspeptic complaints will achieve satiation or develop symptoms at ingested volumes below those typically required to achieve these endpoints in controls. Substantial variation in test performance exists and a greater degree of standardization is required. Additionally, it remains unclear exactly what drink tests measure as correlations with measures of gastric sensation, accomodation and emptying are modest at best. Finally, results of drink tests do not guide therpay. At present, these tests are best reserved for research studies and are not advocated for use in clinical practice.

Key words: Drink test; Dyspepsia; Gastroparesis

Peer reviewer: Benedicte Y De Winter, President, Chief, MD, PhD, Professor, University of Antwerp, Laboratory of Gastroenterology, Campus Drie Eiken, Universiteitsplein 1, 2610 Antwerp, Belgium

Jones MP. Satiety testing: Ready for the clinic? *World J Gastroenterol* 2008; 14(35): 5371-5376 Available from: URL: http://www.wjgnet.com/1007-9327/14/5371.asp DOI: http://dx.doi.org/10.3748/wjg.14.5371

OVERVIEW AND RATIONALE

Drink tests were originally developed as a noninvasive means to assess upper digestive sensation and, perhaps, gastric accommodation. These tests are most commonly performed in patients with symptoms of functional dyspepsia or gastroparesis and many patients with these conditions will achieve satiation or develop dyspeptic symptoms at ingested volumes below those typically required to achieve these endpoints in controls. Drink tests are well tolerated, inexpensive and easy to perform. They are variously performed using either water or nutrient-containing solutions administered at different rates. This variability in test performance has limited our understanding of the exact physiologic parameters measured by the test. Drink tests are often used in clinical studies evaluating patients with functional dyspepsia or gastroparesis. Although patients often report satiation or develop symptoms at substantially smaller ingested volumes than controls, it remains unclear exactly what physiologic processes are assessed by the drink test. Additionally, results of drink tests do not guide therapy. As such, these tests are probably best reserved for research studies and are not advocated for use in clinical practice.

Drink tests and symptoms

Drink tests were originally developed as a symptom provocative technique for patients with dyspeptic complaints. Patients with dyspepsia will generally drink less and report more symptoms than do healthy subjects. Symptom reporting is influenced to a large degree by the endpoint of the drink test. For example, the 5-min water load test asks subjects to drink room temperature water *ad libitum* over a 5 min period until they become full[1]. Patients rate symptoms of fullness, nausea and bloating at the end of the drink test and then again 20 min and 30 min after the conclusion of the test. Not surprisingly, scores for the endpoint of fullness do not differ as greatly between patients with functional dyspepsia and controls while patients with functional dyspepsia do report significantly greater scores for symptoms of bloating and nausea (Figure 1)[2]. Compared with controls, symptoms induced by the drink test are more likely to persist in patients with functional dyspepsia compared with controls.

Boeckxstaens *et al*[3] have evaluated symptom responses to both water and Nutridrink consumed at a rate of 100 mL/min in healthy subjects and patients with functional dyspepsia. After each 100 mL, symptoms of satiety, epigastric bloating, nausea, and pain were scored on a scale from 0 (no sensation) to 5 (discomfort). The test was ended when a score of 5 was reached for at least one of the symptoms and the maximal ingested volume calculated. Subjects also rated these symptoms

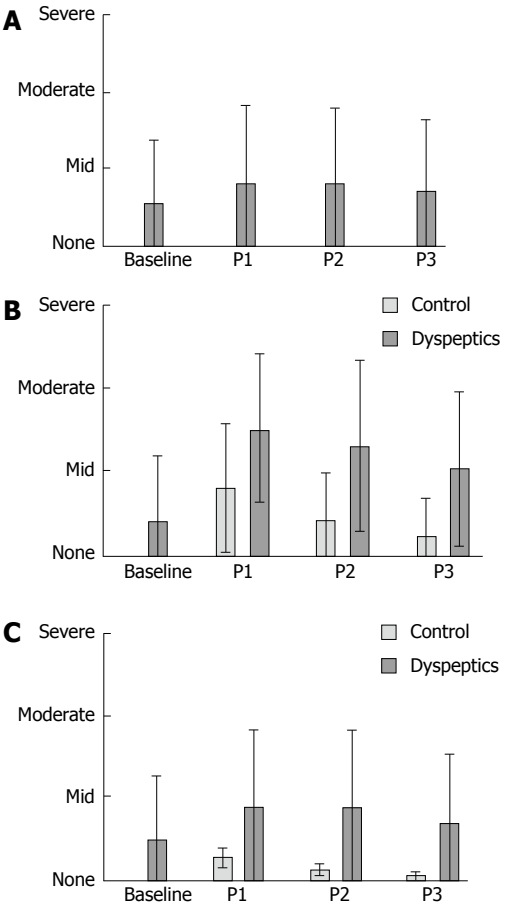


Figure 1 Symptoms before and after a 5-min water load test in controls and patients with functional dyspepsia. Patients with dyspepsia were significantly more symptomatic in terms of nausea (**A**), fullness (**B**), and bloating (**C**) both at baseline and after the water load test. Symptom scores at all time points were significantly different between the two groups. P1: 10 min after WL5; P2: 20 min after WL5; P3: 30 min after WL5. Data are expressed as mean ± SD. Adapted from Jones *et al*[2].

1 and 2 h after the end of the drink test. Again, patients with functional dyspepsia reported greater and more persistent symptoms during the drink test than did controls. Nutridrink was more symptom-provoking than water. Importantly, subjects in this study also underwent gastric barostat testing and were classified as having either normal physiology, visceral hypersensitivity or impaired accommodation. Symptom scores during drink tests were not influenced by the results of the barostat study.

While patients with functional dyspepsia will often achieve satiation at lower drink test volumes than controls and will report greater symptoms during the test, specific dyspeptic symptoms are not associated with an abnormal drink test. Jones and Maganti[4] evaluated the relationship between 15 common dyspeptic symptoms and volume to fullness as measured by a 5-min water load test. The only symptom significantly correlated with volume to fullness was nausea which showed a weak inverse correlation with nausea severity (r = -0.3, P = 0.05). Similarly, Boeckxstaens *et al*[5] reported that while patients with functional dyspepsia were more likely to report more symptoms during drink testing, no specific symptom was more likely to be associated with an abnormal drink test. Finally, Kindt *et al*[6] recently reported that maximal drink test volume was inversely associated with dyspeptic symptom scores prior to the study. For specific dyspeptic symptoms, only early satiety was significantly associated with the endpoint of the first satiety drinking test (r = 0.25, P = 0.02).

Psychiatric comorbidity is common in functional dyspepsia. In healthy subjects, experimentally induced anxiety is associated with decreased gastric compliance and meal-induced accommodation as well as increased symptom scores during a standard nutrient drink challenge[7]. However, in patients with functional dyspepsia, correlations between drink test volumes and general psychiatric distress (measured using either the SCL-90R or the Psychological General Well Being Scale) have been modest at best[2,4]. We do often encounter patients who report fullness at volumes that clearly defy physiologic parameters (< 50 mL), suggesting that central factors clearly influence test results. Finally, patients with functional dyspepsia, compared with controls, patients with gastroparesis or patients with gastroesophageal reflux often demonstrate poor self-efficacy and are less capable of estimating the volume required to produce fullness (Figure 2)[2].

Drink tests and accommodation

Assessment of gastric sensation and accommodation is most rigorously measured using a barostat. This is a cumbersome, expensive device that is decidedly patient unfriendly. Logically, it would seem that incrementally distending the stomach by drinking could achieve a result similar to incrementally distending the stomach using a balloon on the end of a catheter. Indeed, Tack *et al*[8] have reported a good correlation between barostat-measured accommodation and total calories consumed during a nutrient drink test administered at 15 mL/min. For both patients with functional dyspepsia and controls, the correlation was 0.76 (P < 0.001) and the nutrient drink test was calculated to have a sensitivity of 92% and a specificity of 86% in predicting impaired gastric accommodation.

Not all authors have agreed with these findings. Boeckxstaens *et al*[5] found no correlation between drinking capacity and fundal accommodation to a meal. These authors used both water and nutrient drink tests to evaluate subjects. The sensitivity of the water load test and nutrient drink test to detect impaired accommodation was 73% and 81%, respectively. The discrepant results between these two studies may reflect the methods used. Tack *et al*[8] had subjects ingest Nutridrink at a rate of 15 mL/min while Boeckxstaens *et al*[5] had subjects consume Nutridrink or water at 100 mL/min.

Accommodation can also be assessed using single-photon emission computed tomography (SPECT). Using a nutrient drink test administered at a rate of 120 mL/4 min, Gonenne *et al*[9] found that after controlling for covariates in a convenience sample of controls and patients with functional dyspepsia, the maximal tolerated nutrient drink test volume explained

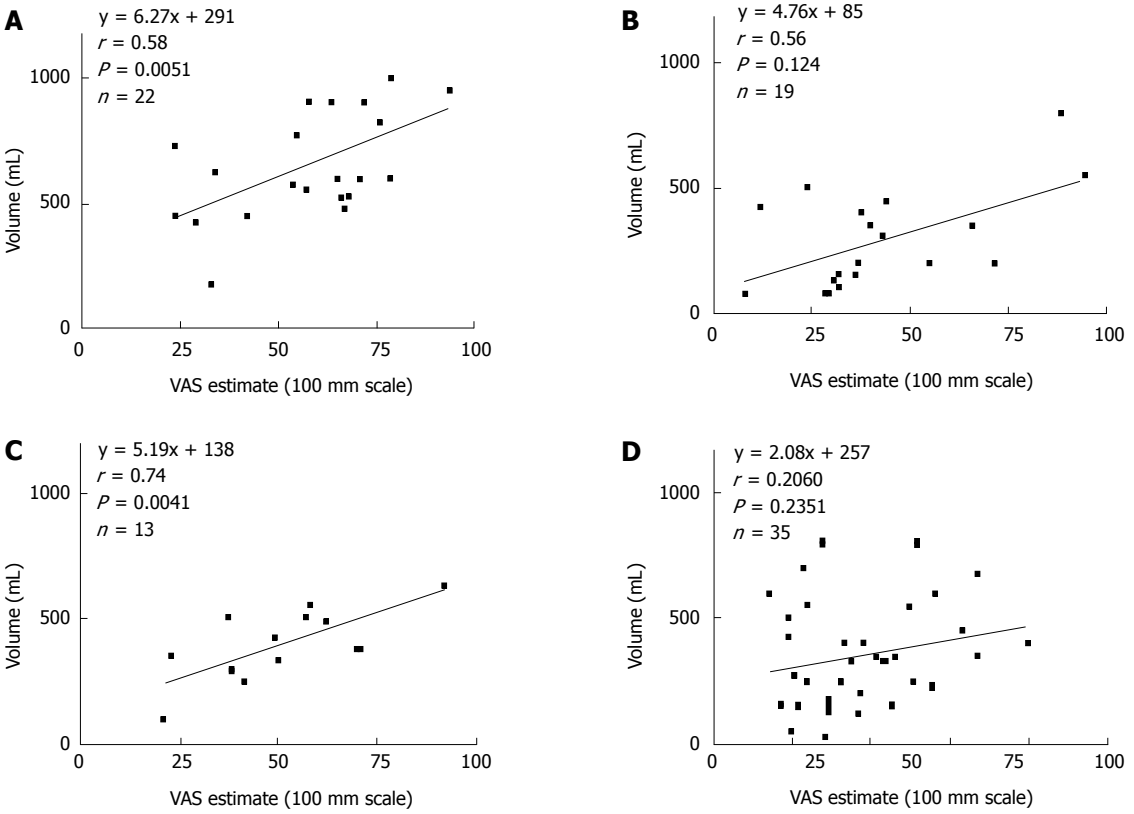


Figure 2 Drink test self-efficacy. Controls (**A**), and patients with either gastroparesis (**B**) or gastroesophageal reflux disease (**C**) are able to accurately estimate drinking capacity, while patients with functional dyspeptics (**D**) cannot. VAS: Visual analog scale. Adapted from Jones *et al*[2].

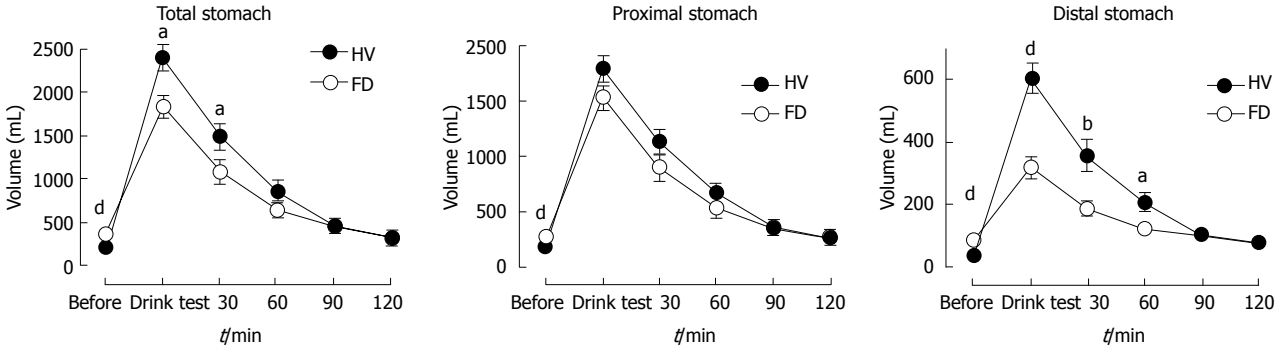


Figure 3 Volumes measured by gastric volume scintigraphy for total, proximal and distal stomach volume over time. Patients with functional dyspepsia had higher fasting volumes but reduced maximal tolerated volumes during drink test and reduced lower distal stomach volumes ($^{a}P < 0.05$, $^{b}P < 0.01$, $^{d}P < 0.001$; Mann-Whitney *U*-test). Adapted from van der Elzen *et al*[10]. HV: Healthy volumes; FD: Functional dyspepsia.

only 13% and 3% of the variations in fasting and postprandial volumes measured by SPECT.

Recently, van den Elzen *et al*[10] have shown that drinking capacity may be more related to distal rather than proximal stomach function. Compared to controls, patients with functional dyspepsia ingested significantly less water ($P < 0.001$) and had reduced filling of the distal stomach ($P < 0.001$) after the drink test (Figure 3).

Drink tests and gastric emptying

Only a few studies have examined the relationship between gastric emptying and maximal tolerated volume and that relationship appears modest at best. Cuomo *et al*[11] reported that in females with functional dyspepsia, the correlation between maximal tolerated drink test volume and the fractional rate of gastric emptying was 0.48 ($P = 0.0003$, Figure 4). Tack *et al*[8] also reported a weak but significant correlation between maximal tolerated volume for the nutrient drink test and the half time of gastric emptying for a solid meal when pooled controls and patients with functional dyspepsia were studied ($r = -0.40$, $P = 0.001$). The correlation was not statistically significant when only evaluating patients with functional dyspepsia. Jones *et al*[2] did not find a correlation between volume to fullness using a 5-min

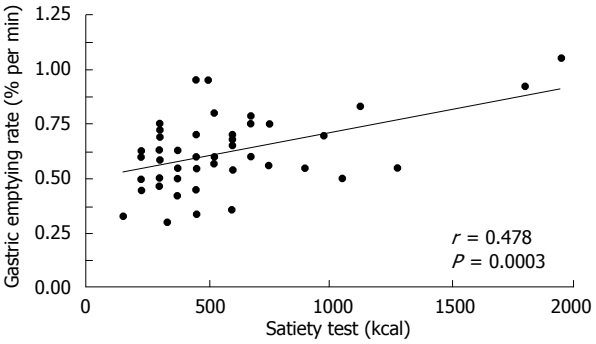


Figure 4 Correlation between nutrient drink test (kcal) and gastric emptying rate (% per min of gastric content) in the dyspeptic patients. Adapted from Cuomo *et al*[11].

water load test and T_{lag} (r = 0.1532, P = 0.4549) or $T_{1/2}$ (r = 0.1489, P = 0.4679) using a stable isotope gastric emptying breath test.

COMPARISONS BETWEEN DRINK TESTS

Drink tests are performed using either water or nutrient-containing beverages which are consumed at various rates. No method has proven superior although nutrient drink tests appear to be performed more often in clinical research. Presently, there is a need for a consensus on drink test methodology so that observations made by various investigators will be uniformly interpretable. Limited data exist regarding the performance characteristics of drink tests. Males ingest greater volumes than females but there appears to be less of an influence with respect to age and BMI[2,5,6,8].

Results of drink tests are reproducible at least in the short term. For healthy subjects, the correlations between 5-min water load tests at baseline and repeated 2 wk and 2 mo later were 0.78 (P < 0.0001) and 0.33 (P = 0.16)[2]. Cuomo *et al*[11] repeated a nutrient drink test between 2 d and 5 d in 10 controls and 5 patients with functional dyspepsia. The resulting inter-day variation of kcal ingested was 4.7% ± 1.5%.

Water loading at different rates produces comparable results in healthy subjects. The correlation between volumes to fullness for the 5-min water load and 100 mL/min water load was 0.79 (P < 0.0001)[2]. For the same subjects, the correlation between the 5-min water load and a 5-min nutrient drink test was 0.20 (P = 0.48). Boeckxstaens *et al*[5] found a significant correlation between the maximal volume ingested in the water test and the nutrient drink test. The correlation was greatest among controls (r = 0.67, P = 0.0001) and weakest among patients with functional dyspepsia (r = 0.57, P = 0.0001).

Using a 15 mL/min Nutridrink test, Kindt *et al*[6] demonstrated excellent test-retest reliability for a group that included 34 controls and 78 patients with FD (r = 0.88, P < 0.0001). During repeat testing, controls tended to consume higher volumes while patients with functional dyspepsia showed less variability.

PUBLISHED VALUES AND RANGES FOR DRINK TESTS

For the 5-min water load test, the mean volume required to produce fullness in a group of 73 controls was 648 ± 204 mL[2]. Males (703 ± 217 mL) drank more than females (611 ± 188 mL), but the difference was not statistically significant (t = 1.907; P = 0.0605). No healthy subject consumed < 300 mL of water and that volume was proposed as a cut-off for an abnormal test. A subsequent study demonstrated that the 300 mL cut-off value for the 5-min water load test discriminated controls from patients with functional dyspepsia with a sensitivity of 98% and a specificity of 46%[12].

For healthy subjects, the mean volume to fullness for the 100 mL/min water load test in controls has been reported as 1128 ± 355 mL[2]. In the same population, the mean volume to fullness for the 5-min nutrient drink test was 688 ± 187 mL. The nutrient drink test was performed using Boost™ (Mead Johnson Nutritionals, Evansville, Indiana) which contains 1.1 kcal/mL and is 70% carbohydrate, 15% fat, and 16% protein. Boost differs somewhat from Nutridrink™ (N.V. Nutricia, Zoetermeer, Netherlands) which contains 1.5 kcal/mL and is 39% fat. Boost™ is comparable to Ensure™ (Abbott Laboratories, Abbott Park, Ill.) which contains 1.06 kcal/mL and is 65% carbohydrate, 20% fat, and 15% protein. These test meals have not been directly compared but Tack *et al*[8] have shown that with increasing caloric density, maximum satiety occurs at progressively higher caloric intakes while satiety scores according to ingested volumes do not differ significantly. This suggests that volume may be a greater stimulus than caloric density. No study has assessed the influence of caloric composition or palatability.

Boeckxstaens *et al*[5] have reported normal values for both Nutridrink and water load administered at 100 mL/min. Males consumed significantly more water (2084 ± 181 mL *vs* 1367 ± 97 mL, P = 0.0001) and Nutridrink (1405 ± 81 mL *vs* 946 ± 74 mL, P = 0.002) than females. Using the 10th percentile as the lower limit of the normal range, volumes < 1100 mL of water for men and < 800 mL of water for women were considered abnormal. Similarly, volumes < 800 mL of Nutridrink for men and < 600 mL for women were considered abnormal. The difference in results for the 100 mL/min water load tests between these two studies likely reflects the fact that Jones *et al*[2] had subjects stop drinking when they first experienced fullness while Boeckxstaens *et al*[5] had patients continue to drink until they developed very severe or uncomfortable sensations of symptoms of satiety, epigastric bloating, nausea or pain.

Using a Nutridrink test administered at a rate of 15 mL/min to healthy volunteers, Tack *et al*[8] reported that maximum satiety occurred after ingestion of 1005 ± 35 mL (mean ± SE) with a lower limit of normal of 653 mL. This observation was supported by a more recent observation from the same group[2]. In this study, controls reported maximum satiety after ingestion of 937 ± 428 mL. Increasing drink test

volumes were associated with male sex and increasing age[6]. Moreover, Chial *et al*[13] used a nutrient drink test adopted from the methodology of Tack *et al*[8]. Subjects consumed 120 mL of Ensure™ every 4 min until full, and the average volume of nutrient drink ingested (mean ± SE) was 1181 ± 50 mL. There was a weak but significant correlation ($r = 0.29$, $P = 0.02$) between volume to fullness and body mass index.

Indications for drink tests

Given that it is unclear exactly what drink tests measure, and that the test remains poorly standardized, the role of drink tests in clinical practice remains to be established[14,15]. The test has most often been employed in clinical research studies evaluating patients with functional dyspepsia. Water loading is also performed as a provocative maneuver during the performance of electrogastrography[1].

Performing a drink test

Patients should be studied in the morning after an overnight fast. While certain medications can alter digestive sensation, accommodation or gastric emptying, we do not routinely stop motility or sensory modifying medications for clinical studies.

The 5-min water load test is performed by having subjects drink room temperature tap water *ad libitum* over a 5-min period until reaching the point of fullness. Water is consumed from an unmarked flask that is taken from the subject and refilled after each drink. The volume required to refill the flask to the initial level is recorded, and the total volume consumed is calculated by summing these volumes. In this way, the flask is "bottomless" and the subject blinded as to the actual volume of water consumed. During the test, patients rate symptoms of fullness, bloating, and nausea using a 4-point Likert scale for each symptom. Scores are recorded at baseline and then every 10 min for a 30-min period after completion of the test. Individual symptoms can therefore receive a total score ranging from 0-12, and the total WL symptom score has a range of 0-36.

Nutrient drink tests can be performed in a several ways. The simplest method is that used by Chial *et al*[13], in which subjects consume 120 mL of Ensure™ every 4 min until full. Ensure is administered in a paper cup that is refilled every 4 min. At 5 min intervals, participants score fullness using a rating scale that combines verbal descriptors on a scale graded 0-5 [0: no symptoms; 1: first sensation of fullness (threshold); 2: mild; 3: moderate; 4: severe; 5: maximum or unbearable fullness]. Participants are told to stop when a score of 5 is obtained. Postprandial symptoms were measured 30 min after completing the test with participants scoring symptoms of bloating, fullness, nausea and pain using a visual analogue scale (VAS) with 100 mm lines and the words "unnoticeable" and "unbearable" as anchors. The sum of the four 100-mm VAS scales for each symptom provides an aggregate symptom score.

The nutrient drink test used by Boeckxstaens *et al*[5] had subjects who consumed Nutridrink™ at a rate of 100 mL/min. Nutridrink™ is given in beakers or paper cups filled with 100 mL aliquots. After each 100 mL, symptoms of satiety, epigastric bloating, nausea, and pain are scored on a 5-point Likert scale (0: no sensation; 1: very mild; 2: mild; 3: moderate; 4: severe; 5: very severe or discomfort). When a score of 5 is reached for any symptom, the test ends and the maximal ingested volume is calculated.

Reporting and interpreting test results

Drink test results are reported as the maximal ingested volume. Occasionally, a patient may experience emesis during the test. If emesis occurs, the volume of emesis should be recorded and subtracted from the total ingested volume. Along with the maximal ingested volume, individual and cumulative symptom scores can be reported.

While the utility of drink tests remains to be determined, we find the test most helpful when it is either normal or glaringly abnormal. In the former scenario, the patient can be reassured that gastric function is likely to be intact. The latter scenario is more subjective. Often patients will report maximal fullness after the consumption of physiologically insignificant volumes (< 50 mL). We have not found results from the water load test to be correlated with measures of psychiatric distress or somatization but maximal ingested volumes are positively correlated with quality of life[2,12]. Others have reported that maximal ingested volumes are reduced in patients with depression[16]. While drink tests are not intended as surrogates for assessing psychosocial factors or quality of life, maximal fullness at extremely low volumes may suggest that extra-gastric or central factors are playing an important role in symptom generation, perpetuation or tolerance.

Since the physiologic parameters that determine maximal ingested volumes are not well known at present, drink test results cannot be reasonably used to guide therapy. Few studies have assessed the impact of commonly used treatments for functional dyspepsia on drink test results. A brief, randomized controlled trial found that 14 d of therapy with nortriptyline, mirtazapine or placebo did not alter either maximal ingested volumes or symptom scores[17]. A similar study also found no effect on maximal ingested volume or symptoms in healthy subjects treated with either citalopram, desipramine or placebo for 11 d[18]. In contrast, in healthy subjects, the kappa-opioid agonist asimadoline has been shown to increase maximal tolerated volumes without altering gastric emptying[19].

A small trial randomized patients with functional dyspepsia to biofeedback (breathing exercises using software for vagal biofeedback) or an educational control group[20]. Drinking capacity and quality of life improved significantly more in the biofeedback group than in the control group without any significant change in baseline autonomic activity or intra-gastric volume.

Combining an incomplete understanding of relevant pathophysiologic alterations that might be measured by drink tests with limited data regarding effects of

therapy on drink test volumes leads us to conclude that drink tests are of limited utility in guiding clinical management. Until our understanding in this area has evolved, drink tests should not routinely be performed in clinical practice.

CONCLUSION

Drink tests are often used in clinical studies evaluating patients with functional dyspepsia or gastroparesis. Although patients often report satiation or develop symptoms at substantially smaller ingested volumes than controls, it remains unclear exactly what physiologic processes are assessed by the drink test. Additionally, results of drink tests do not guide therapy. As such, these tests are probably best reserved for research studies and are not advocated for use in clinical practice.

REFERENCES

1 **Koch KL**, Hong SP, Xu L. Reproducibility of gastric myoelectrical activity and the water load test in patients with dysmotility-like dyspepsia symptoms and in control subjects. *J Clin Gastroenterol* 2000; **31**: 125-129
2 **Jones MP**, Hoffman S, Shah D, Patel K, Ebert CC. The water load test: observations from healthy controls and patients with functional dyspepsia. *Am J Physiol Gastrointest Liver Physiol* 2003; **284**: G896-G904
3 **Boeckxstaens GE**, Hirsch DP, Kuiken SD, Heisterkamp SH, Tytgat GN. The proximal stomach and postprandial symptoms in functional dyspeptics. *Am J Gastroenterol* 2002; **97**: 40-48
4 **Jones MP**, Maganti K. Symptoms, gastric function, and psychosocial factors in functional dyspepsia. *J Clin Gastroenterol* 2004; **38**: 866-872
5 **Boeckxstaens GE**, Hirsch DP, van den Elzen BD, Heisterkamp SH, Tytgat GN. Impaired drinking capacity in patients with functional dyspepsia: relationship with proximal stomach function. *Gastroenterology* 2001; **121**: 1054-1063
6 **Kindt S**, Coulie B, Wajs E, Janssens J, Tack J. Reproducibility and symptomatic predictors of a slow nutrient drinking test in health and in functional dyspepsia. *Neurogastroenterol Motil* 2008; **20**: 320-329
7 **Geeraerts B**, Vandenberghe J, Van Oudenhove L, Gregory LJ, Aziz Q, Dupont P, Demyttenaere K, Janssens J, Tack J. Influence of experimentally induced anxiety on gastric sensorimotor function in humans. *Gastroenterology* 2005; **129**: 1437-1444
8 **Tack J**, Caenepeel P, Piessevaux H, Cuomo R, Janssens J. Assessment of meal induced gastric accommodation by a satiety drinking test in health and in severe functional dyspepsia. *Gut* 2003; **52**: 1271-1277
9 **Gonenne J**, Castillo EJ, Camilleri M, Burton D, Thomforde GM, Baxter KL, Zinsmeister AR. Does the nutrient drink test accurately predict postprandial gastric volume in health and community dyspepsia? *Neurogastroenterol Motil* 2005; **17**: 44-50
10 **van den Elzen BD**, Bennink RJ, Holman R, Tytgat GN, Boeckxstaens GE. Impaired drinking capacity in patients with functional dyspepsia: intragastric distribution and distal stomach volume. *Neurogastroenterol Motil* 2007; **19**: 968-976
11 **Cuomo R**, Sarnelli G, Grasso R, Bruzzese D, Pumpo R, Salomone M, Nicolai E, Tack J, Budillon G. Functional dyspepsia symptoms, gastric emptying and satiety provocative test: analysis of relationships. *Scand J Gastroenterol* 2001; **36**: 1030-1036
12 **Jones MP**, Roth LM, Crowell MD. Symptom reporting by functional dyspeptics during the water load test. *Am J Gastroenterol* 2005; **100**: 1334-1339
13 **Chial HJ**, Camilleri C, Delgado-Aros S, Burton D, Thomforde G, Ferber I, Camilleri M. A nutrient drink test to assess maximum tolerated volume and postprandial symptoms: effects of gender, body mass index and age in health. *Neurogastroenterol Motil* 2002; **14**: 249-253
14 **Bratten J**, Jones MP. New directions in the assessment of gastric function: clinical applications of physiologic measurements. *Dig Dis* 2006; **24**: 252-259
15 **Mimidis K**. Drinking tests in functional dyspepsia: what do they really measure? *Neurogastroenterol Motil* 2007; **19**: 947-950
16 **Strid H**, Norström M, Sjöberg J, Simrén M, Svedlund J, Abrahamsson H, Björnsson ES. Impact of sex and psychological factors on the water loading test in functional dyspepsia. *Scand J Gastroenterol* 2001; **36**: 725-730
17 **Choung RS**, Cremonini F, Thapa P, Zinsmeister AR, Talley NJ. The effect of short-term, low-dose tricyclic and tetracyclic antidepressant treatment on satiation, postnutrient load gastrointestinal symptoms and gastric emptying: a double-blind, randomized, placebo-controlled trial. *Neurogastroenterol Motil* 2008; **20**: 220-227
18 **Talley NJ**, Camilleri M, Chitkara DK, Bouras E, Locke GR 3rd, Burton D, Rucker MJ, Thapa P, Zinsmeister AR. Effects of desipramine and escitalopram on postprandial symptoms induced by the nutrient drink test in healthy volunteers: a randomized, double-blind, placebo-controlled study. *Digestion* 2005; **72**: 97-103
19 **Delgado-Aros S**, Chial HJ, Camilleri M, Szarka LA, Weber FT, Jacob J, Ferber I, McKinzie S, Burton DD, Zinsmeister AR. Effects of a kappa-opioid agonist, asimadoline, on satiation and GI motor and sensory functions in humans. *Am J Physiol Gastrointest Liver Physiol* 2003; **284**: G558-G566
20 **Hjelland IE**, Svebak S, Berstad A, Flatabo G, Hausken T. Breathing exercises with vagal biofeedback may benefit patients with functional dyspepsia. *Scand J Gastroenterol* 2007; **42**: 1054-1062

S- Editor Zhong XY **L- Editor** Kumar M **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5377

World J Gastroenterol 2008 September 21; 14(35): 5377-5384
World Journal of Gastroenterology ISSN 1007-9327

REVIEW

Neuroendocrine tumors of the gastro-entero-pancreatic system

Sara Massironi, Valentina Sciola, Maddalena Peracchi, Clorinda Ciafardini, Matilde Pia Spampatti, Dario Conte

Sara Massironi, Valentina Sciola, Maddalena Peracchi, Clorinda Ciafardini, Matilde Pia Spampatti, Dario Conte, Gastroenterology Unit Ⅱ, Fondazione IRCCS Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Milan 20122, Italy
Author contributions: All co-authors contributed equally to this review.
Correspondence to: Dario Conte, MD, Gastroenterology Unit Ⅱ, Fondazione IRCCS Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Via Sforza 35, Milan 20122, Italy. dario.conte@unimi.it
Telephone: +39-2-55033418 Fax: +39-2-55033644
Received: April 10, 2008 Revised: July 7, 2008
Accepted: July 14, 2008
Published online: September 21, 2008

Abstract

Gastro-entero-pancreatic (GEP) neuroendocrine tumors (NETs) are rare neoplasms, although their prevalence has increased substantially over the past three decades. Moreover, there has been an increased clinical recognition and characterization of these neoplasms. They show extremely variable biological behavior and clinical course. Most NETs have endocrine function and secrete peptides and neuroamines that cause distinct clinical syndromes, including carcinoid syndrome; however, many are clinically silent until late presentation with mass effects. Investigation and management should be individualized for each patient, taking into account the likely natural history of the tumor and general health of the patient. Management strategies include surgery for cure or palliation, and a variety of other cytoreductive techniques, and medical treatment including chemotherapy, and biotherapy to control symptoms due to hormone release and tumor growth, with somatostatin analogues (SSAs) and alpha-interferon. New biological agents and somatostatin-tagged radionuclides are under investigation. Advances in the therapy and development of centers of excellence which coordinate multicenter studies, are needed to improve diagnosis, treatment and therefore survival of patients with GEP NETs.

Key words: Gastro-entero-pancreatic neuroendocrine tumors; Carcinoids; Entero-endocrine tumors; Pancreatic tumors; Medical treatment; Molecular targeted therapy

Peer reviewer: Leonidas G Koniaris, Professor, Alan Livingstone Chair in Surgical Oncology, 3550 Sylvester Comprehensive Cancer Center (310T), 1475 NW 12th Ave, Miami, FL 33136, United States

Massironi S, Sciola V, Peracchi M, Ciafardini C, Spampatti MP, Conte D. Neuroendocrine tumors of the gastro-entero-pancreatic system. *World J Gastroenterol* 2008; 14(35): 5377-5384 Available from: URL: http://www.wjgnet.com/1007-9327/14/5377.asp DOI: http://dx.doi.org/10.3748/wjg.14.5377

INTRODUCTION

Neuroendocrine tumors (NETs) of the gastro-entero-pancreatic (GEP) system are rare and originate from the diffused endocrine system, located in the gastro-intestinal (GI) tract (carcinoids) and in the pancreas (insular tumors), with extremely varying clinical pictures. GEP NETs represent about 2% of all the GI tumors[1], but their prevalence has increased substantially over the past three decades, only in part as a consequence of increased awareness and improved diagnostic techniques[2]. The most recent estimates suggest a global clinical incidence of 2.5-5 cases/100 000 per year[2,3], with an autoptical incidence 2-5 times higher than the clinical one, and a slight predominance in females[4,5].

The term carcinoid (from the German *Karzinoide*) was introduced in 1907 by Oberndorfer to identify some ileal tumors, originating from the enterochromaffin cells (EC) that produce serotonin, characterized by a better prognosis in comparison with adenocarcinomas. Later the term was used to describe NETs, both of the gut and extra-intestinal sites (pancreas, lung and bronchus, liver, thymus), even though the term NET should always be used specifying the tumor's origin site, in order to avoid misunderstanding. The term carcinoid should be used to indicate the serotonin-secreting tumors[6].

The diffused endocrine system of the GEP tract is the widest of the whole organism, with at least 16 different types of endocrine cells that produce more than 50 peptides or amines[2,6,7]. GEP NETs arise within the GI tract, but NETs can also occur elsewhere such as in the bronchus and lung (bronchial epithelium), hypophysis, thyroid, parathyroids, thymus, adrenal cortex and medulla, and paraganglia. GEP NETs can preserve and amplify the activity of the origin cells

characterized by secretion of a number of peptides and neurotransmitters, which can lead to the development of typical clinical syndromes by the so called "functioning" tumors, or they can be biologically inactive ("non-functioning" tumors)[1,2,8] for several reasons (defect of hormonal synthesis/secretion, rapid hormone degradation, synthesis of precursors/inactive hormones, co-secretion of antagonist hormones).

GEP NETs are usually sporadic, but they may also be multiple and may occur in some genetic syndromes such as multiple endocrine neoplasia (MEN) type 1, von Hippel-Lindau syndrome, neurofibromatosis type 1 and tuberous sclerosis[2,9,10]. Their frequency in these syndromes varies from very low (< 1%) for carcinoid to high (80%-100%) for pancreatic endocrine tumors (insulinomas 5%-20%, gastrinomas 25%-30%, non-functioning > 50%)[6].

CLASSIFICATION

As GEP NETs represent a heterogeneous group of tumors, their classification is still a critical point. In the past, GEP NETs were classified according to their embryonic origin and, according to the classification of William and Sandler[11], three distinct groups have been identified: (1) carcinoids derived from the proximal GI tract (foregut), located in the stomach, proximal duodenum, biliary tract and pancreas fed by the celiac tripod; (2) carcinoids derived from intermediate GI tract (midgut), located in the distal duodenum, small intestine, appendix and right colon, fed from the superior mesenteric artery; (3) carcinoids of the distal intestine (hindgut) localized into the descending colon, sigmoid colon and rectum, fed from the inferior mesenteric artery.

The most recent WHO classification[12] (Table 1) categorized all GEP NETs on the basis of clinical-pathological criteria as follow: (1) well-differentiated endocrine tumors, with benign or uncertain behaviour; (2) well-differentiated endocrine carcinomas, with a low-grade malignant behaviour; (3) poorly differentiated endocrine carcinomas (small cells carcinomas), with a high-grade malignant behaviour; (4) mixed endocrine-exocrine carcinomas, with characteristics of both endocrine and exocrine tumors. Each category includes functioning and non-functioning tumors.

However this classification has prognostic limits and a suboptimal reproducibility among pathologists hence TNM classification is being developed for NETs[13,14]. Table 2 provides examples of TNM classification for pancreatic NETs and carcinoids.

CLINICAL FEATURES

Clinical manifestations of GEP NETs are very heterogeneous: indeed, they can either remain asymptomatic for years, or can occur with obstructive symptoms, such as abdominal pain, nausea, vomiting, cholestasis, or can present with metastases, found accidentally, or can occur with typical syndromes due to hormonal hypersecretion. In most cases, because of vagueness of symptoms, the diagnosis is delayed (3-10 years on average), with an increased risk of developing metastases.

Gastrointestinal NETs (carcinoids)

NETs of the small intestine according to the Surveillance, Epidemiology, and End Results (SEER) database have an incidence of 0.15-0.5 cases/100 000 per year[15]. They are usually asymptomatic or characterized by obstructive symptoms, due to the local fibrotic reaction or, rarely, to the mass itself, until liver metastases appear[6]. At this stage, the typical clinical picture is the carcinoid syndrome that occurs in 18% of patients with ileal carcinoid[2,16] and is characterized by flushing, diarrhea, abdominal pain; less frequent events are lacrymation, profuse sweating, telangiectasias, cardiac fibrosis, and cutaneous manifestations pellagra-like due to lack of niacin (Table 3). Carcinoid syndrome is caused by the release of serotonin, which is no longer metabolized in the liver, and other substances, such as tachykinins, prostaglandins, and bradykinins[2,17].

Gastric carcinoids, that account for 4.6% of all carcinoids[15], originate from gastric EC-like mucosal cells, are mostly asymptomatic and occasionally found in the course of gastroscopies[6]; rarely they can cause an atypical carcinoid syndrome (flushing of greater duration than typical, of a red colour, with scialorrea, sweating, tearing, hypotension and itching)[16-18]. These carcinoids are divided into 3 groups: those that occur in chronic hypergastrinemic conditions, such as the type 1, associated with chronic atrophic gastritis, and type 2, associated with Zollinger Ellison syndrome in MEN-1, while type 3 is not associated with hypergastrinemia and is frequently malignant, with distant metastases.

Appendiceal endocrine tumors are often small and are found incidentally during appendectomies, with a frequency of 3-9/1.000 appendectomies and are usually benign[6,19-21]. Colonic carcinoids account for 8.6% of all carcinoids. They are often large and, among the intestinal carcinoids, have the worst prognosis[6,22].

Rectal carcinoids may present as an incidental finding on sigmoidoscopy or colonoscopy (1:2.500). They are typically small, non-functioning and distant metastases are rarely present at diagnosis (probably due to the early diagnosis)[6,22].

Carcinoids have previously been reported to be associated with secondary non-carcinoid malignancies, with rates as high as 46%-55%, more frequently located in the lung, breast, prostate and colon[23,24].

Pancreatic NETs

Endocrine tumors of the pancreas can occur with typical syndromes due to hormonal hypersecretion, such as insulinoma, gastrinoma, VIP-oma, glucagonoma and somatostatinoma (Table 4), but in a percentage of 40%-50% they are non-functioning or secrete peptide with a low biological impact, such as pancreatic polypeptide (PP) and neurotensin. Moreover a metastatic disease can be present at the time of diagnosis in approximately 50% of the cases[1,2,6,8].

Table 1 WHO classification[12]

Site	Well differentiated endocrine tumor		Well-differentiated endocrine carcinoma	Poorly-differentiated endocrine carcinoma
	BB	UB		
Pancreas	< 2 cm	⩾ 2 cm	Local invasion	Small cells
	< 2 mitoses[1]	> 2 mitoses	2-10 mitoses	> 10 mitoses
	< 2% Ki-67	> 2% Ki-67	> 5% Ki-67	> 15% Ki-67
	No vascular invasion	Vascular invasion	Vascular invasion ± metastases	Vascular/perineural invasion
Stomach	Mucosa/Submucosa	Mucosa/Submucosa	Invasion of muscolaris propria ± metastases	Small cells
	⩽ 1 cm	> 1 cm		
	No vascular invasion	Vascular invasion		
Duodenum/	Mucosa/Submucosa	Mucosa/Submucosa	Invasion of muscolaris propria ± metastases	Small cells
Jejunum	⩽ 1 cm	> 1 cm		
	No vascular invasion	Vascular invasion		
Ileum/	Mucosa/Submucosa	Mucosa/Submucosa	Invasion of muscolaris propria ± metastases	Small cells
Colon/	⩽ 1 cm (ileum)	> 1 cm (ileum)		
Rectum	⩽ 2 cm (colon)	> 2 cm (colon)		
	No vascular invasion	Vascular invasion		
Appendix	⩽ 2 cm	> 2 cm	Extensive invasion of mesoappendix ± metastases	Small cells
	No vascular invasion	Vascular invasion		

[1]Mitoses expressed as number/10 high power field. BB: Benign behavior; UB: Uncertain behavior.

Table 2 TNM staging for pancreatic NETs[13], foregut and midgut gastrointestinal carcinoids[14]

	Pancreatic NETs			Foregut and midgut gastrointestinal carcinoids		
T-primary tumor						
Tx	Primary tumor cannot be assessed			Primary tumor cannot be assessed		
T0	No evidence of primary tumor			No evidence of primary tumor		
T1	Tumor limited to the pancreas and size < 2 cm			Tumor invades mucosa or submucosa and size ⩽ 1 cm		
T2	Tumor limited to the pancreas and size 2-4 cm			Tumor invades muscolaris propria and size > 1 cm		
T3	Tumor limited to the pancreas and size > 4 cm or invading duodenum or bile duct			Tumor invades subserosa		
T4	Tumor invading adjacent organs (stomach, spleen, colon, adrenal gland) or the wall of large vessels (celiac axis or superior mesenteric artery)			Tumor invades adjacent structures		
	For any T, add (m) for multiple tumors			For any T, add (m) for multiple tumors		
N-regional lymph nodes						
Nx	Regional lymph nodes cannot be assessed			Regional lymph nodes cannot be assessed		
N0	No regional lymph node metastases			No regional lymph node metastases		
N1	Regional lymph node metastases			Regional lymph node metastases		
M- distant metastases						
Mx	Distant metastases cannot be assessed			Distant metastases cannot be assessed		
M0	No distant metastases			No distant metastases		
M1	Distant metastases			Distant metastases		
Disease stage						
Ⅰ	T1	N0	M0	T1	N0	M0
Ⅱa	T2	N0	M0	T2	N0	M0
Ⅱb	T3	N0	M0	T3	N0	M0
Ⅲa	T4	N0	M0	T4	N0	M0
Ⅲb	Any T	N1	M0	Any T	N1	M0
Ⅳ	Any T	Any N	M1	Any T	Any N	M1

Criteria for carcinoids of the appendix and colon rectum differ only for the tumor size.

Insulinoma and gastrinoma are the most frequent pancreatic NETs. The incidence of insulinomas is 2-4 new cases/1 000 000 per year, whereas that of gastrinoma is 0.5-4 new cases/1 000 000 per year[8,25].

Insulinoma are usually (90%) benign tumors, most are small (> 90% are < 2 cm) and single, 6%-13% are multiple, and 4%-6% are associated with MEN-1. Clinically they are characterized by fasting hypoglycemia and neuroglycopenic symptoms. Moreover the release of catecholamines induced by hypoglycemia produces symptoms such as sweating, tremor and palpitation. Diagnostic procedures are given in Table 4.

Gastrinoma is a NET secreting gastrin. The chronic hypergastrinemia results in marked gastric acid hypersecretion that ultimately causes peptic ulcer disease, often refractory and severe, diarrhea and gastroesophageal reflux disease (Zollinger Ellison Syndrome, ZES).

At the time of diagnosis 50%-60% of gastrinomas are malignant. The tumor is preferentially located in the pancreas (24%-53%) and in the duodenum (13%-49%). Approximately 20% of gastrinomas are part of MEN-1. The diagnosis requires the demonstration of hypergastrinemia with hyperchlorhydria (Table 4).

Table 3 Carcinoid syndrome

Clinical features	Incidence (%)	Characteristics	Mediators
Flushing	90	Foregut tumors: prolonged fit, red-purple, localized to face and trunk. Midgut tumors: quick fit, pink-red.	Serotonin, histamine, P substance, prostaglandins
Diarrhea	70	Secretory	Serotonin, histamine, VIP, prostaglandins, gastrin
Abdominal pain	40	Long lasting	Obstruction, hepatomegaly, intestinal ischemia, fibrosis
Profuse sweating	15		Serotonin, histamine
Telangiectasias	25	Face	Unknown cause
Heart disease	30 (right)	Valvulopathies (tricuspid valve, pulmonary valve). Right heart failure. Dyspnea	P substance, serotonin
	10 (left)		
Pellagra	5	Dermatitis	Deficit of niacin

Table 4 Clinical features of the main endocrine pancreatic tumors

Tumor (syndrome)	Clinical features and diagnostic tests	MEN-1 (%)	Metastases (%)	SnSRS (%)
Insulinoma	Spontaneous or fasting hypoglycemia (Whipple's triad) Positive fasting test (hypoglycemia with hyperinsulinism)	8-10	10	50
Gastrinoma (Zollinger-Ellison syndrome)	Peptic ulcers, diarrhea, GERD, BAO > 15 mEq/h Positive secretin test (serum gastrinemia > 200 ng/L within 10 min from secretin venous infusion, 2 U/kg per min)	30	60	80
VIP-oma (Verner Morrison syndrome)	Severe watery diarrhea (> 1L/die), hypokalemia, hypochlorhydria	Rare	70	80
Glucagonoma	Necrolytic migratory erythema, diabetes, weight loss, anemia, hypoaminoacidemia, venous thrombosis	Rare	60	80
Somatostatinoma	Diarrhea, steatorrhea, weight loss, diabetes, cholelithiasis	Not associated	84	80
CRH/ACTH-oma	Cushing's syndrome	-	90	-
GHRH-oma	Acromegaly	-	-	-

SnSRS: Sensitivity of ^{111}In-Pentetreotide scintigraphy (Octreoscan®).

VIP-omas are NET that secretes VIP, which causes a distinct syndrome (Verner Morrison syndrome) characterized by large volume watery diarrhea, hypokalemia and dehydration. Pancreatic VIP-omas are rare (3%-8% of all pancreatic NETS)[8,25]. They are usually large (72% are > 5 cm) and malignant at the time of diagnosis (64%-92%). Extra-pancreatic VIP-omas may occur in pediatric patients and are neurogenic tumors (ganglioneuromas, ganglioneuroblastomas, neuroblastomas and pheochromocytomas).

Glucagonomas are rare (1/20 000 000 per year)[8,25,26]. They are usually large tumors at diagnosis with a size of 5-10 cm and from 50% to 82% are metastatic. The most common presenting feature is necrolytic migratory erythema, associated with glucose intolerance or diabetes, anemia, weight loss, depression, diarrhea and thromboembolism.

Somatostatinomas are rare tumors of either the pancreas or the upper small intestine, usually duodenum, near the ampulla of Vater. Somatosatinomas can be part of neurofibromatosis 1. Pancreatic tumors are usually large and metastatic (70%-92%) at diagnosis. The clinical symptoms include: diabetes, cholelithiasis, diarrhea with steatorrhea, hypochloridria, abdominal pain, weight loss and anemia.

Other rare tumors include CRH/ACTH-omas, GRF-omas, calcitoninomas and neurotensinomas[26]. Non functioning tumors constitute 30%-50% of all pancreatic NETs and differentiation from pancreatic adenocarcinomas is extremely important because prognosis is clearly different. The tumors are usually large, can be multifocal when are part of MEN-1 and malignancy rate varies from 62% to 92%[25].

DIAGNOSIS

Hormonal dosages

Several circulating or urinary tumor markers can be used for the diagnosis and follow-up of GEP NETs.

Among the generic markers, chromogranin A (CgA), a glycoprotein contained in secretion granules of neuroendocrine cells, has become the most important circulating tumor marker for the diagnosis and follow-up of NETs[27,28]. Elevated circulating levels of CgA are found in about 60%-80% of GEP NETs, both functioning and non-functioning[29], even if other non-neoplastic conditions, such as renal insufficiency, atrophic chronic gastritis, therapy with proton pump inhibitors[30,31] can determine false-positive results, reducing its specificity. Other generic markers include neuron-specific enolase (NSE), PP and human chorionic gonadotropin, with lower diagnostic accuracy than CgA[6,32].

5-hydroxyindoleacetic acid (5-HIAA) is the specific marker for carcinoids producing serotonin[2,6,18,32]; it is a metabolite of serotonin that can be determined in 24 h urines. The sensibility of the urinary 5-HIAA is about 65%-75%, while its specificity between 90%-100%[6].

Certain foods and drugs will affect the urinary excretion of 5-HIAA if they are taken in the 3-5 d before collection

of the urine sample. Bananas, avocados, aubergines, pineapples, plums, walnuts, cough syrup, paracetamol, fluorouracil, methysergide, levodopa, aspirin, 5-aminosalicylic acid (5-ASA), naproxen and caffeine may cause false-positive results. Adrenocorticotrophic hormone (ACTH), glucocorticoids, heparin, isoniazid, methyldopa and phenothiazines may give false-negative results[6].

For functioning NETs, the dosage of the specific hormone that causes the characteristic syndrome represents the specific tumor marker[1,6,8]. In particular in patients with suspected insulinoma, glycemia, insulin, peptide C and pro-insulin must be tested. Further biochemical tests include the prolonged fast (48-72 h), which is the gold standard for establishing the diagnosis of insulinoma. Indeed, 98% of patients with insulinoma will develop symptomatic hypoglycemia within 72 h.

In Zollinger Ellison syndrome, serum gastrin and basal gastric acid output should be evaluated[33,34]. If the gastrin is $\geqslant$ 1000 ng/L and gastric pH $<$ 2.5, the diagnosis is established. The secretin test is the provocative test of choice in patients with gastrin levels $<$ 1000 ng/L (Table 4). Plasma vasointestinal polypeptide (VIP) determination is used to diagnose VIP-oma in the suspicion of Verner-Morrison syndrome, plasma glucagon for glucagonoma, and serum somatostatin for somatostatinoma[1,6,8].

Imaging

Different integrated techniques can be used for diagnosis[1,2,6,35]. Imaging has an important role in localizing the primary tumor, identifying sites of metastatic disease and assessing response to treatment. The gastric and intestinal tumors are usually well studied with endoscopic techniques and endoscopic ultrasound. The tumors of the small intestine may require, besides enforcement of traditional radiological techniques (small bowel barium studies), the use of the most current techniques for studying small bowel (double balloon enteroscopy, video endoscopic capsule). Both for carcinoid and pancreatic tumors, computer tomograghy (CT) and magnetic resonance imaging (MRI) are important in defining the extent of metastatic disease and assessing response to treatment. Both techniques appear to have similar sensitivities for detection of these tumors, ranging from 30% to 94%[35]. Endoscopic ultrasound has an important role in the preoperative assessment of the pancreas where a small functioning tumor or the possibility of multiple tumors is suspected. This technique is very successful in expert hands, with sensitivities as high as 79%-100% being reported[35].

Functional imaging modalities, such as somatostatin receptor scintigraphy (SRS, Octreoscan®), have great impact on patient management by providing tools for better staging of the disease, visualization of occult tumor, and evaluation of eligibility for somatostatin analogue (SSA) treatment. In fact NETs generally express somatostatin receptors and by administering a radiolabelled SSA, the tumor is highlighted by the scintigraphic investigation. The SRS is a highly specific examination with sensitivity, for tumors of more than 1 cm, approximately of 80%-90% (with the exception of insulinoma that expresses somatostatin receptors in only 50% of cases)[1,2,6,36,37]. SRS also detects distant metastases with a sensitivity that can reach 96%[2,6]. It should be also noted that a positive SRS may lead to a possible systemic SSAs treatment or radionuclide therapy. On the other hand, even more sensitive techniques are being developed, based on methods combining PET-CT using [^{18}F] levodopa, 5HTP [^{11}C] or [^{68}Ga] linked to a SSA (^{68}Ga-DOTA-octreotide-PET)[36].

On the contrary, PET with conventional fluoro-deoxy-glucose has not proven advantageous for NET imaging, because of GEP NETs' low metabolic activity, with the exception of tumors with high proliferative activity and low differentiation[36].

Finally angiographic techniques, with the possible establishment of hormonal gradients, are currently used only in special cases and adequately equipped centers.

Pathology

Histopathological examination is the main criterion of the WHO classification[12] (Table 1), which takes into account: tumor size, number of mitosis, presence of cellular atypias, proliferative index, angioinvasion. Immunohistochemistry is also one of the most important techniques for the study of NETs. Several antibodies are available both against general endocrine markers such as NSE, sinaptofisin and CgA, and against specific hormones.

It is also important to discriminate well-differentiated forms from poorly-differentiated carcinomas using malignancy markers. With this aim, the immunohistochemical expression of Ki67 seems as important as the determination of the mitotic index, expressed as the number of mitoses/10 high power fields[6,38].

TREATMENT

Surgical treatment

If possible, radical surgery is the cornerstone of the treatment of primitive GEP NETs. If there is loco-regional or liver metastases a debulking surgery can be performed in patients in whom 90% of the tumor is removable. It is suggested to perform a palliative surgery in the following clinical situations: (1) on the primary tumor with non-operable liver metastases (particularly in functioning tumors) because symptoms correlate with neoplastic mass; (2) if the primary tumor is localized in the small bowel, as it can lead to bowel obstruction; (3) in the case whereby surgery allow a subsequent multimodal treatment.

A combination of several therapies can be performed for liver metastases, such as surgical resection, (chemo) embolization, radiofrequency ablation and, in selected cases, orthotopic liver transplantation may be considered[16,39,40]. Although there are few studies that compare different treatment options on liver metastases, it would seem that different treatments improve survival

Table 5 Results of studies of molecularly targeted agents in patients with neuroendocrine tumours[54,55]

Agent	Response rate (%)	PFS rate (%)/Duration
VEGF monoclonal antibody		
Bevacizumab[56]	18	95 at 18 wk
mTOR inhibitor		
RAD001 (everolimus)	13	71 at 24 wk
Temsirolimus[57]	5.6	50 at 6 mo
VEGF TKI		
Sunitinib	10	Median, 42 wk
Vatalanib	In progress	(time to progression)
Sorafenib	In progress	
Pazopanib	In progress	
PDGFR/Kit/Abl inhibitor		
Imatinib[58]	4	Median, 5.9 mo
EGFR inhibitor		
Gefitinib	4	61 (carcinoids) and 31 (pancreatic tumor) at 6 mo
Other		
Bortezomib[59]	0	Median, 3 mo (Time to treatment failure)

PFS: Progression free survival.

rate at 5 years globally from 30% for the untreated tumor to 50%-70%[39,40].

Medical therapy

Medical treatment of NETs is different depending on whether the tumor is a well-differentiated or a poorly differentiated one. Functioning tumors are usually well differentiated and the first target of therapy is the control of symptoms. As these tumors are generally slow in growth, with a relatively long life expectancy, it is essential to ensure patients a good quality of life.

Treatment of gastrinomas is based on the use of proton pump inhibitors at an appropriate dosage (omeprazole and lansoprazole 40-60 up to 120 mg/d)[41,42]. Insulinomas are treated with diazoxide associated with hydrochlorothiazide; if this therapy is ineffective calcium channel blockers, beta blockers and glucocorticoids can be used[43]. For other well-differentiated cancers therapy is based on the use of SSAs, interferon and, more recently, targeted therapy[44,45].

Somatostatin is a hormone that inhibits the secretion of various hormones and peptides; somatostatin receptors are present in most well-differentiated GEP NETs (70%-95% of tumors), with the exception of insulinoma. SSAs allow control of hormonal-related symptoms and should be used both in a preoperative setting and in inoperable tumors[44]. They are sometimes used as antiproliferative agents, even if clinical studies have given disappointing results with regard to tumor regression and tumor shrinkage is demonstrated in less than 10% of the patients at standard dosage, although about 50% of patients can show stabilization of tumor size[46]. A possible positive effect on tumor volume regression with high-dose SSAs has yet to be demonstrated. Two different SSAs, octreotide and lanreotide, are used clinically. These analogues bind principally to the receptor subtypes 2 and 5. Recently pasireotide, a somatostatin analog with high affinity for all types of somatostatin receptors, has been introduced and has been shown to be effective in patients who do not respond to the currently available SSAs octreotide and lanreotide[47]. However, its use is still restricted to clinical studies. Altogether, SSAs are safe, easy to use, and well tolerated by patients experiencing only mild and infrequent side effects, among which are diarrhea, abdominal pain, steatorrhea, and cholelithiasis[48].

In addition, alpha interferon, such as monotherapy or in combination with SSAs, can be used to inhibit hormone hypersecretion and to stabilize the disease, with variable response rates. There has been biochemical response in 40%-60% of patients, symptomatic improvement in 40%-70% of patients, and significant tumor shrinkage in a median of 10%-15% of patients[48,49]. Interferon is used for the same indications as are SSAs in NETs of the gut, except for carcinoid crisis. Side-effects are generally mild, flu-like syndrome, fatigue, weight loss, polyneuropathy, myositis, thrombocytopenia, anemia, leukopenia, hepatotoxicity and neutralizing antibodies.

Poorly differentiated tumors are generally treated with different chemotherapy schedules. The role of chemotherapy in the treatment of GEP NETs is still uncertain, as variable response rates in different studies have been reported. While well-differentiated tumors are not responsive to chemotherapy (based on streptozotocin, doxorubicin, dacarbazine and 5-flurouracile variously associated with each other)[6,50] with only about 10% of carcinoids having a positive response, the best response rates (40%-70%) have been reported in some studies for anaplastic cancer, using different schemes based on cisplatin and etoposide, although there is no unequivocal evidence of survival improvement[51-53]. Furthermore, randomized controlled trials on chemotherapy *versus* biological treatment (SSAs with/without interferon) are still lacking.

GEP NETs can over express some molecules, such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) and its receptor (VEGFR) or insulin-like growth factor receptor (IGFR), that can be targeted by some new drugs under assessment in early clinical trial (see Table 5)[54-59]. Other molecular therapies currently under investigation include the Raf-kinase inhibitor sorafenib and the inhibitor of the mTOR pathway, everolimus (RAD001)[54,55].

Peptide receptor radionuclide therapy (PRRT)

Another therapeutic approach is PRRT, which uses somatostatin analogs to convey radioactivity within the tumor itself (using generally ^{90}Ittrium, ^{177}Lutetium or ^{111}Indium), through somatostatin receptors[60,61]. PRRT can be considered in patients with inoperable GEP NETs and positive nuclear medicine imaging. According to some studies a stabilization of the disease can be reached in 50%-70% of cases[62-64] and control of symptoms in 70%[60]. Data in the literature, which however are not based on randomized, comparative studies, seem to favor [^{177}Lu-DOTA, Tyr] octreotate as

the most suitable peptide and radionuclide for PRRT[65]. Currently, tolerated dose is defined by the dose tolerated by the critical organs, kidney and bone marrow; it is likely that the dose can be modified in the future by more sophisticated, individually tailored dosimetry models, and by the introduction of new protective agents, different treatment schedules and radionuclides. This treatment has to be carried out in centers properly equipped and is to be reserved for selected cases.

REFERENCES

1 **Warner RR**. Enteroendocrine tumors other than carcinoid: a review of clinically significant advances. *Gastroenterology* 2005; **128**: 1668-1684
2 **Modlin IM**, Oberg K, Chung DC, Jensen RT, de Herder WW, Thakker RV, Caplin M, Delle Fave G, Kaltsas GA, Krenning EP, Moss SF, Nilsson O, Rindi G, Salazar R, Ruszniewski P, Sundin A. Gastroenteropancreatic neuroendocrine tumours. *Lancet Oncol* 2008; **9**: 61-72
3 **Modlin IM**, Lye KD, Kidd M. A 5-decade analysis of 13,715 carcinoid tumors. *Cancer* 2003; **97**: 934-959
4 **Hemminki K**, Li X. Incidence trends and risk factors of carcinoid tumors: a nationwide epidemiologic study from Sweden. *Cancer* 2001; **92**: 2204-2210
5 **Levi F**, Te VC, Randimbison L, Rindi G, La Vecchia C. Epidemiology of carcinoid neoplasms in Vaud, Switzerland, 1974-97. *Br J Cancer* 2000; **83**: 952-955
6 **Ramage JK**, Davies AH, Ardill J, Bax N, Caplin M, Grossman A, Hawkins R, McNicol AM, Reed N, Sutton R, Thakker R, Aylwin S, Breen D, Britton K, Buchanan K, Corrie P, Gillams A, Lewington V, McCance D, Meeran K, Watkinson A. Guidelines for the management of gastroenteropancreatic neuroendocrine (including carcinoid) tumours. *Gut* 2005; **54** Suppl 4: iv1-i16
7 **Rehfeld JF**. The new biology of gastrointestinal hormones. *Physiol Rev* 1998; **78**: 1087-1108
8 **Alexakis N**, Neoptolemos JP. Pancreatic neuroendocrine tumours. *Best Pract Res Clin Gastroenterol* 2008; **22**: 183-205
9 **Duerr EM**, Chung DC. Molecular genetics of neuroendocrine tumors. *Best Pract Res Clin Endocrinol Metab* 2007; **21**: 1-14
10 **Toumpanakis CG**, Caplin ME. Molecular genetics of gastroenteropancreatic neuroendocrine tumors. *Am J Gastroenterol* 2008; **103**: 729-732
11 **Williams ED**, Sandler M. The classification of carcinoid tum ours. *Lancet* 1963; **1**: 238-239
12 **Solcia E**, Klöppel G, Sobin LH. Histological Typing of Endocrine Tumors. WHO International Histological Classification of Tumors. 2nd ed. Berlin: Springer, 2000: 56-70
13 **Rindi G**, Kloppel G, Alhman H, Caplin M, Couvelard A, de Herder WW, Erikssson B, Falchetti A, Falconi M, Komminoth P, Korner M, Lopes JM, McNicol AM, Nilsson O, Perren A, Scarpa A, Scoazec JY, Wiedenmann B. TNM staging of foregut (neuro)endocrine tumors: a consensus proposal including a grading system. *Virchows Arch* 2006; **449**: 395-401
14 **Rindi G**, Kloppel G, Couvelard A, Komminoth P, Koerner M, Lopes JM, McNicol AM, Nilsson O, Perren A, Scarpa A, Scoazec JY, Wiedenmann B. TNM staging of midgut and hindgut (neuro) endocrine tumors: a consensus proposal including a grading system. *Virchows Arch* 2007; **451**: 757-762
15 **Modlin IM**, Lye KD, Kidd M. A 5-decade analysis of 13,715 carcinoid tumors. *Cancer* 2003; **97**: 934-959
16 **Eriksson B**, Kloppel G, Krenning E, Ahlman H, Plockinger U, Wiedenmann B, Arnold R, Auernhammer C, Korner M, Rindi G, Wildi S. Consensus guidelines for the management of patients with digestive neuroendocrine tumors--well-differentiated jejunal-ileal tumor/carcinoma. *Neuroendocrinology* 2008; **87**: 8-19
17 **Modlin IM**, Kidd M, Latich I, Zikusoka MN, Shapiro MD. Current status of gastrointestinal carcinoids. *Gastroenterology* 2005; **128**: 1717-1751
18 **Caplin ME**, Buscombe JR, Hilson AJ, Jones AL, Watkinson AF, Burroughs AK. Carcinoid tumour. *Lancet* 1998; **352**: 799-805
19 **Goede AC**, Caplin ME, Winslet MC. Carcinoid tumour of the appendix. *Br J Surg* 2003; **90**: 1317-1322
20 **Moertel CG**, Weiland LH, Nagorney DM, Dockerty MB. Carcinoid tumor of the appendix: treatment and prognosis. *N Engl J Med* 1987; **317**: 1699-1701
21 **Stinner B**, Rothmund M. Neuroendocrine tumours (carcinoids) of the appendix. *Best Pract Res Clin Gastroenterol* 2005; **19**: 729-738
22 **Ramage JK**, Goretzki PE, Manfredi R, Komminoth P, Ferone D, Hyrdel R, Kaltsas G, Kelestimur F, Kvols L, Scoazec JY, Garcia MI, Caplin ME. Consensus guidelines for the management of patients with digestive neuroendocrine tumours: well-differentiated colon and rectum tumour/carcinoma. *Neuroendocrinology* 2008; **87**: 31-39
23 **Habal N**, Sims C, Bilchik AJ. Gastrointestinal carcinoid tumors and second primary malignancies. *J Surg Oncol* 2000; **75**: 310-316
24 **Tichansky DS**, Cagir B, Borrazzo E, Topham A, Palazzo J, Weaver EJ, Lange A, Fry RD. Risk of second cancers in patients with colorectal carcinoids. *Dis Colon Rectum* 2002; **45**: 91-97
25 **Oberg K**, Eriksson B. Endocrine tumours of the pancreas. *Best Pract Res Clin Gastroenterol* 2005; **19**: 753-781
26 **Doherty GM**. Rare endocrine tumours of the GI tract. *Best Pract Res Clin Gastroenterol* 2005; **19**: 807-817
27 **Campana D**, Nori F, Piscitelli L, Morselli-Labate AM, Pezzilli R, Corinaldesi R, Tomassetti P. Chromogranin A: is it a useful marker of neuroendocrine tumors? *J Clin Oncol* 2007; **25**: 1967-1973
28 **Nobels FR**, Kwekkeboom DJ, Bouillon R, Lamberts SW. Chromogranin A: its clinical value as marker of neuroendocrine tumours. *Eur J Clin Invest* 1998; **28**: 431-440
29 **Peracchi M**, Conte D, Gebbia C, Penati C, Pizzinelli S, Arosio M, Corbetta S, Spada A. Plasma chromogranin A in patients with sporadic gastro-entero-pancreatic neuroendocrine tumors or multiple endocrine neoplasia type 1. *Eur J Endocrinol* 2003; **148**: 39-43
30 **Taupenot L**, Harper KL, O'Connor DT. The chromogranin-secretogranin family. *N Engl J Med* 2003; **348**: 1134-1149
31 **Peracchi M**, Gebbia C, Basilisco G, Quatrini M, Tarantino C, Vescarelli C, Massironi S, Conte D. Plasma chromogranin A in patients with autoimmune chronic atrophic gastritis, enterochromaffin-like cell lesions and gastric carcinoids. *Eur J Endocrinol* 2005; **152**: 443-448
32 **de Herder WW**. Biochemistry of neuroendocrine tumours. *Best Pract Res Clin Endocrinol Metab* 2007; **21**: 33-41
33 **Hoffmann KM**, Furukawa M, Jensen RT. Duodenal neuroendocrine tumors: Classification, functional syndromes, diagnosis and medical treatment. *Best Pract Res Clin Gastroenterol* 2005; **19**: 675-697
34 **Berna MJ**, Hoffmann KM, Long SH, Serrano J, Gibril F, Jensen RT. Serum gastrin in Zollinger-Ellison syndrome: II. Prospective study of gastrin provocative testing in 293 patients from the National Institutes of Health and comparison with 537 cases from the literature. evaluation of diagnostic criteria, proposal of new criteria, and correlations with clinical and tumoral features. *Medicine* (Baltimore) 2006; **85**: 331-364
35 **Rockall AG**, Reznek RH. Imaging of neuroendocrine tumours (CT/MR/US). *Best Pract Res Clin Endocrinol Metab* 2007; **21**: 43-68
36 **Sundin A**, Garske U, Orlefors H. Nuclear imaging of neuroendocrine tumours. *Best Pract Res Clin Endocrinol Metab* 2007; **21**: 69-85
37 **Gibril F**, Reynolds JC, Doppman JL, Chen CC, Venzon

DJ, Termanini B, Weber HC, Stewart CA, Jensen RT. Somatostatin receptor scintigraphy: its sensitivity compared with that of other imaging methods in detecting primary and metastatic gastrinomas. A prospective study. *Ann Intern Med* 1996; **125**: 26-34

38 **Rindi G**, D'Adda T, Froio E, Fellegara G, Bordi C. Prognostic factors in gastrointestinal endocrine tumors. *Endocr Pathol* 2007; **18**: 145-149

39 **Touzios JG**, Kiely JM, Pitt SC, Rilling WS, Quebbeman EJ, Wilson SD, Pitt HA. Neuroendocrine hepatic metastases: does aggressive management improve survival? *Ann Surg* 2005; **241**: 776-783; discussion 783-785

40 **Steinmuller T**, Kianmanesh R, Falconi M, Scarpa A, Taal B, Kwekkeboom DJ, Lopes JM, Perren A, Nikou G, Yao J, Delle Fave GF, O'Toole D. Consensus guidelines for the management of patients with liver metastases from digestive (neuro)endocrine tumors: foregut, midgut, hindgut, and unknown primary. *Neuroendocrinology* 2008; **87**: 47-62

41 **Quatrini M**, Castoldi L, Rossi G, Cesana BM, Peracchi M, Bardella MT. A follow-up study of patients with Zollinger-Ellison syndrome in the period 1966-2002: effects of surgical and medical treatments on long-term survival. *J Clin Gastroenterol* 2005; **39**: 376-380

42 **Metz DC**, Sostek MB, Ruszniewski P, Forsmark CE, Monyak J, Pisegna JR. Effects of esomeprazole on acid output in patients with Zollinger-Ellison syndrome or idiopathic gastric acid hypersecretion. *Am J Gastroenterol* 2007; **102**: 2648-2654

43 **Grant CS**. Insulinoma. *Best Pract Res Clin Gastroenterol* 2005; **19**: 783-798

44 **Oberg K**, Kvols L, Caplin M, Delle Fave G, de Herder W, Rindi G, Ruszniewski P, Woltering EA, Wiedenmann B. Consensus report on the use of somatostatin analogs for the management of neuroendocrine tumors of the gastroenteropancreatic system. *Ann Oncol* 2004; **15**: 966-973

45 **Delaunoit T**, Neczyporenko F, Rubin J, Erlichman C, Hobday TJ. Medical management of pancreatic neuroendocrine tumors. *Am J Gastroenterol* 2008; **103**: 475-483; quiz 484

46 **Plockinger U**, Wiedenmann B. Neuroendocrine tumors. Biotherapy. *Best Pract Res Clin Endocrinol Metab* 2007; **21**: 145-162

47 **Ben-Shlomo A**, Melmed S. Pasireotide--a somatostatin analog for the potential treatment of acromegaly, neuroendocrine tumors and Cushing's disease. *IDrugs* 2007; **10**: 885-895

48 **Frank M**, Klose KJ, Wied M, Ishaque N, Schade-Brittinger C, Arnold R. Combination therapy with octreotide and alpha-interferon: effect on tumor growth in metastatic endocrine gastroenteropancreatic tumors. *Am J Gastroenterol* 1999; **94**: 1381-1387

49 **Oberg K**, Funa K, Alm G. Effects of leukocyte interferon on clinical symptoms and hormone levels in patients with midgut carcinoid tumors and carcinoid syndrome. *N Engl J Med* 1983; **309**: 129-133

50 **Delaunoit T**, Ducreux M, Boige V, Dromain C, Sabourin JC, Duvillard P, Schlumberger M, de Baere T, Rougier P, Ruffie P, Elias D, Lasser P, Baudin E. The doxorubicin-streptozotocin combination for the treatment of advanced well-differentiated pancreatic endocrine carcinoma; a judicious option? *Eur J Cancer* 2004; **40**: 515-520

51 **Moertel CG**, Kvols LK, O'Connell MJ, Rubin J. Treatment of neuroendocrine carcinomas with combined etoposide and cisplatin. Evidence of major therapeutic activity in the anaplastic variants of these neoplasms. *Cancer* 1991; **68**: 227-232

52 **Moertel CG**, Lefkopoulo M, Lipsitz S, Hahn RG, Klaassen D. Streptozocin-doxorubicin, streptozocin-fluorouracil or chlorozotocin in the treatment of advanced islet-cell carcinoma. *N Engl J Med* 1992; **326**: 519-523

53 **Mitry E**, Baudin E, Ducreux M, Sabourin JC, Rufie P, Aparicio T, Aparicio T, Lasser P, Elias D, Duvillard P, Schlumberger M, Rougier P. Treatment of poorly differentiated neuroendocrine tumours with etoposide and cisplatin. *Br J Cancer* 1999; **81**: 1351-1355

54 **Yao JC**. Neuroendocrine tumors. Molecular targeted therapy for carcinoid and islet-cell carcinoma. *Best Pract Res Clin Endocrinol Metab* 2007; **21**: 163-172

55 **Yao JC**, Hoff PM. Molecular targeted therapy for neuroendocrine tumors. *Hematol Oncol Clin North Am* 2007; **21**: 575-581; x

56 **Yao JC**, Phan A, Hoff PM, Chen HX, Charnsangavej C, Yeung SC, Hess K, Ng C, Abbruzzese JL, Ajani JA. Targeting vascular endothelial growth factor in advanced carcinoid tumor: a random assignment phase II study of depot octreotide with bevacizumab and pegylated interferon alpha-2b. *J Clin Oncol* 2008; **26**: 1316-1323

57 **Duran I**, Kortmansky J, Singh D, Hirte H, Kocha W, Goss G, Le L, Oza A, Nicklee T, Ho J, Birle D, Pond GR, Arboine D, Dancey J, Aviel-Ronen S, Tsao MS, Hedley D, Siu LL. A phase II clinical and pharmacodynamic study of temsirolimus in advanced neuroendocrine carcinomas. *Br J Cancer* 2006; **95**: 1148-1154

58 **Yao JC**, Zhang JX, Rashid A, Yeung SC, Szklaruk J, Hess K, Xie K, Ellis L, Abbruzzese JL, Ajani JA. Clinical and in vitro studies of imatinib in advanced carcinoid tumors. *Clin Cancer Res* 2007; **13**: 234-240

59 **Shah MH**, Young D, Kindler HL, Webb I, Kleiber B, Wright J, Grever M. Phase II study of the proteasome inhibitor bortezomib (PS-341) in patients with metastatic neuroendocrine tumors. *Clin Cancer Res* 2004; **10**: 6111-6118

60 **Forrer F**, Valkema R, Kwekkeboom DJ, de Jong M, Krenning EP. Neuroendocrine tumors. Peptide receptor radionuclide therapy. *Best Pract Res Clin Endocrinol Metab* 2007; **21**: 111-129

61 **Kwekkeboom DJ**, Bakker WH, Kam BL, Teunissen JJ, Kooij PP, de Herder WW, Feelders RA, van Eijck CH, de Jong M, Srinivasan A, Erion JL, Krenning EP. Treatment of patients with gastro-entero-pancreatic (GEP) tumours with the novel radiolabelled somatostatin analogue [177Lu-DOTA(0),Tyr3]octreotate. *Eur J Nucl Med Mol Imaging* 2003; **30**: 417-422

62 **Esser JP**, Krenning EP, Teunissen JJ, Kooij PP, van Gameren AL, Bakker WH, Kwekkeboom DJ. Comparison of [(177)Lu-DOTA(0),Tyr(3)]octreotate and [(177)Lu-DOTA(0),Tyr(3)]octreotide: which peptide is preferable for PRRT? *Eur J Nucl Med Mol Imaging* 2006; **33**: 1346-1351

63 **Valkema R**, De Jong M, Bakker WH, Breeman WA, Kooij PP, Lugtenburg PJ, De Jong FH, Christiansen A, Kam BL, De Herder WW, Stridsberg M, Lindemans J, Ensing G, Krenning EP. Phase I study of peptide receptor radionuclide therapy with [In-DTPA]octreotide: the Rotterdam experience. *Semin Nucl Med* 2002; **32**: 110-122

64 **Anthony LB**, Woltering EA, Espenan GD, Cronin MD, Maloney TJ, McCarthy KE. Indium-111-pentetreotide prolongs survival in gastroenteropancreatic malignancies. *Semin Nucl Med* 2002; **32**: 123-132

65 **Kwekkeboom DJ**, de Herder WW, Kam BL, van Eijck CH, van Essen M, Kooij PP, Feelders RA, van Aken MO, Krenning EP. Treatment with the radiolabeled somatostatin analog [177 Lu-DOTA 0,Tyr3]octreotate: toxicity, efficacy, and survival. *J Clin Oncol* 2008; **26**: 2124-2130

S-Editor Li DL **L- Editor** Alpini GD **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5385

World J Gastroenterol 2008 September 21; 14(35): 5385-5402
World Journal of Gastroenterology ISSN 1007-9327

RESEARCH FRONTIER

"Rescue" regimens after *Helicobacter pylori* treatment failure

Javier P Gisbert

Javier P Gisbert, Gastroenterology Unit, Hospital Universitario de la Princesa and Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas, Madrid, Spain
Author contributions: Gisbert JP wrote the paper.
Supported by The Instituto de Salud Carlos Ⅲ
Correspondence to: Javier P Gisbert, MD, Gastroenterology Unit, Hospital de la Princesa, 28006 Madrid, Spain. gisbert@meditex.es
Telephone: +34-91-5202254 Fax: +34-914022299
Received: April 8, 2008 Revised: July 20, 2008
Accepted: July 27, 2008
Published online: September 21, 2008

Abstract

Helicobacter pylori (*H pylori*) infection is the main cause of gastritis, gastroduodenal ulcer disease, and gastric cancer. After more than 20 years of experience in *H pylori* treatment, in my opinion, the ideal regimen to treat this infection is still to be found. Currently, apart from having to know first-line eradication regimens well, we must also be prepared to face treatment failures. Therefore, in designing a treatment strategy we should not focus on the results of primary therapy alone, but also on the final (overall) eradication rate. The choice of a "rescue" treatment depends on which treatment is used initially. If a clarithromycin-based regimen was used initially, a subsequent metronidazole-based treatment (quadruple therapy) may be used afterwards, and then a levofloxacin-based combination would be a third "rescue" option. Alternatively, it has recently been suggested that levofloxacin-based rescue therapy constitutes an encouraging second-line strategy, representing an alternative to quadruple therapy in patients with previous PPI-clarithromycin-amoxicillin failure, with the advantage of efficacy, simplicity and safety. In this case, a quadruple regimen may be reserved as a third-line rescue option. Finally, rifabutin-based rescue therapy constitutes an encouraging empirical fourth-line strategy after multiple previous eradication failures with key antibiotics such as amoxicillin, clarithromycin, metronidazole, tetracycline, and levofloxacin. Even after two consecutive failures, several studies have demonstrated that *H pylori* eradication can finally be achieved in almost all patients if several rescue therapies are consecutively given. Therefore, the attitude in *H pylori* eradication therapy failure, even after two or more unsuccessful attempts, should be to fight and not to surrender.

Key words: *Helicobacter pylori*; Rescue; Salvage; Rifabutin; Levofloxacin

Peer reviewers: Yoshio Yamaoka, MD, PhD, Associate Professor, Department of Medicine/Gastroenterology, Baylor College of Medicine and VA Medical Center (111D), 2002 Holcombe Blvd, Houston, Texas 77030, United States; Tadashi Shimoyama, MD, Hirosaki University, 5 Zaifu-cho, Hirosaki 036-8562, Japan

Gisbert JP. "Rescue" regimens after *Helicobacter pylori* treatment failure. *World J Gastroenterol* 2008; 14(35): 5385-5402 Available from: URL: http://www.wjgnet.com/1007-9327/14/5385.asp DOI: http://dx.doi.org/10.3748/wjg.14.5385

INTRODUCTION

Helicobacter pylori (*H pylori*) infection is the main cause of gastritis, gastroduodenal ulcer disease, and gastric cancer. After more than 20 years of experience in *H pylori* treatment, in my opinion, the ideal regimen to treat this infection is still to be found. Consensus conferences have recommended therapeutic regimens that achieve *H pylori* cure rates higher than 80% on an intention-to-treat basis[1-3]. However, several large clinical trials and meta-analyses have shown that the most commonly used first-line therapies-including proton pump inhibitors (PPIs) plus two antibiotics-may fail in up to 20% of patients[4,5], and in the clinical routine setting, the treatment failure rate might be even higher. Moreover, during the last few years, the efficacy of PPI-based regimens seems to be decreasing, and several studies have reported intention-to-treat eradication rates lower than 75%[6-14] and even lower than 50%[15,16]. Antibiotic resistance to clarithromycin has been identified as one of the major factors affecting our ability to cure *H pylori* infection, and the rate of resistance to this antibiotic seems to be increasing in many geographical areas[17,18].

Reports dealing with retreatment of *H pylori* after failure are difficult to analyze for several reasons[19]. Firstly, patients who fail with their first-line treatment

probably include a higher percentage of individuals who are unreliable tablet takers, others have resistant organisms and there is the "constitutional" group, where failure will be inevitable. On the other hand, some patients submitted for rescue therapy have already had more than one previous treatment for *H pylori*, and this circumstance is not always clarified in the protocols. Furthermore, the original primary treatments vary among the different studies, not only with respect to the antibiotic type, but also with respect to the dose and duration of the regimen. Finally, only a few studies have directly compared, in the same protocol, two or more second therapies[20,21].

Several rescue therapies have been recommended, but they still fail to eradicate *H pylori* in more than 20% of cases[20], and these patients constitute a therapeutic dilemma[21]. Patients who are not cured with two consecutive treatments, including clarithromycin and metronidazole, will have at least single, and usually double, resistance[18]. Furthermore, bismuth salts are not available worldwide anymore and, therefore, management of first-line eradication failures is becoming challenging. Currently, a standard third-line therapy is lacking, and European guidelines recommend a culture for these patients to select a third-line treatment according to microbial sensitivity to antibiotics[2,3]. However, cultures are often carried out only in research centers, and the use of this procedure as "routine practice" in patients who failed several treatments seems not to be feasible[20-22]. Therefore, the evaluation of drugs without cross-resistance to nitroimidazole or macrolides as components of retreatment combination therapies would be worthwhile.

All these issues are important at the present time, but they will be even more relevant in the near future, as therapy for *H pylori* infection is becoming more and more frequently prescribed. Therefore, the evaluation of second or third rescue regimens for these problematic cases seems to be worthwhile. In designing a treatment strategy, we should not focus on the results of primary therapy alone; an adequate strategy for treating this infection should use several therapies which, if consecutively prescribed, come as close to the 100% cure rate as possible[20,21,23,24].

The aim of the present manuscript will be to review the experience dealing with "non-responders" to *H pylori* eradication therapy. As, at present, the current most prescribed first-line regimens include a combination of PPI plus two antibiotics, the present review will focus only on rescue regimens when these triple combinations fail. Bibliographical searches were performed in the PubMed (Internet) database including studies available until March 2008, looking for the following words (all fields): pylori AND (retreatment OR re-treatment OR rescue OR failure OR salvage OR second-line OR third-line OR fourth-line). References of reviews on *H pylori* eradication treatment, and from the articles selected for the study, were also examined in search of articles meeting inclusion criteria (that is, dealing with *H pylori* rescue therapies).

IS IT NECESSARY TO PERFORM CULTURE AFTER FAILURE OF THE FIRST ERADICATION TREATMENT?

Pretreatment antibiotic resistance is the most important factor in nonresponse to initial treatment[25-29]. Thus, the choice of a second-line treatment depends on which treatment was used initially, as it would appear that retreatment with the same regimen cannot be recommended[30]. If a clarithromycin-based regimen was used, a metronidazole-based treatment (or at least a clarithromycin-free regimen) should be used afterwards, and *vice versa*[31]. This recommendation is based on the observation that acquired bacterial resistance to metronidazole or clarithromycin results primarily from the previous treatment failure[26,32,33], and therefore rescue therapies should avoid these antibiotics and use different combinations.

An antimicrobial susceptibility test for *H pylori* before second-line treatment is sometimes performed, although whether the test is truly necessary remains unknown. Some authors have evaluated the efficacy of susceptibility-guided *vs* empiric retreatment for *H pylori* after a treatment failure. In the study by Yahav *et al*[34], patients in whom at least one treatment regimen for *H pylori* eradication had failed underwent gastric biopsy and culture, and were retreated according to the *in vitro* susceptibility results. Findings were compared with those of control patients (where culture was unavailable). Susceptibility-guided retreatment was associated with better eradication rates (86%) than empiric treatment (63%). However, several methodological drawbacks exist in this study. Firstly, more than 50% of the patients received first-line eradication treatment with both clarithromycin and metronidazole (instead of including clarithromycin and amoxicillin), which is not the generally recommended combination; consequently, no logical empirical treatment remained afterwards (levofloxacin-based regimens were not available at that time). In this respect, when only the eradication rates in control (culture unavailable) patients treated with a first regimen of PPI-amoxicillin-clarithromycin followed by a second *empiric* quadruple regimen were considered (the generally recommended first and second-line strategies), the success figures were not significantly different from those reported in patients receiving susceptibility-guided retreatment. Secondly, because this study was nonrandomized, there might have been heterogeneity among the two groups with respect to the treatment regimens prescribed by the treating physicians. Finally, this study was limited by the lack of susceptibility data for the controls, which restricted the ability to analyze the reasons why empiric therapy did not work as well as the susceptibility-guided protocol.

In a French multicenter study[35], patients in whom one previous *H pylori* eradication therapy (mainly with PPI-amoxicillin-clarithromycin) had failed were randomized to receive one of three empirical triple therapy regimens or a strategy based on antibiotic susceptibility. The empirical regimens

were PPI-amoxicillin-clarithromycin (for 7 d or 14 d) or PPI-amoxicillin-metronidazole (for 14 d). In the susceptibility-based strategy, patients with clarithromycin-susceptible strains received PPI-amoxicillin-clarithromycin, whilst the others received PPI-amoxicillin-metronidazole. The eradication rates for empirical therapies were low, while the cure rate was higher (74%) for the susceptibility-based treatment. If the *H pylori* strain was clarithromycin-susceptible (which occurred in approximately 1/3 of the cases), a high success rate was obtained with the PPI-clarithromycin-amoxicillin rescue regimen. The study, however, was done in France, where bismuth is banned, so that the use of quadruple therapy with a PPI, bismuth, tetracycline, and metronidazole as recommended by the updated Maastricht Consensus Report[3], was not tested. In fact, as will be reviewed later, several studies have obtained relatively good results with this quadruple regimen empirically prescribed, with a mean eradication rate of 77%, which is similar to the 74% achieved for the susceptibility-based treatment in the present study. Thus, in this study, instead of not readministering any of the antibiotics against which *H pylori* had probably become resistant, the authors insist on prescribing again clarithromycin (or metronidazole) for the second-line treatment. Furthermore, statistically significant differences were not demonstrated when comparing the efficacy of the empirical PPI-amoxicillin-metronidazole and the susceptibility-based strategy, suggesting that the metronidazole-based combination may be an effective empirical alternative after failure of a clarithromycin-based combination.

In the updated Maastricht Consensus Report[3], it was recommended that culture and antimicrobial sensitivity testing should be routinely performed only after two treatment failures with different antibiotics. According to this statement, some studies have suggested that an antimicrobial susceptibility test for *H pylori* before administering second-line treatment is not necessary. In this respect, in the study by Avidan *et al*[36], after failure of first-line eradication treatment, half of the patients were randomly assigned to treatment with a different PPI-based triple regimen regardless of the culture obtained, and the other half was assigned to treatment with PPI and two antibacterial agents chosen according to a susceptibility test; the authors found that the culture results did not influence the treatment protocol employed. Similarly, in the study by Miwa *et al*[37], patients with *H pylori* infection for whom first-line treatment with a PPI-amoxicillin-clarithromycin regimen had failed were randomly assigned to two groups: those having or not having the susceptibility test before retreatment. For those patients in the susceptibility-test group, the authors used what they considered the best regimen based on susceptibility testing; while for those patients in the group with no susceptibility testing, PPI-amoxicillin-metronidazole was prescribed. The cure rates in the groups with and without susceptibility testing were not different.

SECOND-LINE *H PYLORI* RESCUE THERAPY AFTER FAILURE OF ONE ERADICATION TREATMENT

Rescue regimen after PPI-clarithromycin-amoxicillin failure

PPI, amoxicillin and metronidazole: After failure of a combination of PPI, amoxicillin and clarithromycin, a theoretically correct alternative would be the use, as second option, of other PPI-based triple therapy including amoxicillin (which does not induce resistance) and metronidazole (an antibiotic not used in the first trial), and several authors have reported encouraging results with this strategy[37-44]. However, in our experience, when this therapy has been administered twice-daily for one week, eradication rates lower than 50% have been obtained[45]; the subsequent use of higher (three times per day) antibiotic doses was followed only by a mild increase in eradication rate (58%), which was still unacceptable[45]. However, if ranitidine bismuth citrate (RBC) is used instead of PPI, also plus amoxicillin and nitroimidazole, encouraging results have been reported (81% cure rate), although in this protocol antibiotics were administered for 14 d instead of 7 d[46]. In this same study, the readministration of clarithromycin, even when co-prescribed with RBC, was associated with poor eradication rates. In the same way, Nagahara *et al*[47] studied a group of patients who, after failure of first-line PPI-clarithromycin-amoxicillin therapy, had received second-line therapy with the same regimen (for 14 d) or had received PPI-amoxicillin-metronidazole (for 10 d). The eradication rates for second-line therapy with the same regimen (thus readministering clarithromycin) was only 53%, while it was 81% with PPI-amoxicillin-metronidazole. These observations underlie the idea that antibiotics, and specifically clarithromycin, should not be readministered in successive treatments.

Quadruple therapy: Another alternative, the use of a quadruple regimen (i.e. PPI, bismuth, tetracycline and metronidazole), has been generally used as an optimal second-line therapy after PPI-clarithromycin-amoxicillin failure, and has been the recommended rescue regimen in several guidelines[3,48-50]. Several studies have obtained relatively good results with this quadruple regimen, and the results are summarized in Table 1[45,51-71]. Thus, the weighted mean eradication rate with this rescue therapy, calculated from the studies included in the table, is 77%. In this combination regimen, PPI should be prescribed in the usual dose for twice a day, colloidal bismuth subcitrate 120 mg four times per day, tetracycline 500 mg four times per day, and metronidazole is probably best prescribed at high doses (i.e. 500 mg three times per day). The study with the lowest efficacy[57] administered metronidazole at low doses (250 mg four times per day). Limited experience suggests that quadruple therapy may also be effective when the first (failed) regimen included RBC instead of PPI. Thus, Beales *et al*[72] reported that four of the five patients

Table 1 Eradication rates with quadruple therapy (proton pump inhibitor, bismuth, tetracycline and a nitroimidazole) as "rescue" therapy for proton pump inhibitor-clarithromycin-amoxicillin failure

Author	Number of patients	Duration (d)	Eradication rate (%)
Baena Diez *et al*[51]	31	14	90
Bilardi *et al*[52]	46	7	37
Elizalde *et al*[53]	31	7	87
Choung *et al*[54]	56	7	77
Choung *et al*[54]	99	14	88
Chung *et al*[55]	87	7	84
Gasbarrini *et al*[56]	9	7	88
Gisbert *et al*[57]	30	7	57
Gisbert *et al*[45]	9	7	78
Gomollón *et al*[58]	21	7	95
Lee *et al*[59]	20	7	68
Lee *et al*[60]	63	7	75
Marko *et al*[61]	27	7	63
Michopoulos *et al*[62]	38	14	76
Navarro-Jarabo *et al*[63]	54	7	70
Nista *et al*[64]	70	7	63
Nista *et al*[64]	70	14	68
Orsi *et al*[65]	50	12	88
Perri *et al*[66]	45	10	67
Perri *et al*[67]	60	7	83
Sicilia *et al*[68]	21	10	83
Uygun *et al*[69]	100	14	82
Wong *et al*[70]	53	7	91
Wu *et al*[71]	47	7	77

Eradication rates by intention-to-treat analysis when available. *H pylori* eradication rate (weighted mean) with quadruple therapy is 77%.

initially failing RBC-clarithromycin-amoxicillin therapy were successfully treated with quadruple therapy. Seven-day treatment duration seems to be sufficient when quadruple therapy is used after a failed first regimen, as quite similar eradication rates with 7, 10 and 14 d have been reported (mean figures, calculated from Table 1, of 74%, 72% and 81%, respectively). Furthermore, in a recent retrospective study, patients who failed the standard triple therapy (PPI, amoxicillin, clarithromycin) received 1 or 2 wk quadruple therapy, and the eradication rate was similar between the two regimens[54]. These results are in agreement with those reported previously with quadruple therapy as a first-line regimen, where 1-wk therapy appeared sufficient, and prolonging treatment did not increase efficacy[73]. Finally, although PPIs are generally prescribed as the antisecretors in quadruple therapy, some authors have shown, in a randomized study, that omeprazole 20 mg *b.i.d.* and ranitidine 300 mg *b.i.d.* were equally effective as antisecretory agents combined in a second-line quadruple eradication regimen after failure with previous regimens without metronidazole[62]. Nevertheless, these regimens were administered over 14 d and, therefore, it remains to be demonstrated whether the equivalence between both antisecretors-PPIs and H_2-blockers- is also observable with 7 d regimens.

The question may be suggested whether treatment with PPI-clarithromycin-amoxicillin followed by rescue with quadruple therapy if initial failure occurs is preferable to the inverse strategy. To analyze this interesting aspect, Gomollón *et al*[74] randomized consecutive patients to one of two strategies: (1) treatment during 7 d with quadruple therapy, and if failure occurs then second-line treatment with omeprazole-clarithromycin-amoxicillin during 7 d; and (2) initial treatment with omeprazole-clarithromycin-amoxicillin and if failure occurs then treatment with quadruple therapy. Direct and indirect costs were estimated, and a cost-effectiveness analysis using a decision-tree model was undertaken after real clinical data. Eradication was obtained (intention-to-treat) in 73% with the first strategy, *versus* 92% with the second strategy. Furthermore, cost per case eradicated was lower in the second group (320 *versus* 296 euros). However, in a similar but more recent study, Marko *et al*[61] assessed the usefulness and the cost-effectiveness of these two treatment strategies, performing a decision analysis. The effectiveness of "triple first" and "quadruple first" strategies was similar, although the latter seemed slightly more cost-effective.

RBC, tetracycline and metronidazole: More recently, it has been reported that replacing the PPI and the bismuth compound of the quadruple therapy by RBC also achieves good results as a rescue regimen[57,75-80]. RBC is a compound that has, on the one hand, the antisecretory activity of ranitidine, and, on the other hand, the mucosal protective and anti-*H pylori* effects of certain other bismuth salts[81-83]. To date, several studies have evaluated 7-14 d RBC-based second-line regimens after PPI-based triple therapy failures, achieving encouraging results, with eradication rates of 67%[77], 68%[79], 76%[84], 82%[75], 83%[57], 86%[80], and 96%[76]. Furthermore, one randomized study has demonstrated that triple RBC-based therapy, when prescribed to patients with previous PPI-clarithromycin-amoxicillin failure, achieved an even higher efficacy than quadruple therapy, with additional advantages of a lower number of drugs and a simpler dose scheme[57]. Nevertheless, the eradication rate with a quadruple regimen in this last study was remarkably low, which was explained by the low dose of metronidazole prescribed. The favorable results obtained with RBC in the aforementioned studies were explained, at least in part, by the fact that RBC-based therapies may overcome the impact of metronidazole resistant and clarithromycin resistant strains on *H pylori* eradication treatment[18,81-83]. In summary, due to the aforementioned encouraging results, quadruple therapy (as well as RBC-based regimens) may be considered as the preferred regimen after initial treatment failure with PPI-clarithromycin-amoxicillin[3,20,78,85]. However, bismuth salts, including RBC, are no longer available worldwide, and some National Guidelines have been changed accordingly[86].

PPI, amoxicillin and levofloxacin: As previously mentioned, after failure of a combination of a PPI-based triple regimen, the use of the quadruple therapy has been generally recommended as the optimal second-line therapy based on the relatively good results reported

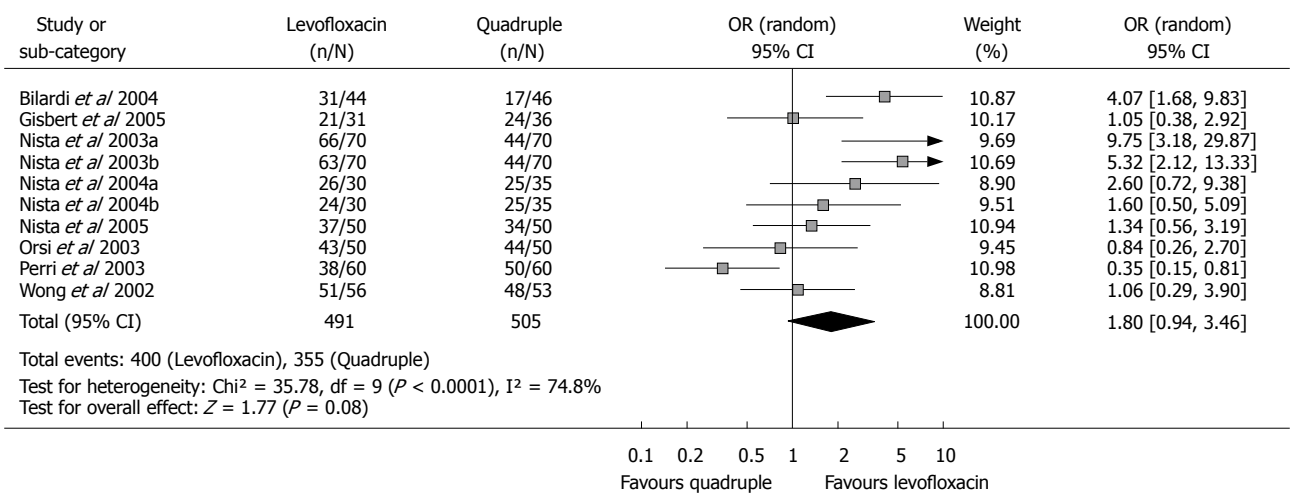


Figure 1 Meta-analysis comparing *H pylori* eradication efficacy with levofloxacin-based triple regimens *versus* quadruple therapy, as second-line "rescue" regimen after failure of a proton pump inhibitor-amoxicillin-clarithromycin.

by several authors[3,20,78,85]. However, this quadruple regimen requires the administration of 4 drugs with a complex scheme (bismuth and tetracycline usually prescribed every 6 h, and metronidazole every 8 h) and is associated with a relatively high incidence of adverse effects[20]. Furthermore, this quadruple regimen still fails to eradicate *H pylori* in approximately 20% to 30% of the patients, and these cases constitute a therapeutic dilemma, as patients who are not cured with two consecutive treatments including clarithromycin and metronidazole will usually have double resistance[20].

Levofloxacin is a fluoroquinolone antibacterial agent with a broad spectrum of activity against Gram-positive and Gram-negative bacteria and atypical respiratory pathogens[87]. Recently, some studies have evaluated the efficacy of new fluoroquinolones, such as levofloxacin, that could prove to be a valid alternative to standard antibiotics, not only as first-line therapies, but more interestingly, as second-line regimens[21,88-90]. In this respect, levofloxacin-based second-line therapies represent an encouraging strategy for eradication failures, as some studies have demonstrated that levofloxacin has, *in vitro*, remarkable activity against *H pylori*[91], and that primary resistances to such an antibiotic are (still) relatively infrequent (when compared with metronidazole or clarithromycin)[92-96]. A recent *in vitro* study also showed a synergistic effect of quinolone antimicrobial agents and PPIs on strains of *H pylori*[97]. Furthermore, it has been shown *in vitro* that levofloxacin retains its activity when *H pylori* strains are resistant to clarithromycin and metronidazole[95,98,99]. These favorable results have been confirmed *in vivo*, indicating that most of the patients with both metronidazole and clarithromycin resistance are cured with the levofloxacin-based regimen[52,94,100].

A combination of a PPI, amoxicillin and levofloxacin, as a first-line regimen, has been associated with favorable results, with mean eradication rates of about 90%[95,101-106]. Subsequently, other authors studied this same regimen in patients with one previous eradication failure, also reporting exciting results, with *H pylori* cure rates ranging from 60% to 94%[52,64,65,67,70,98, 100,106-111]. A recent systematic review showed a mean eradication rate with levofloxacin-based rescue regimens (combined with amoxicillin and a PPI in most studies) of 80%, which represents a relatively high figure when considering that this regimen was evaluated as a rescue therapy[89]. This systematic review found higher *H pylori* cure rates with a 10-d rather than a 7-d regimen, both in general (81% *vs* 73%) and also with the levofloxacin-amoxicillin-PPI combination in particular (80% *vs* 68%), suggesting that the longer (10-d) therapeutic scheme should be chosen.

Furthermore, two recent meta-analyses have suggested that after *H pylori* eradication failure, a levofloxacin-based rescue regimen is more effective than the generally recommended quadruple therapy[88,89]. In one of these meta-analyses[89], higher *H pylori* cure rates with the levofloxacin-based triple regimens than with the quadruple combinations were found (81% *vs* 70%), but with borderline statistical significance (Figure 1). Nevertheless, results were heterogeneous, mainly due to the discordant results of the study by Perri *et al*[67], who reported a cure rate of only 63% with the levofloxacin-regimen, which is the lowest reported in the literature, and is a figure that contrasts with the mean eradication rate of 80% calculated in a systematic review[89]. Nevertheless, when that single outlier study[67] was excluded from the meta-analysis, the difference between cure rates with both regimens reached statistical significance and heterogeneity markedly decreased. Furthermore, when only high-quality studies were considered, the advantage of the levofloxacin regimen over the quadruple regimen increased (88% *vs* 64%), also achieving statistical significance, and heterogeneity among studies almost disappeared[89].

As previously mentioned, the quadruple regimen requires the administration of a complex scheme[20]. On the contrary, levofloxacin-based regimens (with amoxicillin and PPIs administered twice daily, and levofloxacin every 12 or 24 h) represents an encouraging alternative to quadruple therapy, with the advantage of simplicity. Furthermore, the quadruple regimen is associated with a relatively high incidence of adverse

effects[20]. In contrast, levofloxacin is generally well tolerated, and most adverse events associated with its use are mild to moderate in severity and transient[87]. The most frequent adverse effects affect the gastrointestinal tract[87]. Occasional cases of tendinitis and tendon rupture have been reported in the literature with levofloxacin therapy[52,87]. However, data derived from more than 15 million prescriptions in the US indicated the rate is fewer than 4 per million prescriptions[112]. In the aforementioned systematic review[89], adverse effects were reported, overall, by 18% of the patients treated with levofloxacin-based therapies, and these adverse effects were severe (defined so by the authors or explaining treatment discontinuation) in only 3% of the cases. Furthermore, the incidence of adverse effects was not different when levofloxacin-amoxicillin-PPI was administered for 7 d or 10 d, supporting the aforementioned recommendation of prescribing the more effective 10-d regimen. Moreover, two meta-analyses have demonstrated a lower incidence of adverse effects with levofloxacin-based treatments than with the quadruple combinations[88,89].

Unfortunately, it has been shown that resistance to quinolones is easily acquired, and in countries with a high consumption of these drugs, the resistance rate is increasing and is already relatively high[94,103,107,113-123]. More importantly, it has been demonstrated that the presence of levofloxacin resistance significantly reduce the eradication rate following a therapy with this antibiotic[94,103,121,124]. Therefore, it has been suggested to reserve levofloxacin for rescue treatment to avoid the increase of the resistance phenomenon.

Rescue regimen after PPI-amoxicillin-nitroimidazole failure

After PPI-amoxicillin-nitroimidazole failure, retreatment with PPI-amoxicillin-clarithromycin has proved to be very effective, and it seems to be a logical strategy, as while amoxicillin is maintained (which does not induce resistance), clarithromycin is substituted for metronidazole. Furthermore, the absence of cross-resistance among nitroimidazoles and clarithromycin favors this position. With this therapy, some authors[45] have achieved *H pylori* eradication in 85% of cases, while others have reported success rates of 86%[125] or even 100%[126]. In favor of this strategy is the study by Magaret *et al*[127], who studied a group of 48 patients after failure of previous *H pylori* therapy with a metronidazole-containing regimen, and randomized them to either lansoprazole, amoxicillin and clarithromycin twice daily for 14 d, which is the logical approach with triple therapy not repeating metronidazole, or to lansoprazole, bismuth, metronidazole and tetracycline for 14 d, which is the quadruple therapy repeating metronidazole. Intention-to-treat efficacies were 75% for a triple regimen and 71% for a quadruple. Although this difference did not reach statistical significance, the small sample size of this study does not preclude the possibility of a small but clinically significant difference in efficacy between the regimens. Finally, preliminary studies have suggested that RBC may be used instead of PPI in this triple second-line strategy (i.e., RBC-clarithromycin-amoxicillin), with similar or even better results[72].

Rescue regimen after PPI-clarithromycin-nitroimidazole failure

As previously mentioned, acquired bacterial resistance to metronidazole or clarithromycin results primarily from the previous treatment failure[26,32], and therefore the first choice probably should not be a regimen that combines these two antibiotics in the same regimen[23,24,128]. Although this regimen is very effective[4], patients who are not cured will probably have double resistance[26,129], and no logical empirical treatment remains afterwards (although, more recently, the levofloxacin-based regimens may represent an option). Thus, some authors have demonstrated that initial regimens containing both clarithromycin and nitroimidazole are associated with significantly worse results overall, with lower eradication rates after logically chosen second-line therapy and sensitivity-directed third-line therapy; these poor results were due to the emergence of multiple resistant strains as evidenced by the results of culture testing after the second failed course[72]. In summary, due to problems with resistance it could be suggested that both key antibiotics-clarithromycin and metronidazole- should not be used together until a valid empirical back up regimen is available[23].

Nevertheless, if culture is not performed after failure of PPI-clarithromycin-metronidazole, and hence antibiotic susceptibility is unknown, several rescue options may be suggested. Firstly, omeprazole plus amoxicillin, with a high dose of both the antibiotic and the antisecretor, could, in theory, be recommended[128,130]; however, we must remember that this "old-fashioned" dual combination has achieved disappointing results in many countries. Therefore, a second antibiotic should be added, and at this point a difficult decision appears, as both antibiotics used in the first trial (clarithromycin and metronidazole) are capable of inducing secondary resistance to *H pylori*, playing a negative role in future efficacy[25-29,131]. Nevertheless, the following possibilities exist:

Readministering metronidazole: Due to the fact that metronidazole resistance is frequent and clinically relevant[25-28], if this antibiotic is readministered, it should be used within a bismuth-based quadruple regimen (thus PPI might reduce the negative effect of metronidazole resistance[28,58,132,133]). With this regimen, eradication rates up to 80% have been achieved[45]. RBC, which may overcome the impact of resistance to metronidazole[81], may also play a role in this regimen. Thus, some authors have reported an 88% cure rate with a 2-wk regimen or RBC-tetracycline-tinidazole in patients who had previously failed a clarithromycin-tinidazole based triple therapy[76].

Readministering clarithromycin: Several studies have underlined the relevance of clarithromycin

resistance[25-27,29], and advise against readministering this antibiotic. Therefore, a further option which has been proposed, is to add (for example to PPI-amoxicillin-clarithromycin) a fourth medication (such as bismuth) with a bactericidal effect against *H pylori*, with which a 70% eradication rate has been achieved[45].

Readministering no antibiotic: A final alternative, obviously, consists of no readministering of either metronidazole or clarithromycin. Although only published in abstract form, one study has prescribed RBC, tetracycline and amoxicillin for 2 wk and has reported eradication in 89% of the cases which had previously failed PPI, clarithromycin and tinidazole[134]. These encouraging results may be due, at least in part, to the use of RBC instead of bismuth in this regimen, as "classic" triple therapy with bismuth, tetracycline and amoxicillin have been previously considered relatively ineffective. Finally, although not specifically evaluated in PPI-clarithromycin-metronidazole failures, rifabutin or levofloxacin-based regimens (e.g. PPI, amoxicillin and either levofloxacin or rifabutin) could play a role in this difficult situation.

IS IT NECESSARY TO PERFORM CULTURE AFTER FAILURE OF THE SECOND ERADICATION TREATMENT?

As previously mentioned, it has been generally recommended that performing culture after a first eradication failure is not necessary, and therefore assessing *H pylori* sensitivity to antibiotics only after failure of the second treatment may be suggested in clinical practice[3,23,48,135]. However, the utility of the culture (with consequent antibiotic susceptibility testing) and the moment when it must be performed after eradication failure are both controversial[21]. It is evident that, as pretreatment, antibiotic resistance is the most important factor in nonresponse to initial treatment[25-29], and knowledge of the organism's antibiotic susceptibility may represent an aid in selecting the therapy regimen. However, performing culture systematically after the second eradication failure also has some limitations, which are summarized as follows:

(1) Culture implies, obviously, the performance of endoscopic exploration, which has several disadvantages: it is not free of risk, and, since endoscopy centers have been meeting increasing demand, culture usually involves prolonged waiting times.

(2) *H pylori* culture is expensive, due to the cost of the procedure itself, but mainly the costs of the associated endoscopy, which is necessary to obtain biopsy samples.

(3) Culture is time-consuming, as *H pylori* is a rather "fastidious" bacterium at culture, especially when a low bacterial load is present, as generally occurs after eradication failure[22].

(4) Culture is not always available on a routine basis.

(5) The sensitivity of bacterial culture is not 100%, and therefore the antimicrobial susceptibility cannot be obtained in all cases[136]. Indeed, even in the optimal conditions usually encountered in therapeutic trials- when both gastroenterologist and microbiologist are thoroughly motivated- a culture sensitivity of "only" approximately 90% has been achieved in patients not previously treated[22]. Furthermore, in several studies enrolling patients who had failed one or more eradication treatments, the bacterium was isolated in less than 80% of cases[22]. Therefore, an even lower probability of isolating the bacterium is to be expected in routine clinical practice. This indicates that, even in the hands of experts, antimicrobial sensitivity would not be obtained in several eradication failure patients, who had undergone an upper endoscopy solely for bacterial culture[22].

(6) Antibiotic susceptibility testing in clinical practice yields useful information only regarding a few antibiotics. Antibiotics effective and generally used against *H pylori* are mainly the following four: amoxicillin, clarithromycin, metronidazole, and tetracycline. Resistance to amoxicillin has been estimated to be less than 1% in most studies[18,22]. Hence, its role in clinical practice may even be marginalized. Similarly, resistance to tetracycline is also very low, or even absent, in most countries[18,22]. Therefore, it may even be assumed that antibiotic susceptibility testing in clinical practice yields useful information only regarding the latter two antibiotics, namely clarithromycin and metronidazole[22].

(7) *In vitro* antibiotic susceptibility does not necessarily lead to eradication *in vivo*. Even knowing the susceptibility of *H pylori*, eradication rates do not achieve 100%, as the results observed *in vivo* by following *in vitro* susceptibility to anti-*H pylori* antibiotics are often disappointing[137]. Some discrepancies between antibiotic susceptibility and *H pylori* eradication may occur, due for example, to the possibility of co-infection with different *H pylori* strains[138]. Thus, a variable proportion of non-eradicated patients is made of subjects who harbor strains sensitive to the administered drugs, and in these patients the reasons for treatment failure are unclear[139]. For example, Gomollón *et al*[140] reported how third-line treatment often (in 50% of the cases) failed to eradicate *H pylori* infection, in spite of giving a 14-d, full-dose, quadruple culture-guided combination, showing that *in vitro* susceptibility did not predict eradication success. In the same way, Vicente *et al*[141] determined the effectiveness of a third, culture-guided, treatment of *H pylori* infection after two unsuccessful attempts. Patients received a two-week quadruple culture-guided therapy, and overall eradication was achieved in only 60% of the patients. In fact, paradoxically, the lowest eradication rate was obtained in patients with *H pylori* strains sensitive to all antibiotics. In summary, it seems that despite the use of culture-guided combinations of drugs, a third treatment is frequently unsuccessful, indicating that other factors, different from *in vitro* antibiotic susceptibility, influence eradication rates. On the other hand, the reverse situation is also possible, as *H pylori* eradication may, nonetheless, be achieved in the presence of metronidazole- or clarithromycin-

resistant strains, even with a drug combination including these antibiotics. Therefore, *in vitro* resistance to either clarithromycin or metronidazole could be overcome *in vivo* in a significant proportion of patients by prescribing the same antibiotics[22].

(8) When a repeat (rescue) therapy must be selected, we have several data that will aid us in suspecting resistance to a particular antibiotic, without the necessity of a culture, based on the observation that acquired bacterial resistance to metronidazole or clarithromycin results primarily from previous treatment failure[26,32]. Thus, when a therapy with clarithromycin fails, resistance to this antibiotic appears in most cases, and the same is true when a nitroimidazole is the antibiotic first used[18,29,131,142]. Even if resistance to these antibiotics does not appear, it remains uncertain whether their readministration is adequate, as they were not efficacious (for unknown reasons) for the first time. Some studies suggest that retreatment of *H pylori* infection with the same combination is still a choice when the status of bacterial resistance to antibiotics is unknown, however, full doses and a longer treatment duration must be used and a poor eradication rate has usually been reported[143]. Therefore, the position in the case of therapy failure would be clear: do not readminister any of the antibiotics against which *H pylori* has probably become resistant[1,49].

(9) Finally, relatively high eradication rates have been obtained with *empirical* third-line treatment after two consecutive failures in several studies[76,144-156].

However, limited experience suggests that endoscopy with culture and susceptibility testing may be appropriate after failure of two eradication therapies; in this situation, a non-randomized retrospective study suggests that third-line therapy directed by the results of sensitivity testing improve eradication compared to further empirical antibiotics, demonstrating that the success rate of sensitivity-directed therapy is superior to PPI-amoxicillin-rifabutin triple therapy, and therefore suggesting that endoscopy and sensitivity testing at this point may be worthwhile rather than more widespread use of rifabutin-based regimens[72]. Cammarota *et al*[122] assessed the efficacy of a third-line, culture-guided treatment approach for the eradication of *H pylori*. After the first two eradication attempts, all patients were resistant to metronidazole, and 95% were resistant to clarithromycin. Consequently, most patients (89 out of 94) received a quadruple regimen including PPI, bismuth, tetracycline and amoxicillin, and *H pylori* eradication was obtained in 90% of the cases. Although the authors concluded that a culture-guided, third-line therapeutic approach is effective for the eradication of *H pylori*, it would seem more appropriate to conclude, in fact, that the tetracycline- and amoxicillin-based quadruple regimen may be a good *empirical* third-line rescue treatment option (as to choose such a regimen, which implies not readministering metronidazole or clarithromycin, it would not be necessary to know antibiotic susceptibilities).

In summary, when critically reviewing the role of culture in the management of *H pylori* infection in clinical practice it may be concluded, in coincidence with other authors, that *H pylori* culture is an invasive, time-consuming method, offering quite low sensitivity, requiring significant cost, and which, in practice, tests very few antibiotics, with a questionable contribution to the management of non-responder patients[22,157]. Obviously, the importance of *H pylori* culture remains unaltered both in epidemiological and pharmacological research fields. However, whether patients should undergo an upper endoscopy for bacterial culture after second-line therapy failure remains a debatable matter, and the role of culture in clinical practice requires a critical reappraisal[22,157]. As it has been brilliantly expressed by Zullo *et al*, regrettably, gastroenterologists need to accept that gastric biopsy culture is not as simple as filling a sample bottle![22]

Nevertheless, it is recommended that those prescribing *H pylori* eradication therapies continually assess their success rate and adjust the relevant local practices and policies in line with the results and local bacterial resistance patterns. Thus, it would be recommendable that culture should be routinely performed after eradication failure in some specialized centers with special interest in *H pylori* research and treatment, with the intention to study the incidence of resistances after failures and also to evaluate the influence of such resistances on the efficacy of rescue regimens[158]. Data coming from this experience on *H pylori* resistance will be used as a reference for the corresponding population. This preventive approach has been recommended to avoid an increase in refractory *H pylori* infection in the future[158].

EMPIRICAL THIRD-LINE *H PYLORI* RESCUE THERAPY AFTER FAILURE OF TWO ERADICATION TREATMENTS

If it is decided, finally, not to perform culture before the administration of a third-line rescue treatment after failure of the first two trials (generally including clarithromycin and metronidazole), different possibilities for *empirical* treatment may be suggested. As eradication regimens may be less efficacious for retreatment, as compared to their efficacy when used as primary treatment, it may be suggested that the course of the rescue therapy should be extended to 10-14 d, at least when rescue therapy fails and third-line regimens are therefore prescribed[159]. As several studies have underlined the relevance of metronidazole[25-28] and clarithromycin[25-27,29] resistance, these two antibiotics should not be readministered, and several regimens have been evaluated in this scenario.

Amoxicillin ± tetracycline-based regimens

In a recent study, patients with at least one treatment failure who were infected with *H pylori* resistant to both metronidazole and clarithromycin, were treated with high doses of omeprazole (4 × 40 mg) and amoxicillin (4 × 750 mg) for 14 d, and the infection was cured

Table 2 Rifabutin-based "rescue" therapies (rifabutin-amoxicillin-proton pump inhibitor) in patients with previously failed eradication treatments and/or resistance to clarithromycin and nitroimidazoles

Author	Number of patients	Drugs and doses	Duration of treatment (d)	Eradication rate (%)
Beales *et al*[72]	10	Rifabutin 300 mg *o.d.* Amoxicillin 1 g *b.i.d.* Omeprazole 20 mg *b.i.d.*	14	60
Bock *et al*[152]	25	Rifabutin 150 mg *b.i.d.* Amoxicillin 1 g *b.i.d.* Lansoprazole 30 mg *b.i.d.*	7	72
Borody *et al*[169]	67	Rifabutin 150 mg *b.i.d.* Amoxicillin 1-1.5 g *t.i.d.* Pantoprazole 60 mg *t.i.d*	12	90
Canducci *et al*[153]	10	Rifabutin 300 mg *o.d.* Amoxicillin 1 g *b.i.d.* Omeprazole 20 mg *b.i.d.*	10	70
Gisbert *et al*[149]	14	Rifabutin 150 mg *b.i.d.* Amoxicillin 1 g *b.i.d.* Omeprazole 20 mg *b.i.d.*	14	79
Gisbert *et al*[155]	20	Rifabutin 150 mg *b.i.d.* Amoxicillin 1 g *b.i.d.* Omeprazole 20 mg *b.i.d.*	10	45
Gonzalez Carro[170]	92	Rifabutin 150 mg *b.i.d.* Amoxicillin 1 g *b.i.d.* Pantoprazole 40 mg *b.i.d*	10	61
Miehlke *et al*[130]	73	Rifabutin 150 mg *b.i.d.* Amoxicillin 1 g *b.i.d.* Esomeprazole 20 mg *b.i.d.*	7	74
Navarro-Jarabo *et al*[63]	45	Rifabutin 150 mg *b.i.d.* Amoxicillin 1 g *b.i.d.* Omeprazole 20 mg *b.i.d.*	7	44
Perri *et al*[151]	41	Rifabutin 300 mg *o.d.* Amoxicillin 1 g *b.i.d.* Pantoprazole 40 mg *b.i.d.*	7	71
Toracchio *et al*[171]	65	Rifabutin 150 mg *b.i.d.* Amoxicillin 1 g *b.i.d.* Pantoprazole 40 mg *b.i.d.*	10	78
Van der Poorten *et al*[172]	44	Rifabutin 150 mg *b.i.d.* Amoxicillin 1 g *b.i.d.* PPI *b.i.d.*	10	68

PPI: Proton pump inhibitor (omeprazole, pantoprazole, rabeprazole or esomeprazole) at the usual dose. Eradication rates by intention-to-treat analysis when available. *H pylori* eradication rate (weighted mean) with rifabutin-based "rescue" therapy is 69%.

in 76% of the cases[160]. This study suggests that, although the "old-fashioned" dual combination of omeprazole plus amoxicillin is generally considered quite ineffective as a first-line regimen, it may be associated with relatively good results if prescribed at high doses, even for *H pylori* resistant to both metronidazole and clarithromycin, in patients who experienced previous treatment failures. Another possibility to avoid retreatment with clarithromycin or metronidazole is to prescribe a quadruple combination of PPI, bismuth, tetracycline and amoxicillin (instead of metronidazole), which has been used by some authors with favorable results[161]. Nevertheless, this regimen has been tested only as second-line (and not third-line) therapy, and only after failure of PPI-clarithromycin-amoxicillin (and not after metronidazole-based therapy), emphasizing that the experience should be extended to patients with two previous eradication failures containing both clarithromycin and metronidazole. Finally, as previously mentioned, one study prescribed RBC, tetracycline and amoxicillin for 2 wk and achieved eradication in 89% of the cases which had previously failed PPI, clarithromycin and tinidazole[134].

Levofloxacin-based rescue regimens

It has been suggested that levofloxacin-based therapies may also represent an alternative when two (or more) consecutive eradication treatments have failed to eradicate the infection[52,94,118,147,154,155,162-164]. As an example, a recent study by Zullo *et al*[147] aimed to evaluate the efficacy of a levofloxacin-amoxicillin-PPI combination in patients who previously had failed two or more therapeutic attempts, and they found the eradication rate was 83% (intention-to-treat analysis). More recently, Gisbert *et al*[155] evaluated, in a multicenter study including 100 patients, the efficacy of a third-line levofloxacin-based regimen in patients with two consecutive *H pylori* eradication failures. An intention-to-treat eradication rate was 66%, which represents a relatively high figure when considering that this regimen was evaluated as a third-line therapy. Other alternative rescue therapies, different from levofloxacin-based regimens, have been suggested. Rifabutin-based rescue therapy, as will be reviewed in the following section, also constitutes a possible strategy after previous eradication failures, although it has been recently shown that a 10 d triple levofloxacin-based regimen is more effective than the same combination with rifabutin as a rescue regimen[155]. In summary, levofloxacin-based rescue therapy constitutes an encouraging empirical third-line strategy after multiple previous *H pylori* eradication failures with key antibiotics (such as amoxicillin, clarithromycin, metronidazole and tetracycline).

Rifabutin-based rescue regimens

As previously mentioned, the evaluation of drugs without cross-resistance to nitroimidazole or macrolides as components of retreatment combination therapies seem to be worthwhile. *H pylori* has been proved to be highly susceptible *in vitro* to rifabutin, a rifamycin derivate of the established tuberculostatic drug[165-167]. Moreover, rifabutin is chemically stable at a wide pH range and its antibacterial activity is likely not to be hampered by the acidic environment of the stomach[168]. Furthermore, selection of resistant *H pylori* strains has been low in experimental conditions. Thus, until now, no rifabutin resistant strain has been isolated from patients who were either treated or untreated for *H pylori* infection[166].

As summarized Table 2, rifabutin-based rescue therapy constitutes an encouraging strategy after multiple previous eradication failures[63,72,130,149,151-153,155,169-172]. As an example, Perri *et al*[151,173] used a 1-wk regimen of PPI, amoxicillin and rifabutin in patients who were still *H pylori* infected after two or more courses of PPI-based triple therapies, and achieved an eradication rate of 71% by intention-to-treat analysis. Gisbert *et al*[149], in a prospective multicenter study, included patients in whom a first eradication trial with PPI, clarithromycin and amoxicil-

lin and a second trial with PPI, bismuth, tetracycline and metronidazole had failed. A third 14 d eradication regimen with rifabutin, amoxicillin and a PPI was effective in 79% of the patients (intention-to-treat analysis). However, these encouraging results were not confirmed in a more recent study by these same authors[155]. In the largest study on rifabutin[170], 92 consecutive patients diagnosed with *H pylori* infection resistant to two previous treatment regimens were treated with a PPI, rifabutin and amoxicillin for 10 d and the intention-to-treat eradication rate was 61%. In summary, the weighted mean eradication rate with rifabutin-based rescue therapy, calculated from the studies included in the Table 2, is 69%.

These findings suggest that new rifabutin-based combinations are effective for *H pylori* strains resistant to antibiotics, and specifically to clarithromycin or metronidazole[174]. Furthermore, rifabutin-based therapies have been compared with the widely used "classic" quadruple therapy. Perri *et al*[66] performed a randomized study where three groups of patients were treated for 10 d with pantoprazole, amoxicillin, and rifabutin 150 mg *o.d.*, or 300 mg *o.d.*, and quadruple therapy. On intention-to-treat analysis, eradication rates were 67% in the rifabutin 150 mg and quadruple groups, and higher (87%) in the rifabutin 300 mg group. Finally, in this comparative study, side-effects were less frequent in rifabutin-treated patients than in those on quadruple therapy[66].

Several concerns still remain, however, regarding rifabutin treatment. Firstly, this drug is very expensive. Secondly, severe leucopoenia and trombocytopenia have been reported in one patient treated with rifabutin, with myelotoxicity demonstrated by bone marrow aspirate[153]. Although blood cell count returned to normal at day 15 after discontinuation of therapy, physicians should be aware of the risk of major side-effects arising during a rifabutin-based regimen[149,155]. Finally, there is some concern about wide-spread use of rifabutin, a member of a class of established antimycobacterial drugs, in patients with *H pylori* infection. Because multiresistant strains of *Mycobacterium tuberculosis* increase in numbers, indications for these drugs should be chosen very carefully to avoid further acceleration of development of resistance[152]. At present, therefore, rifabutin should be considered only as the last option (e.g. restricted to infected patients even after several eradication regimens including, among them, levofloxacin).

Furazolidone-based rescue regimens

Furazolidone is an antimicrobial drug that belongs to synthetic nitrofurans and is active against a broad spectrum of gram-negative and gram-positive bacteria and protozoa. This antibiotic has demonstrated a high antimicrobial activity against *H pylori* if given as a single drug[175], and the majority of first-line furazolidone-based combination therapies revealed eradication rates above 80%[92]. Primary resistance to furazolidone is virtually absent[158,176,177], and its potential to develop resistance is as low as for bismuth compounds or amoxicillin[178]. Moreover, this drug has no cross-resistance potential to metronidazole[176]. Triple therapy in which furazolidone is used instead of metronidazole achieves high eradication rates, even in populations with a high prevalence of nitroimidazole resistance[179-182]. In this respect, a recent study has evaluated furazolidone-based triple therapy (combined with bismuth and tetracycline) in the eradication of *H pylori* resistant to metronidazole, with favorable results (86% eradication rate)[183]. A few years ago, some authors tested a quadruple combination of furazolidone, bismuth, tetracycline and PPI as a second-line eradication therapy, and reported encouraging results[184]. More recently, Treiber *et al*[145] investigated whether this quadruple regimen containing furazolidone could be effective as a third-line therapy in patients with *H pylori* treatment failure after first-line (clarithromycin-metronidazole ± amoxicillin) and second-line (PPI-bismuth-tetracycline-metronidazole) regimens, and *H pylori* infection was cured in up to 90% of the cases. Furthermore, a 7 d triple-regimen comprising furazolidone, amoxicillin and a PPI achieved an eradication rate of 60% in 10 patients who failed first-line, second-line and even rifabutin-based triple therapy[185].

A recent systematic review and meta-analysis of the effect of furazolidone- and nitrofurantoin-based regimens in the eradication of *H pylori* infection has been performed[186]. The pooled eradication rate of primary PPI-based regimens containing furazolidone was 76%. Second-line schedules containing furazolidone obtained eradication rates of 76%. Finally, third-line rescue therapies were effective in 65% of the cases. In summary, a quadruple regimen including furazolidone, bismuth, tetracycline and PPI seems to represent a promising alternative after two consecutive failures with regimens including both metronidazole and clarithromycin.

CUMULATIVE ERADICATION RATES WITH THREE (OR MORE) CONSECUTIVE ERADICATION TREATMENTS

In patients with conditions where the indication for *H pylori* eradication is definitively accepted, as is the case of peptic ulcer disease (or gastric MALT lymphoma), rescue treatment after first-line failure is clearly advisable. Furthermore, if the second therapy fails, a third or even a fourth regimen should be prescribed, as infected patients continue to have high risk of ulcer recurrence and ulcer complications and are in an obviously disadvantageous situation in view of the enormous benefits that follow *H pylori* eradication in peptic ulcer disease: increased ulcer healing, less ulcer recurrence, and less ulcer bleeding. However, multiple repeated antibiotic treatment of patients where benefits of *H pylori* eradication has not been so clearly established, such as those with functional dyspepsia[174,187], may not be completely justified.

Some authors have evaluated, in the same study,

different regimens after failure of two eradication treatments, which provide interesting information about cumulative, and not only absolute, eradication rates[21]. For example, in the study by Gasbarrini *et al*[188], a total of 2606 patients were administered a PPI, tinidazole and clarithromycin for 1 wk. Patients with continuing infection were then given a second 1-wk course of amoxicillin, clarithromycin and RBC. Finally, patients still infected after the second course underwent upper gastrointestinal endoscopy with *H pylori* culture, and then received a 1-wk quadruple scheme established on antibiotic sensitivity. Eradication rates after the first, second and third treatment, were, respectively, 79%, 77%, and 52%. This algorithm led to overall per-protocol eradication rates of 99%. Chan *et al*[146] prescribed quadruple therapy to a group of patients who had failed to respond to RBC-based regimens (as first regimen) and PPI-clarithromycin-amoxicillin combination (as second regimen), and achieved successful eradication in 83% of the cases receiving a quadruple regimen, finally achieving a 99% cumulative eradication rate. Beales *et al*[72] evaluated 469 patients receiving eradication therapy in routine clinical practice. Second-line therapy was chosen empirically, using whichever of clarithromycin or metronidazole was not used initially. All patients requiring third-line therapy underwent endoscopy, choice of therapy being guided by sensitivities. Overall success after one, two and three courses of therapy were 73%, 94% and 98%, respectively. Zullo *et al*[76] reported 83% cure rate in patients who had previously failed two courses of clarithromycin-amoxicillin and clarithromycin-tinidazole based triple therapies. Gomollón *et al*[140] studied the effectiveness of third-line treatment of *H pylori* infection with two-week quadruple, culture-guided regimens. The combination of omeprazole, tetracycline, bismuth and clarithromycin showed an eradication rate of only 36%, but if amoxicillin was used the rate was 67%. In the study by Vicente *et al*[141], after two unsuccessful attempts at eradication, all patients underwent endoscopy and culture, and patients received a quadruple culture-guided therapy. Cumulative *H pylori* eradication rate with this strategy was as high as 99.6%. Treiber *et al*[145] investigated whether a quadruple regimen containing furazolidone could be effective as a third-line therapy in patients with two previous *H pylori* treatment failures. Cure of *H pylori* was achieved in 90% of the patients nonresponsive to a second eradication trial, which gave a final eradication rate of 99%. In the study by Qasim *et al*[185], 3280 patients received standard first-line eradication therapy, which was successful in 77% of the cases. Second-line therapy (bismuth-based quadruple) or triple therapy (altering constituent antibiotics) was successful in 56% of treated patients. Subsequent eradication attempts using rifabutin-based regimen was successful in 38% of patients, giving a cumulative eradication rate of 94%. Gisbert *et al*[150] included consecutive patients in whom two eradication regimens had failed to eradicate *H pylori*, prescribed empirical third-line rescue regimens, and achieved *H pylori* eradication in 71% of the cases (intention-to-treat analysis). Based on these results, with estimated efficacy of 85%, 75% and 71%, respectively with first, second and third regimens, *H pylori* eradication could finally be achieved in 99% of the patients. Finally, Gisbert *et al*[156] evaluated the efficacy of different rescue therapies empirically prescribed during 10 years to 500 patients in whom at least one eradication regimen had failed to cure *H pylori* infection. Antibiotic susceptibility was unknown (therefore rescue regimens were chosen empirically). Overall, *H pylori* cure rates with the second and third-line rescue regimens were 70% and 74%, giving a cumulative eradication rate as high as 98%.

Therefore, a wider perspective of the benefits of retreating *H pylori* infection can be obtained if cumulative eradication rates with successive treatments are taken into account. Thus, as represented in Figure 2, it can be concluded that *H pylori* eradication can finally be achieved in almost 100% of the patients if three rescue therapies are consecutively given[72,76,140,141,144-146,150,156,185,188].

Furthermore, these encouraging (cumulative) results have been obtained when more than three consecutive treatments have been prescribed[21]. As an example, Seppälä *et al*[144] reported a cumulative eradication rate of 93% (intention-to-treat analysis) and even 100% (per-protocol analysis) after four empirical retreatments. We have recently confirmed that a levofloxacin-based regimen can also be administered with good results after three previous eradication failures with antibiotics, such as amoxicillin, clarithromycin, metronidazole, tetracycline, and even rifabutin[163]. Thus, we prospectively evaluated 10 patients with three consecutive *H pylori* eradication failures (1st treatment with PPI-clarithromycin-amoxicillin, 2nd treatment with RBC-tetracycline-metronidazole, and 3rd treatment with PPI-amoxicillin-rifabutin). A fourth eradication regimen with 10 d levofloxacin, amoxicillin and PPI was prescribed, and intention-to-treat eradication rates were 70%. When we reviewed our experience with different rescue therapies empirically prescribed during 10 years to 500 patients, the cumulative *H pylori* eradication rate with 4 successive treatments was 99.5%[156].

Finally, reports of "ineradicable" *H pylori* infection after more than four eradicating treatments failed have been recently published. Dore *et al*[148] prescribed a quadruple combination of PPI, bismuth, tetracycline, and metronidazole to patients who had failed two or more treatment courses of *H pylori* eradication therapy (33 patients had failed prior treatment twice, 19 had failed three times, and 16 had failed four or more times); despite this *a priori* difficult task, *H pylori* eradication was finally achieved in 93% of the patients. Tucci *et al*[189] reported their experience of 13 patients with at least 5 eradication failures and *H pylori* strains resistant to both clarithromycin and nitroimidazoles. The treatment was organized into three sequential schedules employing partially different drug combinations (to face the various resistant strains), suspension formulations were preferred to tablets (to improve the dispersal of the drugs into the stomach), antibiotics were administered after meals and

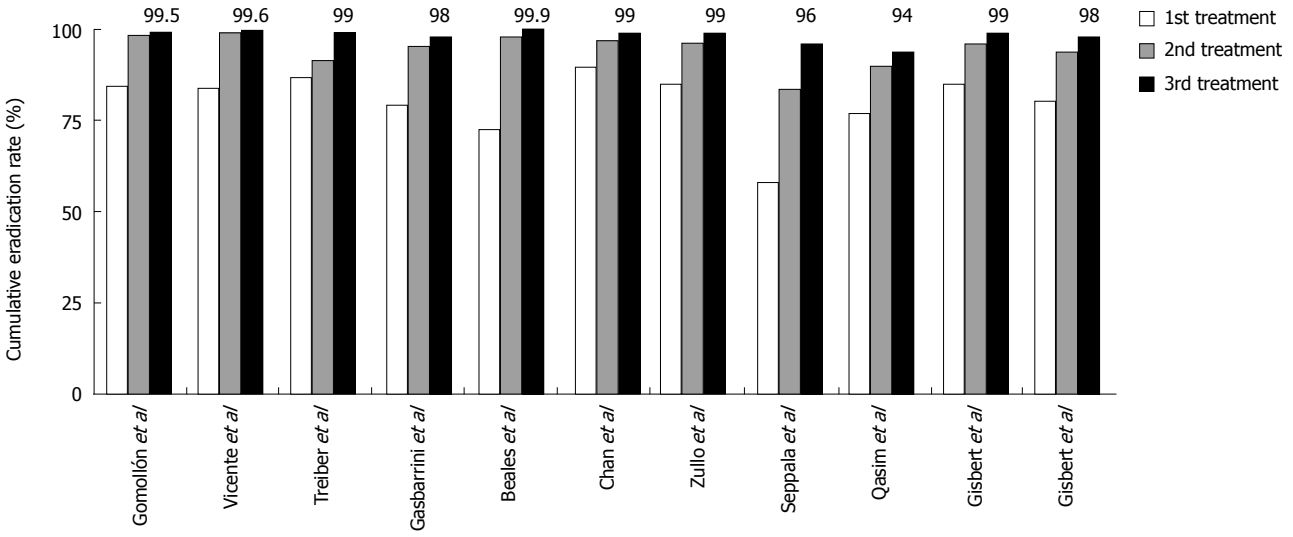


Figure 2 Cumulative *H pylori* eradication rates with three consecutive eradication treatments.

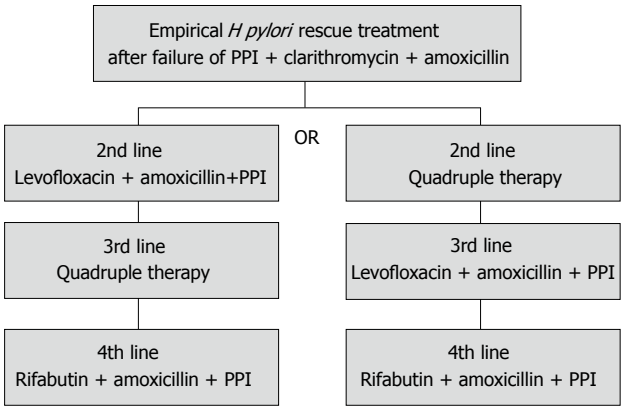


Figure 3 Choice of a empirical retreatment regimen, without culture and antimicrobial sensitivity testing, after failure of proton pump inhibitor (PPI), amoxicillin and clarithromycin combination. Quadruple therapy: Combination of PPI, bismuth, tetracycline and nitroimidazole (metronidazole or tinidazole).

a variation on a standard diet exceeding the normal fat composition was given (to increase the time of contact of the antimicrobials with gastric mucosa), and patients were invited to lie down after the meals, changing their position every 5 min (to facilitate the penetration of drugs amid the anfractuosities of fundic mucosa). With this particular therapy, eradication was successful in 70% of the patients. In another example of "ineradicable" *H pylori* infection, levofloxacin-amoxicillin combination was successfully employed in a patient with a clarithromycin- and metronidazole-resistant strain, who previously failed eight consecutive therapeutic attempts[162].

CONCLUSION

Even with the current most effective treatment regimens, ⩾ 20% of patients will fail to eradicate *H pylori* infection. This issue seems important at the present time, as therapy for *H pylori* infection is becoming more and more frequently prescribed. Currently, apart from having to know first-line eradication regimens well, we must also be prepared to face treatment failures. Therefore, in designing a treatment strategy we should not focus on the results of primary therapy alone, but also on the final (overall) eradication rate.

The choice of a rescue treatment depends on which treatment is used initially. If a first-line clarithromycin-based regimen was used, a second-line metronidazole-based treatment (such as the quadruple therapy) may be used afterwards, and then a levofloxacin-based combination would be a third-line rescue option. Alternatively, it has recently been suggested that levofloxacin-based rescue therapy constitutes an encouraging second-line strategy, representing an alternative to quadruple therapy in patients with previous PPI-clarithromycin-amoxicillin failure, with the advantage of efficacy, simplicity and safety. In this case, quadruple regimen may be reserved as a third-line rescue option. Finally, rifabutin-based rescue therapy constitutes an encouraging empirical fourth-line strategy after multiple previous eradication failures with key antibiotics such as amoxicillin, clarithromycin, metronidazole, tetracycline, and levofloxacin (Figure 3).

Even after two consecutive failures, several studies have demonstrated that *H pylori* eradication can finally be achieved in almost all patients if several rescue therapies are consecutively given. As a final conclusion, therefore, the attitude in *H pylori* eradication therapy failure, even after two or more unsuccessful attempts, should be to fight and not to surrender[190].

REFERENCES

1 **Howden CW**, Hunt RH. Guidelines for the management of Helicobacter pylori infection. Ad Hoc Committee on Practice Parameters of the American College of Gastroenterology. *Am J Gastroenterol* 1998; **93**: 2330-2338

2 **Malfertheiner P**, Megraud F, O'Morain C, Hungin AP, Jones R, Axon A, Graham DY, Tytgat G. Current concepts in the management of Helicobacter pylori infection--the Maastricht 2-2000 Consensus Report. *Aliment Pharmacol Ther* 2002; **16**: 167-180

3 **Malfertheiner P**, Megraud F, O'Morain C, Bazzoli F, El-

Omar E, Graham D, Hunt R, Rokkas T, Vakil N, Kuipers EJ. Current concepts in the management of Helicobacter pylori infection: the Maastricht III Consensus Report. *Gut* 2007; **56**: 772-781

4 **Gisbert JP**, Gonzalez L, Calvet X, Garcia N, Lopez T, Roque M, Gabriel R, Pajares JM. Proton pump inhibitor, clarithromycin and either amoxycillin or nitroimidazole: a meta-analysis of eradication of Helicobacter pylori. *Aliment Pharmacol Ther* 2000; **14**: 1319-1328

5 **Gisbert JP**, Pajares R, Pajares JM. Evolution of Helicobacter pylori therapy from a meta-analytical perspective. *Helicobacter* 2007; **12** Suppl 2: 50-58

6 **Hawkey CJ**, Atherton JC, Treichel HC, Thjodleifsson B, Ravic M. Safety and efficacy of 7-day rabeprazole- and omeprazole-based triple therapy regimens for the eradication of Helicobacter pylori in patients with documented peptic ulcer disease. *Aliment Pharmacol Ther* 2003; **17**: 1065-1074

7 **Paoluzi P**, Iacopini F, Crispino P, Nardi F, Bella A, Rivera M, Rossi P, Gurnari M, Caracciolo F, Zippi M, Pica R. 2-week triple therapy for Helicobacter pylori infection is better than 1-week in clinical practice: a large prospective single-center randomized study. *Helicobacter* 2006; **11**: 562-568

8 **Veldhuyzen Van Zanten S**, Machado S, Lee J. One-week triple therapy with esomeprazole, clarithromycin and metronidazole provides effective eradication of Helicobacter pylori infection. *Aliment Pharmacol Ther* 2003; **17**: 1381-1387

9 **Vakil N, Lanza F**, Schwartz H, Barth J. Seven-day therapy for Helicobacter pylori in the United States. *Aliment Pharmacol Ther* 2004; **20**: 99-107

10 **Laine L**, Fennerty MB, Osato M, Sugg J, Suchower L, Probst P, Levine JG. Esomeprazole-based Helicobacter pylori eradication therapy and the effect of antibiotic resistance: results of three US multicenter, double-blind trials. *Am J Gastroenterol* 2000; **95**: 3393-3398

11 **Laine L**, Suchower L, Frantz J, Connors A, Neil G. Twice-daily, 10-day triple therapy with omeprazole, amoxicillin, and clarithromycin for Helicobacter pylori eradication in duodenal ulcer disease: results of three multicenter, double-blind, United States trials. *Am J Gastroenterol* 1998; **93**: 2106-2112

12 **Calvet X**, Ducons J, Bujanda L, Bory F, Montserrat A, Gisbert JP. Seven versus ten days of rabeprazole triple therapy for Helicobacter pylori eradication: a multicenter randomized trial. *Am J Gastroenterol* 2005; **100**: 1696-1701

13 **Gisbert JP**, Dominguez-Munoz A, Dominguez-Martin A, Gisbert JL, Marcos S. Esomeprazole-based therapy in Helicobacter pylori eradication: any effect by increasing the dose of esomeprazole or prolonging the treatment? *Am J Gastroenterol* 2005; **100**: 1935-1940

14 **Della Monica P**, Lavagna A, Masoero G, Lombardo L, Crocella L, Pera A. Effectiveness of Helicobacter pylori eradication treatments in a primary care setting in Italy. *Aliment Pharmacol Ther* 2002; **16**: 1269-1275

15 **Altintas E**, Sezgin O, Ulu O, Aydin O, Camdeviren H. Maastricht II treatment scheme and efficacy of different proton pump inhibitors in eradicating Helicobacter pylori. *World J Gastroenterol* 2004; **10**: 1656-1658

16 **Gumurdulu Y**, Serin E, Ozer B, Kayaselcuk F, Ozsahin K, Cosar AM, Gursoy M, Gur G, Yilmaz U, Boyacioglu S. Low eradication rate of Helicobacter pylori with triple 7-14 days and quadriple therapy in Turkey. *World J Gastroenterol* 2004; **10**: 668-671

17 **Vakil N**, Hahn B, McSorley D. Clarithromycin-resistant Helicobacter pylori in patients with duodenal ulcer in the United States. *Am J Gastroenterol* 1998; **93**: 1432-1435

18 **Megraud F**. H pylori antibiotic resistance: prevalence, importance, and advances in testing. *Gut* 2004; **53**: 1374-1384

19 **Axon A**. Quadruple therapy should be second-line treatment for Helicobacter pylori infection. In: Hunt RH, Tytgat GNJ, eds. Helicobacter pylori: Basic Mechanisms to Clinical Cure. London: Kluwer, 2000: 631-635

20 **Gisbert JP**, Pajares JM. Review article: Helicobacter pylori "rescue" regimen when proton pump inhibitor-based triple therapies fail. *Aliment Pharmacol Ther* 2002; **16**: 1047-1057

21 **Gisbert JP**, Pajares JM. Helicobacter pylori "rescue" therapy after failure of two eradication treatments. *Helicobacter* 2005; **10**: 363-372

22 **Zullo A**, Hassan C, Lorenzetti R, Winn S, Morini S. A clinical practice viewpoint: to culture or not to culture Helicobacter pylori? *Dig Liver Dis* 2003; **35**: 357-361

23 **de Boer WA**, Tytgat GN. Regular review: treatment of Helicobacter pylori infection. *BMJ* 2000; **320**: 31-34

24 **Calvet X**, Gene E, Sanfeliu I. [Therapeutic strategies in Helicobacter pylori infection] *Med Clin* (Barc) 2001; **116**: 239

25 **Houben MH**, van de Beek D, Hensen EF, Craen AJ, Rauws EA, Tytgat GN. A systematic review of Helicobacter pylori eradication therapy--the impact of antimicrobial resistance on eradication rates. *Aliment Pharmacol Ther* 1999; **13**: 1047-1055

26 **Peitz U**, Hackelsberger A, Malfertheiner P. A practical approach to patients with refractory Helicobacter pylori infection, or who are re-infected after standard therapy. *Drugs* 1999; **57**: 905-920

27 **Dore MP**, Leandro G, Realdi G, Sepulveda AR, Graham DY. Effect of pretreatment antibiotic resistance to metronidazole and clarithromycin on outcome of Helicobacter pylori therapy: a meta-analytical approach. *Dig Dis Sci* 2000; **45**: 68-76

28 **van der Wouden EJ**, Thijs JC, van Zwet AA, Sluiter WJ, Kleibeuker JH. The influence of in vitro nitroimidazole resistance on the efficacy of nitroimidazole-containing anti-Helicobacter pylori regimens: a meta-analysis. *Am J Gastroenterol* 1999; **94**: 1751-1759

29 **Tompkins DS**, Perkin J, Smith C. Failed treatment of Helicobacter pylori infection associated with resistance to clarithromycin. *Helicobacter* 1997; **2**: 185-187

30 **Parente F**, Cucino C, Bianchi Porro G. Treatment options for patients with Helicobacter pylori infection resistant to one or more eradication attempts. *Dig Liver Dis* 2003; **35**: 523-528

31 **Battaglia G**, Di Mario F, Vigneri S, Vianello F, Benvenuti ME, Donisi PM, Stracca-Pansa V, Pasquino M. Strategy for the retreatment of failed Helicobacter pylori eradication therapy: a case series. *Ital J Gastroenterol Hepatol* 1998; **30**: 370-374

32 **Deltenre M**, Ntounda R, Jonas C, De Koster E. Eradication of Helicobacter pylori: why does it fail? *Ital J Gastroenterol Hepatol* 1998; **30** Suppl 3: S326-S328

33 **Kobayashi I**, Saika T, Muraoka H, Murakami K, Fujioka T. Helicobacter pylori isolated from patients who later failed H. pylori eradication triple therapy readily develop resistance to clarithromycin. *J Med Microbiol* 2006; **55**: 737-740

34 **Yahav J**, Samra Z, Niv Y, Evans CT, Passaro DJ, Dinari G, Shmuely H. Susceptibility-guided vs. empiric retreatment of Helicobacter pylori infection after treatment failure. *Dig Dis Sci* 2006; **51**: 2316-2321

35 **Lamouliatte H**, Megraud F, Delchier JC, Bretagne JF, Courillon-Mallet A, De Korwin JD, Fauchere JL, Labigne A, Flejou JF, Barthelemy P. Second-line treatment for failure to eradicate Helicobacter pylori: a randomized trial comparing four treatment strategies. *Aliment Pharmacol Ther* 2003; **18**: 791-797

36 **Avidan B**, Melzer E, Keller N, Bar-Meir S. The effect of culture results for Helicobacter pylori on the choice of treatment following failure of initial eradication. *Isr Med Assoc J* 2001; **3**: 163-165

37 **Miwa H**, Nagahara A, Kurosawa A, Ohkusa T, Ohkura R, Hojo M, Enomoto N, Sato N. Is antimicrobial susceptibility testing necessary before second-line treatment for Helicobacter pylori infection? *Aliment Pharmacol Ther* 2003; **17**: 1545-1551

38 **Shimoyama T**, Fukuda S, Mikami T, Fukushi M, Munakata A. Efficacy of metronidazole for the treatment of clarithromycin-resistant Helicobacter pylori infection in a

Japanese population. *J Gastroenterol* 2004; **39**: 927-930
39 **Nagahara A**, Miwa H, Kawabe M, Kurosawa A, Asaoka D, Hojo M, Iijima K, Terai T, Ohkusa T, Miyazaki A, Sato N. Second-line treatment for Helicobacter pylori infection in Japan: proton pump inhibitor-based amoxicillin and metronidazole regimen. *J Gastroenterol* 2004; **39**: 1051-1055
40 **Murakami K**, Okimoto T, Kodama M, Sato R, Watanabe K, Fujioka T. Evaluation of three different proton pump inhibitors with amoxicillin and metronidazole in retreatment for Helicobacter pylori infection. *J Clin Gastroenterol* 2008; **42**: 139-142
41 **Shirai N**, Sugimoto M, Kodaira C, Nishino M, Ikuma M, Kajimura M, Ohashi K, Ishizaki T, Hishida A, Furuta T. Dual therapy with high doses of rabeprazole and amoxicillin versus triple therapy with rabeprazole, amoxicillin, and metronidazole as a rescue regimen for Helicobacter pylori infection after the standard triple therapy. *Eur J Clin Pharmacol* 2007; **63**: 743-749
42 **Isomoto H**, Inoue K, Furusu H, Enjoji A, Fujimoto C, Yamakawa M, Hirakata Y, Omagari K, Mizuta Y, Murase K, Shimada S, Murata I, Kohno S. High-dose rabeprazole-amoxicillin versus rabeprazole-amoxicillin-metronidazole as second-line treatment after failure of the Japanese standard regimen for Helicobacter pylori infection. *Aliment Pharmacol Ther* 2003; **18**: 101-107
43 **Murakami K**, Okimoto T, Kodama M, Sato R, Miyajima H, Ono M, Inoue K, Watanabe K, Otsu S, Fujioka T. Comparison of amoxicillin-metronidazole plus famotidine or lansoprazole for amoxicillin-clarithromycin-proton pump inhibitor treatment failures for Helicobacter pylori infection. *Helicobacter* 2006; **11**: 436-440
44 **Matsuhisa T**, Kawai T, Masaoka T, Suzuki H, Ito M, Kawamura Y, Tokunaga K, Suzuki M, Mine T, Takahashi S, Sakaki N. Efficacy of metronidazole as second-line drug for the treatment of Helicobacter pylori Infection in the Japanese population: a multicenter study in the Tokyo Metropolitan Area. *Helicobacter* 2006; **11**: 152-158
45 **Gisbert JP**, Boixeda D, Bermejo F, Nieves Rincon M, Jesus Higes M, Angeles Arpa M, Garcia Arata I, Martin de Argila C, Garcia Plaza A. Re-treatment after Helicobacter pylori eradication failure. *Eur J Gastroenterol Hepatol* 1999; **11**: 1049-1054
46 **Perri F**, Villani MR, Quitadamo M, Annese V, Niro GA, Andriulli A. Ranitidine bismuth citrate-based triple therapies after failure of the standard 'Maastricht triple therapy': a promising alternative to the quadruple therapy? *Aliment Pharmacol Ther* 2001; **15**: 1017-1022
47 **Nagahara A**, Miwa H, Ohkura R, Yamada T, Sato K, Hojo M, Sato N. Strategy for retreatment of therapeutic failure of eradication of Helicobacter pylori infection. *J Gastroenterol Hepatol* 2001; **16**: 613-618
48 **European Helicobacter Pylori Study Group.** Current European concepts in the management of Helicobacter pylori infection. The Maastricht Consensus Report. *Gut* 1997; **41**: 8-13
49 **Lam SK**, Talley NJ. Report of the 1997 Asia Pacific Consensus Conference on the management of Helicobacter pylori infection. *J Gastroenterol Hepatol* 1998; **13**: 1-12
50 **Gisbert JP**, Calvet X, Gomollon F, Sainz R. [Treatment for the eradication of Helicobacter pylori. Recommendations of the Spanish Consensus Conference] *Med Clin* (Barc) 2000; **114**: 185-195
51 **Baena Diez JM**, Lopez Mompo C, Rams Rams F, Garcia Lareo M, Rosario Hernandez Ibanez M, Teruel Gila J. [Efficacy of a multistep strategy for Helicobacter pylori eradication: quadruple therapy with omeprazole, metronidazole, tetracycline and bismuth after failure of a combination of omeprazole, clarithromycin and amoxycillin] *Med Clin* (Barc) 2000; **115**: 617-619
52 **Bilardi C**, Dulbecco P, Zentilin P, Reglioni S, Iiritano E, Parodi A, Accornero L, Savarino E, Mansi C, Mamone M, Vigneri S, Savarino V. A 10-day levofloxacin-based therapy in patients with resistant Helicobacter pylori infection: a controlled trial. *Clin Gastroenterol Hepatol* 2004; **2**: 997-1002
53 **Elizalde IR**, Borda F, Jara C, Martínez A, Rodríguez C, Jiménez J. Eficacia de dos tratamientos consecutivos en la erradicación de Helicobacter pylori. *Anales Sis San Navarra* 1998; **21** Supl 2: 83-88
54 **Choung RS**, Lee SW, Jung SW, Han WS, Kim MJ, Jeen YT, Park JJ, Lee HS, Chun HJ, Um SH, Choi JH, Kim CD, Ryu HS, Hyun JH. [Comparison of the effectiveness of quadruple salvage regimen for Helicobacter pylori infection according to the duration of treatment] *Korean J Gastroenterol* 2006; **47**: 131-135
55 **Chung SJ**, Lee DH, Kim N, Jung SH, Kim JW, Hwang JH, Park YS, Lee KH, Jung HC, Song IS. Eradication rates of helicobacter pylori infection with second-line treatment: non-ulcer dyspepsia compared to peptic ulcer disease. *Hepatogastroenterology* 2007; **54**: 1293-1296
56 **Gasbarrini A**, Ojetti V, Pitocco D, Armuzzi A, Silveri NG, Pola P, Ghirlanda G, Gasbarrini G. Efficacy of different Helicobacter pylori eradication regimens in patients affected by insulin-dependent diabetes mellitus. *Scand J Gastroenterol* 2000; **35**: 260-263
57 **Gisbert JP**, Gisbert JL, Marcos S, Gravalos RG, Carpio D, Pajares JM. Seven-day 'rescue' therapy after Helicobacter pylori treatment failure: omeprazole, bismuth, tetracycline and metronidazole vs. ranitidine bismuth citrate, tetracycline and metronidazole. *Aliment Pharmacol Ther* 1999; **13**: 1311-1316
58 **Gomollón F**, Ducons JA, Ferrero M, Garcia Cabezudo J, Guirao R, Simon MA, Montoro M. Quadruple therapy is effective for eradicating Helicobacter pylori after failure of triple proton-pump inhibitor-based therapy: a detailed, prospective analysis of 21 consecutive cases. *Helicobacter* 1999; **4**: 222-225
59 **Lee JM**, Breslin NP, Hyde DK, Buckley MJ, O'Morain CA. Treatment options for Helicobacter pylori infection when proton pump inhibitor-based triple therapy fails in clinical practice. *Aliment Pharmacol Ther* 1999; **13**: 489-496
60 **Lee JH**, Cheon JH, Park MJ, Kim N, Lee DH, Kim JM, Kim JS, Jung HC, Song IS. [The trend of eradication rates of second-line quadruple therapy containing metronidazole for Helicobacter pylori infection: an analysis of recent eight years] *Korean J Gastroenterol* 2005; **46**: 94-98
61 **Marko D**, Calvet X, Ducons J, Guardiola J, Tito L, Bory F. Comparison of two management strategies for Helicobacter pylori treatment: clinical study and cost-effectiveness analysis. *Helicobacter* 2005; **10**: 22-32
62 **Michopoulos S**, Tsibouris P, Bouzakis H, Balta A, Vougadiotis J, Broutet N, Kralios N. Randomized study comparing omeprazole with ranitidine as anti-secretory agents combined in quadruple second-line Helicobacter pylori eradication regimens. *Aliment Pharmacol Ther* 2000; **14**: 737-744
63 **Navarro-Jarabo JM**, Fernandez N, Sousa FL, Cabrera E, Castro M, Ramirez LM, Rivera R, Ubina E, Vera F, Mendez I, Rivas-Ruiz F, Moreno JL, Perea-Milla E. Efficacy of rifabutin-based triple therapy as second-line treatment to eradicate helicobacter pylori infection. *BMC Gastroenterol* 2007; **7**: 31
64 **Nista EC**, Candelli M, Cremonini F, Cazzato IA, Di Caro S, Gabrielli M, Santarelli L, Zocco MA, Ojetti V, Carloni E, Cammarota G, Gasbarrini G, Gasbarrini A. Levofloxacin-based triple therapy vs. quadruple therapy in second-line Helicobacter pylori treatment: a randomized trial. *Aliment Pharmacol Ther* 2003; **18**: 627-633
65 **Orsi P**, Pinazzi O, Aragona G, Di Mario F. Rabeprazole/Levofloxacin based triple therapy as a Salvatage Treatment after Failure of H pylori eradication with standard regimens. *Helicobacter* 2003; **8**: 339-493
66 **Perri F**, Festa V, Clemente R, Villani MR, Quitadamo M, Caruso N, Bergoli ML, Andriulli A. Randomized study of two "rescue" therapies for Helicobacter pylori-infected

patients after failure of standard triple therapies. *Am J Gastroenterol* 2001; **96**: 58-62
67 **Perri F**, Festa V, Merla A, Barberani F, Pilotto A, Andriulli A. Randomized study of different 'second-line' therapies for Helicobacter pylori infection after failure of the standard 'Maastricht triple therapy'. *Aliment Pharmacol Ther* 2003; **18**: 815-820
68 **Sicilia B**, Sierra E, Lago A, Villar M, Garcia S, Gomollon F. [High eradication rates in Helicobacter pylori infection in patients with duodenal ulcer who failed previous eradication therapy] *Med Clin* (Barc) 2000; **115**: 641-643
69 **Uygun A**, Ozel AM, Yildiz O, Aslan M, Yesilova Z, Erdil A, Bagci S, Gunhan O. Comparison of three different second-line quadruple therapies including bismuth subcitrate in Turkish patients with non-ulcer dyspepsia who failed to eradicate Helicobacter pylori with a 14-day standard first-line therapy. *J Gastroenterol Hepatol* 2008; **23**: 42-45
70 **Wong WM**, Gu Q, Lam SK, Fung FM, Lai KC, Hu WH, Yee YK, Chan CK, Xia HH, Yuen MF, Wong BC. Randomized controlled study of rabeprazole, levofloxacin and rifabutin triple therapy vs. quadruple therapy as second-line treatment for Helicobacter pylori infection. *Aliment Pharmacol Ther* 2003; **17**: 553-560
71 **Wu DC**, Hsu PI, Chen A, Lai KH, Tsay FW, Wu CJ, Lo GH, Wu JY, Wu IC, Wang WM, Tseng HH. Randomized comparison of two rescue therapies for Helicobacter pylori infection. *Eur J Clin Invest* 2006; **36**: 803-809
72 **Beales IL**. Efficacy of Helicobacter pylori eradication therapies: a single centre observational study. *BMC Gastroenterol* 2001; **1**: 7
73 **de Boer WA**, Driessen WM, Potters VP, Tytgat GN. Randomized study comparing 1 with 2 weeks of quadruple therapy for eradicating Helicobacter pylori. *Am J Gastroenterol* 1994; **89**: 1993-1997
74 **Gomollón F**, Valdeperez J, Garuz R, Fuentes J, Barrera F, Malo J, Tirado M, Simon MA. [Cost-effectiveness analysis of 2 strategies of Helicobacter pylori eradication: results of a prospective and randomized study in primary care] *Med Clin* (Barc) 2000; **115**: 1-6
75 **Rinaldi V**, Zullo A, De Francesco V, Hassan C, Winn S, Stoppino V, Faleo D, Attili AF. Helicobacter pylori eradication with proton pump inhibitor-based triple therapies and re-treatment with ranitidine bismuth citrate-based triple therapy. *Aliment Pharmacol Ther* 1999; **13**: 163-168
76 **Zullo A**, Hassan C, Campo SM, Lorenzetti R, Febbraro I, De Matthaeis M, Porto D, Morini S. A triple therapy regimen after failed Helicobacter pylori treatments. *Aliment Pharmacol Ther* 2001; **15**: 1193-1197
77 **Gisbert JP**, Fuentes J, Carpio D, Tito L, Guardiola J, Tomas A, Olivares D, Calvet X. 7-day rescue therapy with ranitidine bismuth citrate after Helicobacter pylori treatment failure. *Aliment Pharmacol Ther* 2005; **21**: 1249-1253
78 **Hojo M**, Miwa H, Nagahara A, Sato N. Pooled analysis on the efficacy of the second-line treatment regimens for Helicobacter pylori infection. *Scand J Gastroenterol* 2001; **36**: 690-700
79 **Gisbert JP**, Gisbert JL, Marcos S, Moreno-Otero R, Pajares JM. Levofloxacin- vs. ranitidine bismuth citrate-containing therapy after H. pylori treatment failure. *Helicobacter* 2007; **12**: 68-73
80 **Koksal AS**, Parlak E, Filik L, Yolcu OF, Odemis B, Ulker A, Sasmaz N, Ozden A, Sahin B. Ranitidine bismuth citrate-based triple therapies as a second-line therapy for Helicobacter pylori in Turkish patients. *J Gastroenterol Hepatol* 2005; **20**: 637-642
81 **Gisbert JP**, Pajares JM, Valle J. Ranitidine bismuth citrate therapy regimens for treatment of Helicobacter pylori infection: a review. *Helicobacter* 1999; **4**: 58-66
82 **Van Oijen AH**, Verbeek AL, Jansen JB, De Boer WA. Review article: treatment of Helicobacter pylori infection with ranitidine bismuth citrate- or proton pump inhibitor-based triple therapies. *Aliment Pharmacol Ther* 2000; **14**: 991-999
83 **Gisbert JP**, Gonzalez L, Calvet X. Systematic review and meta-analysis: proton pump inhibitor vs. ranitidine bismuth citrate plus two antibiotics in Helicobacter pylori eradication. *Helicobacter* 2005; **10**: 157-171
84 **Thong-Ngam D**, Mahachai V. 14-day quadruple therapy with ranitidine bismuth citrate after Helicobacter pylori treatment failure in Thailand. *J Med Assoc Thai* 2006; **89** Suppl 3: S119-S125
85 **Kearney DJ**. Retreatment of Helicobacter pylori infection after initial treatment failure. *Am J Gastroenterol* 2001; **96**: 1335-1339
86 **Caselli M**, Zullo A, Maconi G, Parente F, Alvisi V, Casetti T, Sorrentino D, Gasbarrini G. "Cervia II Working Group Report 2006": guidelines on diagnosis and treatment of Helicobacter pylori infection in Italy. *Dig Liver Dis* 2007; **39**: 782-789
87 **Croom KF**, Goa KL. Levofloxacin: a review of its use in the treatment of bacterial infections in the United States. *Drugs* 2003; **63**: 2769-2802
88 **Saad RJ**, Schoenfeld P, Kim HM, Chey WD. Levofloxacin-based triple therapy versus bismuth-based quadruple therapy for persistent Helicobacter pylori infection: a meta-analysis. *Am J Gastroenterol* 2006; **101**: 488-496
89 **Gisbert JP**, Morena F. Systematic review and meta-analysis: levofloxacin-based rescue regimens after Helicobacter pylori treatment failure. *Aliment Pharmacol Ther* 2006; **23**: 35-44
90 **Vaira D**, Ricci C, Lanzini A, Perna F, Romano A, Corinaldesi R. How to proceed in Helicobacter pylori-positive chronic gastritis refractory to first- and second-line eradication therapy. *Dig Dis* 2007; **25**: 203-205
91 **Sanchez JE**, Saenz NG, Rincon MR, Martin IT, Sanchez EG, Martinez MJ. Susceptibility of Helicobacter pylori to mupirocin, oxazolidinones, quinupristin/dalfopristin and new quinolones. *J Antimicrob Chemother* 2000; **46**: 283-285
92 **Xia HH**, Yu Wong BC, Talley NJ, Lam SK. Alternative and rescue treatment regimens for Helicobacter pylori eradication. *Expert Opin Pharmacother* 2002; **3**: 1301-1311
93 **Zou J**, Yang ZX, Qin ZM. [Laboratory and clinical study of levofloxacin against Helicobacter pylori] *Zhonghua Yixue Zazhi* 2003; **83**: 1778-1781
94 **Gatta L**, Zullo A, Perna F, Ricci C, De Francesco V, Tampieri A, Bernabucci V, Cavina M, Hassan C, Ierardi E, Morini S, Vaira D. A 10-day levofloxacin-based triple therapy in patients who have failed two eradication courses. *Aliment Pharmacol Ther* 2005; **22**: 45-49
95 **Antos D**, Schneider-Brachert W, Bastlein E, Hanel C, Haferland C, Buchner M, Meier E, Trump F, Stolte M, Lehn N, Bayerdorffer E. 7-day triple therapy of Helicobacter pylori infection with levofloxacin, amoxicillin, and high-dose esomeprazole in patients with known antimicrobial sensitivity. *Helicobacter* 2006; **11**: 39-45
96 **Kumala W**, Rani A. Patterns of Helicobacter pylori isolate resistance to fluoroquinolones, amoxicillin, clarithromycin and metronidazoles. *Southeast Asian J Trop Med Public Health* 2006; **37**: 970-974
97 **Tanaka M**, Isogai E, Isogai H, Hayashi S, Hirose K, Kimura K, Sugiyama T, Sato K. Synergic effect of quinolone antibacterial agents and proton pump inhibitors on Helicobacter pylori. *J Antimicrob Chemother* 2002; **49**: 1039-1040
98 **Watanabe Y**, Aoyama N, Shirasaka D, Maekawa S, Kuroda K, Miki I, Kachi M, Fukuda M, Wambura C, Tamura T, Kasuga M. Levofloxacin based triple therapy as a second-line treatment after failure of helicobacter pylori eradication with standard triple therapy. *Dig Liver Dis* 2003; **35**: 711-715
99 **Yahav J**, Shmuely H, Niv Y, Bechor J, Samra Z. In vitro activity of levofloxacin against Helicobacter pylori isolates from patients after treatment failure. *Diagn Microbiol Infect Dis* 2006; **55**: 81-83
100 **Matsumoto Y**, Miki I, Aoyama N, Shirasaka D, Watanabe

Y, Morita Y, Toyoda M, Mitani T, Miyachi H, Tamura T, Kasuga M. Levofloxacin- versus metronidazole-based rescue therapy for H. pylori infection in Japan. *Dig Liver Dis* 2005; **37**: 821-825

101 **Cammarota G**, Cianci R, Cannizzaro O, Cuoco L, Pirozzi G, Gasbarrini A, Armuzzi A, Zocco MA, Santarelli L, Arancio F, Gasbarrini G. Efficacy of two one-week rabeprazole/levofloxacin-based triple therapies for Helicobacter pylori infection. *Aliment Pharmacol Ther* 2000; **14**: 1339-1343

102 **Di Caro S**, Zocco MA, Cremonini F, Candelli M, Nista EC, Bartolozzi F, Armuzzi A, Cammarota G, Santarelli L, Gasbarrini A. Levofloxacin based regimens for the eradication of Helicobacter pylori. *Eur J Gastroenterol Hepatol* 2002; **14**: 1309-1312

103 **Marzio L**, Coraggio D, Capodicasa S, Grossi L, Cappello G. Role of the preliminary susceptibility testing for initial and after failed therapy of Helicobacter pylori infection with levofloxacin, amoxicillin, and esomeprazole. *Helicobacter* 2006; **11**: 237-242

104 **Rispo A**, Di Girolamo E, Cozzolino A, Bozzi R, Morante A, Pasquale L. Levofloxacin in first-line treatment of Helicobacter pylori infection. *Helicobacter* 2007; **12**: 364-365

105 **Gisbert JP**, Fernandez-Bermejo M, Molina-Infante J, Perez-Gallardo B, Prieto-Bermejo AB, Mateos-Rodriguez JM, Robledo-Andres P, Gonzalez-Garcia G. First-line triple therapy with levofloxacin for Helicobacter pylori eradication. *Aliment Pharmacol Ther* 2007; **26**: 495-500

106 **Lee JH**, Hong SP, Kwon CI, Phyun LH, Lee BS, Song HU, Ko KH, Hwang SG, Park PW, Rim KS, Kim S. [The efficacy of levofloxacin based triple therapy for Helicobacter pylori eradication] *Korean J Gastroenterol* 2006; **48**: 19-24

107 **Wong WM**, Gu Q, Chu KM, Yee YK, Fung FM, Tong TS, Chan AO, Lai KC, Chan CK, Wong BC. Lansoprazole, levofloxacin and amoxicillin triple therapy vs. quadruple therapy as second-line treatment of resistant Helicobacter pylori infection. *Aliment Pharmacol Ther* 2006; **23**: 421-427

108 **Gisbert JP**, Bermejo F, Castro-Fernandez M, Perez-Aisa A, Fernandez-Bermejo M, Tomas A, Barrio J, Bory F, Almela P, Sanchez-Pobre P, Cosme A, Ortiz V, Nino P, Khorrami S, Benito LM, Carneros JA, Lamas E, Modolell I, Franco A, Ortuno J, Rodrigo L, Garcia-Duran F, O'Callaghan E, Ponce J, Valer MP, Calvet X. Second-line rescue therapy with levofloxacin after H. pylori treatment failure: a Spanish multicenter study of 300 patients. *Am J Gastroenterol* 2008; **103**: 71-76

109 **Giannini EG**, Bilardi C, Dulbecco P, Mamone M, Santi ML, Testa R, Mansi C, Savarino V. A study of 4- and 7-day triple therapy with rabeprazole, high-dose levofloxacin and tinidazole rescue treatment for Helicobacter pylori eradication. *Aliment Pharmacol Ther* 2006; **23**: 281-287

110 **Page RL 2nd**, Ferguson D, Cantu M. An alternative salvage regimen for Helicobacter pylori-resistant patients with heart failure. *Cardiology* 2008; **110**: 112-115

111 **Lee YC**, Wu HM, Chen TH, Liu TY, Chiu HM, Chang CC, Wang HP, Wu MS, Chiang H, Wu MC, Lin JT. A community-based study of Helicobacter pylori therapy using the strategy of test, treat, retest, and re-treat initial treatment failures. *Helicobacter* 2006; **11**: 418-424

112 **Kahn JB**. Latest industry information on the safety profile of levofloxacin in the US. *Chemotherapy* 2001; **47** Suppl 3: 32-37; discussion 44-48

113 **Cabrita J**, Oleastro M, Matos R, Manhente A, Cabral J, Barros R, Lopes AI, Ramalho P, Neves BC, Guerreiro AS. Features and trends in Helicobacter pylori antibiotic resistance in Lisbon area, Portugal (1990-1999). *J Antimicrob Chemother* 2000; **46**: 1029-1031

114 **Miyachi H**, Miki I, Aoyama N, Shirasaka D, Matsumoto Y, Toyoda M, Mitani T, Morita Y, Tamura T, Kinoshita S, Okano Y, Kumagai S, Kasuga M. Primary levofloxacin resistance and gyrA/B mutations among Helicobacter pylori in Japan. *Helicobacter* 2006; **11**: 243-249

115 **Bogaerts P**, Berhin C, Nizet H, Glupczynski Y. Prevalence and mechanisms of resistance to fluoroquinolones in Helicobacter pylori strains from patients living in Belgium. *Helicobacter* 2006; **11**: 441-445

116 **Carothers JJ**, Bruce MG, Hennessy TW, Bensler M, Morris JM, Reasonover AL, Hurlburt DA, Parkinson AJ, Coleman JM, McMahon BJ. The relationship between previous fluoroquinolone use and levofloxacin resistance in Helicobacter pylori infection. *Clin Infect Dis* 2007; **44**: e5-e8

117 **Cattoir V**, Nectoux J, Lascols C, Deforges L, Delchier JC, Megraud F, Soussy CJ, Cambau E. Update on fluoroquinolone resistance in Helicobacter pylori: new mutations leading to resistance and first description of a gyrA polymorphism associated with hypersusceptibility. *Int J Antimicrob Agents* 2007; **29**: 389-396

118 **Coelho LG**, Moretzsohn LD, Vieira WL, Gallo MA, Passos MC, Cindr JM, Cerqueira MC, Vitiello L, Ribeiro ML, Mendonca S, Pedrazzoli-Junior J, Castro LP. New once-daily, highly effective rescue triple therapy after multiple Helicobacter pylori treatment failures: a pilot study. *Aliment Pharmacol Ther* 2005; **21**: 783-787

119 **Kim JM**, Kim JS, Kim N, Kim SG, Jung HC, Song IS. Comparison of primary and secondary antimicrobial minimum inhibitory concentrations for Helicobacter pylori isolated from Korean patients. *Int J Antimicrob Agents* 2006; **28**: 6-13

120 **Glocker E**, Stueger HP, Kist M. Quinolone resistance in Helicobacter pylori isolates in Germany. *Antimicrob Agents Chemother* 2007; **51**: 346-349

121 **Perna F**, Zullo A, Ricci C, Hassan C, Morini S, Vaira D. Levofloxacin-based triple therapy for Helicobacter pylori re-treatment: role of bacterial resistance. *Dig Liver Dis* 2007; **39**: 1001-1005

122 **Cammarota G**, Martino A, Pirozzi G, Cianci R, Branca G, Nista EC, Cazzato A, Cannizzaro O, Miele L, Grieco A, Gasbarrini A, Gasbarrini G. High efficacy of 1-week doxycycline- and amoxicillin-based quadruple regimen in a culture-guided, third-line treatment approach for Helicobacter pylori infection. *Aliment Pharmacol Ther* 2004; **19**: 789-795

123 **Zullo A**, Perna F, Hassan C, Ricci C, Saracino I, Morini S, Vaira D. Primary antibiotic resistance in Helicobacter pylori strains isolated in northern and central Italy. *Aliment Pharmacol Ther* 2007; **25**: 1429-1434

124 **Krakowka S**, Ringler SS, Eaton KA, Green WB, Leunk R. Manifestations of the local gastric immune response in gnotobiotic piglets infected with Helicobacter pylori. *Vet Immunol Immunopathol* 1996; **52**: 159-173

125 **Reilly TG**, Ayres RC, Poxon V, Walt RP. Helicobacter pylori eradication in a clinical setting: success rates and the effect on the quality of life in peptic ulcer. *Aliment Pharmacol Ther* 1995; **9**: 483-490

126 **Lerang F**, Moum B, Haug JB, Berge T, Tolas P, Sandvei PK, Torp R, Tonnesen T. Highly effective second-line anti-Helicobacter pylori therapy in patients with previously failed metronidazole-based therapy. *Scand J Gastroenterol* 1997; **32**: 1209-1214

127 **Magaret N**, Burm M, Faigel D, Kelly C, Peterson W, Fennerty MB. A randomized trial of lansoprazole, amoxycillin, and clarithromycin versus lansoprazole, bismuth, metronidazole and tetracycline in the retreatment of patients failing initial Helicobacter pylori therapy. *Dig Dis* 2001; **19**: 174-178

128 **Axon AT**. Treatment of Helicobacter pylori: an overview. *Aliment Pharmacol Ther* 2000; **14** Suppl 3: 1-6

129 **Peitz U**, Sulliga M, Wolle K, Leodolter A, Von Arnim U, Kahl S, Stolte M, Borsch G, Labenz J, Malfertheiner P. High rate of post-therapeutic resistance after failure of macrolide-nitroimidazole triple therapy to cure Helicobacter pylori infection: impact of two second-line therapies in a randomized study. *Aliment Pharmacol Ther* 2002; **16**: 315-324

130 **Miehlke S**, Hansky K, Schneider-Brachert W, Kirsch C, Morgner A, Madisch A, Kuhlisch E, Bastlein E, Jacobs E,

Bayerdorffer E, Lehn N, Stolte M. Randomized trial of rifabutin-based triple therapy and high-dose dual therapy for rescue treatment of Helicobacter pylori resistant to both metronidazole and clarithromycin. *Aliment Pharmacol Ther* 2006; **24**: 395-403

131 **Pilotto A**, Franceschi M, Rassu M, Leandro G, Bozzola L, Furlan F, Di Mario F. Incidence of secondary Helicobacter pylori resistance to antibiotics in treatment failures after 1-week proton pump inhibitor-based triple therapies: a prospective study. *Dig Liver Dis* 2000; **32**: 667-672

132 **de Boer W**, Driessen W, Jansz A, Tytgat G. Effect of acid suppression on efficacy of treatment for Helicobacter pylori infection. *Lancet* 1995; **345**: 817-820

133 **Graham DY**, Osato MS, Hoffman J, Opekun AR, Anderson SY, Kwon DH, El-Zimaity HM. Metronidazole containing quadruple therapy for infection with metronidazole resistant Helicobacter pylori: a prospective study. *Aliment Pharmacol Ther* 2000; **14**: 745-750

134 **Cudia B**, Romano M, Gioè FP, Barbera N, Lo Gerfo D, Montalto R. "Rescue" therapy including ranitidine bismuth citrate + minocicline + amoxicillin for eradication of Helicobacter pylori in previous H.P. treatment failure. *Gut* 1997; **41** suppl 1: A103

135 **The report of the Digestive Health InitiativeSM International Update Conference on Helicobacter pylori**. *Gastroenterology* 1997; 113: S4-S8

136 **Technical annex: tests used to assess Helicobacter pylori infection**. Working Party of the European Helicobacter pylori Study Group. *Gut* 1997; **41** Suppl 2: S10-S18

137 **Guslandi M**. Review article: alternative antibacterial agents for Helicobacter pylori eradication. *Aliment Pharmacol Ther* 2001; **15:** 1543-1547

138 **Kim JJ**, Kim JG, Kwon DH. Mixed-infection of antibiotic susceptible and resistant Helicobacter pylori isolates in a single patient and underestimation of antimicrobial susceptibility testing. *Helicobacter* 2003; **8**: 202-206

139 **Ali' A**, Menegatti M, Gatta L, Landi F, Ricci C, Acciardi C, Miglioli M, Vaira D, Holton J. A second-line anti-Helicobacter pylori therapy in patients with previously failed treatment. *Am J Gastroenterol* 1999; **94**: 2321-2323

140 **Gomollón F**, Sicilia B, Ducons JA, Sierra E, Revillo MJ, Ferrero M. Third line treatment for Helicobacter pylori: a prospective, culture-guided study in peptic ulcer patients. *Aliment Pharmacol Ther* 2000; **14**: 1335-1338

141 **Vicente R**, Sicilia B, Gallego S, Revillo MJ, Ducons J, Gomollon F. [Helicobacter pylori eradication in patients with peptic ulcer after two treatment failures: a prospective culture-guided study] *Gastroenterol Hepatol* 2002; **25**: 438-442

142 **Adamek RJ**, Suerbaum S, Pfaffenbach B, Opferkuch W. Primary and acquired Helicobacter pylori resistance to clarithromycin, metronidazole, and amoxicillin--influence on treatment outcome. *Am J Gastroenterol* 1998; **93**: 386-389

143 **Huang JQ**, Hunt RH. Treatment after failure: the problem of "non-responders". *Gut* 1999; **45** Suppl 1: I40-I44

144 **Seppala K**, Kosunen TU, Nuutinen H, Sipponen P, Rautelin H, Sarna S, Hyvarinen H, Farkkila M, Miettinen TA. Cure of Helicobacter pylori infection after failed primary treatment: one-center results from 120 patients. *Scand J Gastroenterol* 2000; **35**: 929-934

145 **Treiber G**, Ammon S, Malfertheiner P, Klotz U. Impact of furazolidone-based quadruple therapy for eradication of Helicobacter pylori after previous treatment failures. *Helicobacter* 2002; **7**: 225-231

146 **Chan FK**, Sung JJ, Suen R, Wu JC, Ling TK, Chung SC. Salvage therapies after failure of Helicobacter pylori eradication with ranitidine bismuth citrate-based therapies. *Aliment Pharmacol Ther* 2000; **14**: 91-95

147 **Zullo A**, Hassan C, De Francesco V, Lorenzetti R, Marignani M, Angeletti S, Ierardi E, Morini S. A third-line levofloxacin-based rescue therapy for Helicobacter pylori eradication. *Dig Liver Dis* 2003; **35**: 232-236

148 **Dore MP**, Marras L, Maragkoudakis E, Nieddu S, Manca A, Graham DY, Realdi G. Salvage therapy after two or more prior Helicobacter pylori treatment failures: the super salvage regimen. *Helicobacter* 2003; **8**: 307-309

149 **Gisbert JP**, Calvet X, Bujanda L, Marcos S, Gisbert JL, Pajares JM. 'Rescue' therapy with rifabutin after multiple Helicobacter pylori treatment failures. *Helicobacter* 2003; **8**: 90-94

150 **Gisbert JP**, Gisbert JL, Marcos S, Pajares JM. Empirical Helicobacter pylori "rescue" therapy after failure of two eradication treatments. *Dig Liver Dis* 2004; **36**: 7-12

151 **Perri F**, Festa V, Clemente R, Quitadamo M, Andriulli A. Rifabutin-based 'rescue therapy' for Helicobacter pylori infected patients after failure of standard regimens. *Aliment Pharmacol Ther* 2000; **14**: 311-316

152 **Bock H**, Koop H, Lehn N, Heep M. Rifabutin-based triple therapy after failure of Helicobacter pylori eradication treatment: preliminary experience. *J Clin Gastroenterol* 2000; **31**: 222-225

153 **Canducci F**, Ojetti V, Pola P, Gasbarrini G, Gasbarrini A. Rifabutin-based Helicobacter pylori eradication 'rescue therapy'. *Aliment Pharmacol Ther* 2001; **15**: 143

154 **Gisbert JP**, Castro-Fernandez M, Bermejo F, Perez-Aisa A, Ducons J, Fernandez-Bermejo M, Bory F, Cosme A, Benito LM, Lopez-Rivas L, Lamas E, Pabon M, Olivares D. Third-line rescue therapy with levofloxacin after two H. pylori treatment failures. *Am J Gastroenterol* 2006; **101**: 243-247

155 **Gisbert JP**, Gisbert JL, Marcos S, Moreno-Otero R, Pajares JM. Third-line rescue therapy with levofloxacin is more effective than rifabutin rescue regimen after two Helicobacter pylori treatment failures. *Aliment Pharmacol Ther* 2006; **24**: 1469-1474

156 **Gisbert JP**, Gisbert JL, Marcos S, Jimenez-Alonso I, Moreno-Otero R, Pajares JM. Empirical rescue therapy after Helicobacter pylori treatment failure: a 10-year single-centre study of 500 patients. *Aliment Pharmacol Ther* 2008; **27**: 346-354

157 **Cianci R**, Montalto M, Pandolfi F, Gasbarrini GB, Cammarota G. Third-line rescue therapy for Helicobacter pylori infection. *World J Gastroenterol* 2006; **12**: 2313-2319

158 **Megraud F**, Lamouliatte H. Review article: the treatment of refractory Helicobacter pylori infection. *Aliment Pharmacol Ther* 2003; **17**: 1333-1343

159 **de Boer WA**, Borody TJ. Treatment failures and secondary resistance to antibiotics. A growing concern in Helicobacter pylori therapy. *Dig Liver Dis* 2000; **32**: 673-675

160 **Miehlke S**, Kirsch C, Schneider-Brachert W, Haferland C, Neumeyer M, Bastlein E, Papke J, Jacobs E, Vieth M, Stolte M, Lehn N, Bayerdorffer E. A prospective, randomized study of quadruple therapy and high-dose dual therapy for treatment of Helicobacter pylori resistant to both metronidazole and clarithromycin. *Helicobacter* 2003; **8**: 310-319

161 **Chi CH**, Lin CY, Sheu BS, Yang HB, Huang AH, Wu JJ. Quadruple therapy containing amoxicillin and tetracycline is an effective regimen to rescue failed triple therapy by overcoming the antimicrobial resistance of Helicobacter pylori. *Aliment Pharmacol Ther* 2003; **18**: 347-353

162 **Zullo A**, Hassan C, Lorenzetti R, Morini S. Helicobacter pylori eradication: do we have another ace up our sleeve? *Dig Liver Dis* 2001; **33**: 805-806

163 **Gisbert JP**. Rescue therapy with levofloxacin after multiple H. pylori treatment failures. *Aliment Pharmacol Ther* 2005; **22**: 653-654; author reply 654-655

164 **Rokkas T**, Sechopoulos P, Robotis J, Pistiolas D. Triple levofloxacin-based rescue therapy is an accepted empirical third-line treatment. *Am J Gastroenterol* 2006; **101**: 1938; author reply 1938-1938; author reply 1939

165 **Akada JK**, Shirai M, Fujii K, Okita K, Nakazawa T. In vitro anti-Helicobacter pylori activities of new rifamycin derivatives, KRM-1648 and KRM-1657. *Antimicrob Agents Chemother* 1999; **43**: 1072-1076

166 **Heep M**, Beck D, Bayerdorffer E, Lehn N. Rifampin and rifabutin resistance mechanism in Helicobacter pylori. *Antimicrob Agents Chemother* 1999; **43**: 1497-1499

167 **Brogden RN**, Fitton A. Rifabutin. A review of its antimicrobial activity, pharmacokinetic properties and therapeutic efficacy. *Drugs* 1994; **47**: 983-1009

168 **Rossi G**. [An update on the antibiotic therapy of tuberculosis] *Recenti Prog Med* 1999; **90**: 241-243

169 **Borody TJ**, Pang G, Wettstein AR, Clancy R, Herdman K, Surace R, Llorente R, Ng C. Efficacy and safety of rifabutin-containing 'rescue therapy' for resistant Helicobacter pylori infection. *Aliment Pharmacol Ther* 2006; **23**: 481-488

170 **Gonzalez Carro P**, Perez Roldan F, De Pedro Esteban A, Legaz Huidobro ML, Soto Fernandez S, Roncero Garcia Escribano O, Esteban Lopez-Jamar JM, Pedraza Martin C, Ruiz Carrillo F. Efficacy of rifabutin-based triple therapy in Helicobacter pylori infected patients after two standard treatments. *J Gastroenterol Hepatol* 2007; **22**: 60-63

171 **Toracchio S**, Capodicasa S, Soraja DB, Cellini L, Marzio L. Rifabutin based triple therapy for eradication of H. pylori primary and secondary resistant to tinidazole and clarithromycin. *Dig Liver Dis* 2005; **37**: 33-38

172 **Van der Poorten D**, Katelaris PH. The effectiveness of rifabutin triple therapy for patients with difficult-to-eradicate Helicobacter pylori in clinical practice. *Aliment Pharmacol Ther* 2007; **26**: 1537-1542

173 **Perri F**, Festa V, Andriulli A. Treatment of antibiotic-resistant Helicobacter pylori. *N Engl J Med* 1998; **339**: 53

174 **Moayyedi P**, Soo S, Deeks J, Delaney B, Harris A, Innes M, Oakes R, Wilson S, Roalfe A, Bennett C, Forman D. Eradication of Helicobacter pylori for non-ulcer dyspepsia. *Cochrane Database Syst Rev* 2006; CD002096

175 **Xiao SD**, Liu WZ, Xia DH, Jiang SJ, Wang RN, Zhang ZH, Xu WW. The efficacy of furazolidone and metronidazole in the treatment of chronic gastritis associated with Helicobacter (Campylobacter) pylori--a randomized double-blind placebo-controlled clinical trial. *Hepatogastroenterology* 1990; **37**: 503-506

176 **Haas CE**, Nix DE, Schentag JJ. In vitro selection of resistant Helicobacter pylori. *Antimicrob Agents Chemother* 1990; **34**: 1637-1641

177 **Kwon DH**, Lee M, Kim JJ, Kim JG, El-Zaatari FA, Osato MS, Graham DY. Furazolidone- and nitrofurantoin-resistant Helicobacter pylori: prevalence and role of genes involved in metronidazole resistance. *Antimicrob Agents Chemother* 2001; **45**: 306-308

178 **Treiber G**, Wittig J, Ammon S, Walker S, van Doorn LJ, Klotz U. Clinical outcome and influencing factors of a new short-term quadruple therapy for Helicobacter pylori eradication: a randomized controlled trial (MACLOR study). *Arch Intern Med* 2002; **162**: 153-160

179 **Xiao SD**, Liu WZ, Hu PJ, Ouyang Q, Wang JL, Zhou LY, Cheng NN. A multicentre study on eradication of Helicobacter pylori using four 1-week triple therapies in China. *Aliment Pharmacol Ther* 2001; **15**: 81-86

180 **Nijevitch AA**, Shcherbakov PL, Sataev VU, Khasanov RSh, Al Khashash R, Tuygunov MM. Helicobacter pylori eradication in childhood after failure of initial treatment: advantage of quadruple therapy with nifuratel to furazolidone. *Aliment Pharmacol Ther* 2005; **22**: 881-887

181 **Eisig JN**, Silva FM, Rodriguez TN, Hashimoto CL, Barbuti RC. A furazolidone-based quadruple therapy for Helicobacter pylori retreatment in patients with peptic ulcer disease. *Clinics* 2005; **60**: 485-488

182 **Ebrahimi-Dariani N**, Mirmomen S, Mansour-Ghanaei F, Noormohammadpoor P, Sotodehmanesh R, Haghpanah B, Bahrami H. The efficacy of furazolidone-based quadruple therapy for eradication of Helicobacter pylori infection in Iranian patients resistant to metronidazole-based quadruple therapy. *Med Sci Monit* 2003; **9**: PI105-PI108

183 **Isakov V**, Domareva I, Koudryavtseva L, Maev I, Ganskaya Z. Furazolidone-based triple 'rescue therapy' vs. quadruple 'rescue therapy' for the eradication of Helicobacter pylori resistant to metronidazole. *Aliment Pharmacol Ther* 2002; **16**: 1277-1282

184 **Sotoudehmanesh R**, Malekzadeh R, Vahedi H, Dariani NE, Asgari AA, Massarrat S. Second-line Helicobacter pylori eradication with a furazolidone-based regimen in patients who have failed a metronidazole-based regimen. *Digestion* 2001; **64**: 222-225

185 **Qasim A**, Sebastian S, Thornton O, Dobson M, McLoughlin R, Buckley M, O'Connor H, O'Morain C. Rifabutin- and furazolidone-based Helicobacter pylori eradication therapies after failure of standard first- and second-line eradication attempts in dyspepsia patients. *Aliment Pharmacol Ther* 2005; **21**: 91-96

186 **Buzas GM**, Jozan J. Nitrofuran-based regimens for the eradication of Helicobacter pylori infection. *J Gastroenterol Hepatol* 2007; **22**: 1571-1581

187 **Laine L**, Schoenfeld P, Fennerty MB. Therapy for Helicobacter pylori in patients with nonulcer dyspepsia. A meta-analysis of randomized, controlled trials. *Ann Intern Med* 2001; **134**: 361-369

188 **Gasbarrini A**, Ojetti V, Armuzzi A, Branca G, Canducci F, Torre ES, Candelli M, Pastorelli A, Anti M, Fedeli G, Fadda G, Pola P, Gasbarrini G. Efficacy of a multistep strategy for Helicobacter pylori eradication. *Aliment Pharmacol Ther* 2000; **14**: 79-83

189 **Tucci A**, Poli L, Caletti G. Treatment of the "ineradicable" Helicobacter pylori infection. *Am J Gastroenterol* 1999; **94**: 1713-1715

190 **Gisbert JP**. [The attitude in a failure to eradicate Helicobacter pylori: surrender or fight?] *Med Clin* (Barc) 1998; **111**: 778-782

S- Editor Zhong XY **L- Editor** Lutze M **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5403

World J Gastroenterol 2008 September 21; 14(35): 5403-5411
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Anti-tumor activity of erlotinib in the BxPC-3 pancreatic cancer cell line

Ying-Ying Lu, Da-Dao Jing, Ming Xu, Kai Wu, Xing-Peng Wang

Ying-Ying Lu, Da-Dao Jing, Kai Wu, Xing-Peng Wang, Department of Gastroenterology, Affiliated First People's Hospital, Shanghai Jiao Tong University, Shanghai 200080, China
Ming Xu, Department of Gastroenterology, Wuxi Second People's Hospital, Wuxi 214002, Jiangsu Province, China
Author contributions: Lu YY and Jing DD contributed equally to this work; Jing DD and Wang XP designed the research; Lu YY, Xu M and Wu K performed the research; Lu YY analyzed the data and wrote the paper.
Correspondence to: Dr. Da-Dao Jing, Department of Gastroenterology, Shanghai First People's Hospital, Shanghai Jiao Tong University, 100 Haining RD, Hongkou District, Shanghai 200080, China. jingdadao@medmail.com.cn
Telephone: +86-21-63240090-3141 Fax: +86-21-66302029
Received: April 26, 2008 Revised: June 23, 2008
Accepted: June 30, 2008
Published online: September 21, 2008

Abstract

AIM: To investigate the effect and mechanism of action of erlotinib, an epidermal growth factor receptor (EGFR) small molecule tyrosine kinase inhibitor (TKI), in the human pancreatic cancer cell line BxPC-3 both *in vitro* and *in vivo*.

METHODS: *In vitro*, human pancreatic cancer cell line BxPC-3 was exposed to varying concentrations of erlotinib, and its effects on proliferation, cell cycle distribution, apoptosis and the expression of pro- and antiapoptotic factors such as bcl-2, bcl-xl, bax and bak, and the expression of vascular endothelial cell growth factor (VEGF) were measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, flow cytometric analysis, terminal deoxynucleotidyl transferase-mediated nick end labeling assay (TUNEL), and reverse transcription-polymerase chain reaction (RT-PCR). Potential effect of erlotinib on angiogenesis was examined by tube formation assay. Tumor growth suppression was observed in xenografted nude mice with pancreatic cancer *in vivo*. Immunohistochemical (IHC) staining for EGFR and factor Ⅷ-related antigen was undertaken to detect the microvessel density and VEGF expression in tumor tissue in xenograft nude mice.

RESULTS: Erlotinib, as a single agent, repressed BxPC-3 cell growth in a dose-dependent manner, triggered G_1 arrest and induced cell apoptosis, and suppressed capillary formation of endothelium *in vitro*. Expressions of VEGF were significantly down-regulated at a high concentration of 200 μmol/L, however, the expressions of bcl-2 and bcl-xl were decreased at 50 μmol/L. *In vivo*, Erlotinib-treated mice demonstrated a reduced tumor volume, weight and microvessel density as compared to the control. IHC staining showed decreased expression of EGFR and RT-PCR had lower VEGF expression in treated mice.

CONCLUSION: The *in vitro* and *in vivo* findings provide evidence that BxPC-3 cells are inhibited with erlotinib treatment. Inhibition of EGFR may be a promising adjuvant chemotherapy strategy in pancreatic cancer treatment.

Key words: Pancreatic cancer; Erlotinib; Epidermal growth factor receptor; Human xenograft model; Angiogenesis

Peer reviewers: Francesco Feo, Professor, Dipartimento di Scienze Biomediche, Sezione di Patologia Sperimentale e Oncologia, Università di Sassari, Via P, Manzella 4, Sassari 07100, Italy; Sharon DeMorrow, Division of Research and Education, Scott and White Hospital and The Texas A&M University System, Health Science Center College of Medicine, Temple, Texas 76504, United States

Lu YY, Jing DD, Xu M, Wu K, Wang XP. Anti-tumor activity of erlotinib in the BxPC-3 pancreatic cancer cell line. *World J Gastroenterol* 2008; 14(35): 5403-5411 Available from: URL: http://www.wjgnet.com/1007-9327/14/5403.asp DOI: http://dx.doi.org/10.3748/wjg.14.5403

INTRODUCTION

Pancreatic cancer is one of the most lethal human cancers and continues to be a major unsolved health problem[1,2]. Recently, several orally bioavailable compounds aimed at specific molecular targets have been developed in hopes of improving survival in this dismal disease. Tumor development and progression depend on cellular changes like overexpression of oncogenic tyrosine kinase receptors. Many gastrointestinal tumors, including pancreatic cancer, have been shown to overexpress the epidermal growth factor receptor (EGFR)[3,4]. The overexpression of the EGFR and its ligands correlates with rapidly progressive disease and resistance to chemotherapy.

EGFR is a 170-kDa transmembrane protein with intrinsic tyrosine kinase activity. Stimulation of the EGFR results in activation of multiple intracellular signaling cascades that increase cellular proliferation and prevent programmed cell death[5]. Multiple therapeutic strategies designed to manipulate this receptor have been developed, including specific antibodies and low molecular EGFR tyrosine kinase inhibitors (TKIs). Erlotinib is a small molecule TKI that efficiently blocks EGFR. Preliminary results of a phase Ⅲ trial of gemcitabine with or without erlotinib in pancreatic cancer revealed a modest improvement in survival with the addition of erlotinib. Treatment with anti-EGFR agents is used as a potential therapeutic strategy for pancreatic cancer, but the mechanisms are not yet precisely understood.

The aim of this study was to investigate the growth inhibitory effects of erlotinib in pancreatic cancer cells *in vitro* and *in vivo*, to determine the mechanisms involved and to examine the effects of erlotinib on the regulation of angiogenesis.

MATERIALS AND METHODS

Cell culture and reagents

Human pancreatic cancer cell lines BxPC-3, obtained from Shanghai Institute of Biochemistry and Cell Biology, and ECV 304, a cell line derived from human umbilical vein endothelial cells from ATCC were maintained in RPMI-1640 (Gibco) medium supplemented with 10% fetal calf serum in a humidified atmosphere containing 5% CO_2 at 37℃. The EGFR-selective TKI erlotinib was provided by DeBioChem (Nanjing, China). The agent was dissolved in DMSO (Sigma) or carboxymethylcellulose sodium at appropriate concentrations for *in vitro* or *in vivo* studies, respectively.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was extracted with TRIzol (Life Technologies, INC) following the manufacturer's instructions. Reverse transcription was performed starting with 2 μg of total RNA, using oligo (dT) primer and other reagents, and procedures contained in the MMulv RT-PCR kit (Promega) to form cDNA. cDNA (2 μL), 2 μL of 50 pmol/L of each primer, 10 mmol/L dNTP Mix 1 μL, 1 μL of Taq DNA polymerase (Sangon, China) were used for PCR analysis. The PCR amplification cycles consisted of denaturation at 94℃ for 5 min, 35 cycles of denaturation at 94℃ for 60 s, annealing [54℃ for *bcl-2*, *bcl-xl*, *bax*, *bak*, vascular endothelial cell growth factor (VEGF), 56℃ for GAPDH] for 60 s, extension at 72℃ for 60 s, and a final elongation at 72℃ for 10 min. The PCR products were separated on a 1.5% agarose gel, stained with 0.5 mg/mL ethidium bromide, and visualized by UV light. The primer sequences are listed in Table 1.

Tube formation assay

A well established method was used for the process of *in vitro* angiogenesis assay[6] with a kit from Chemicon (Temecula, California, USA). A 96-well tissue culture plate was coated with Matrigel (50 μL/well). After matrix solution gelled, ECV304 cells were premixed with RPMI-1640 (control), erlotinib (100 μmol/L) and then seed at a concentration of 1×10^4 per well onto the surface of the polymerized gel. Four wells were used for each treatment. After 18 h of incubation at 37℃ and 5% CO_2, the status of capillary tube formation by ECV304 cells was recorded using a CCD camera attached to an inverted light microscope (40 × objective lens).

Cell viability assay

The viability of BxPC-3 cells treated with erlotinib was determined by the standard 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. BxPC-3 cells were plated (5×10^3 per well) in 96-well plates and incubated overnight at 37℃. Erlotinib was dissolved in DMSO and added to the cell culture medium at a concentration not exceeding 0.1% (v/v). The effects of erlotinib on cell proliferation were studied at various concentrations (0, 1, 5, 10, 50 and 100 μmol/L) and at different time points (24, 48, 72 and 96 h) with a certain concentration (50 μmol/L). The MTT assay was done in quadruplicate for each drug concentration used. At appropriate intervals, 100 μg MTT solution was added to each well and incubated for 4 h at 37℃, 5% CO_2. The supernatant was removed, and 150 μL of DMSO was then added. Plates were then read at 490 nm wavelength using a microplate reader (BIO-RAD550, USA). Percentage of inhibition was determined by comparing the cell density in the drug-treated cells with that in the untreated cell controls in the same incubation period [percentage of inhibition = (1-cell density of a treated group)/cell density of the control group]. All experiments were repeated three times.

Cell cycle analysis and apoptosis assays

The effects of EGFR TKIs erlotinib on both cell cycle and apoptosis in BxPC-3 cells were analyzed using flow cytometry. Cells were plated into 12-well plates and the following day, erlotinib (50 μmol/L) was added and kept for 48 h. Cell floating in the medium combined with adherent layer were trypsinized and fixed with 2 mL of Citrate buffer for 1 h. Cells were then incubated with RNase A (1500 μL) and stained with propidium iodide (1500 μL). Samples were immediately analyzed by flow cytometry for cell cycle and apoptosis assays. Immunocytochemical (ICC) detection of apoptotic cells was carried out with terminal deoxynucleotidyl transferase-mediated nick end labeling assay (TUNEL), in which residues of digoxigenin-labeled dUTP were catalytically incorporated into the DNA by terminal deoxynucleotidyl transferase Ⅱ. After treatment with erlotinib (50 μmol/L) for 48 h, slides were fixed and washed thrice in 0.01 mol/L PBS, the following procedures were performed according to the manufacturer instructions (Boster, Wuhan, China). The positive particles of DAB staining were viewed under microscope (Olympus Japan). The number of apoptotic cells was viewed and counted

Table 1 PCR primers

Target genes		Primer sequence	Size (bp)	Annealing temperature (℃)	Cycles
Bcl-2	Sense	5'-GGTGCCACCTGTGGTCCACCT-3'	458	54	35
	Antisense	5'-CCTCACTTGTGGCCCAGATAGG-3'			
Bax	Sense	5'-CTGACATGTTTTCTGACGGC-3'	289	54	35
	Antisense	5'-TCAGCCCATCTTCTTCCAGA-3'			
Bcl-xl	Sense	5'-TTGGACAATGGACTGGTTG-3'	765	54	35
	Antisense	5'-GTAGAGTGGATGGTCAGTG-3'			
Bak	Sense	5'-TGAAAAATGGCTTCGGGGCAAGGC-3'	642	54	35
	Antisense	5'-TCATGATTTGAAGAATCTTCGTACC-3'			
GAPDH	Sense	5'-CATGCCAGTGAGCTTCCCGTT-3'	408	56	35
	Antisense	5'-GTGGAGTCTACTGGCGTCTTC-3'			
VEGF	Sense	5'-ATGAACTTTCTGCTGTCTTG-3'	382	54	35
	Antisense	5'-TGCATGGTGATGTTGGAC-3'			

under microscope (40 × objective lens, Olympus Japan) and expressed as the Apoptotic Index (AI = number of apoptotic body/1000 cells).

Development of nude mice xenografts of pancreatic cancer

BALB/C nu/nu female mice, aged 4-6 wk, weighing about 20 g, were maintained pathogen free at the Shanghai Experimental Animals Centre of Chinese Academy of Sciences. BxPC-3 cells (1×10^7, suspended in 200 μL of PBS) were implanted *s.c.* in the hind flank of each mouse. Once palpable tumors were established, animals were randomly divided into two groups so that all groups had similar starting mean tumor volumes of 100-150 mm^3. The mice in each group were orally gavaged with the vehicle control (0.5% CMC-Na, $n = 6$) and erlotinib (100 mg/kg, $n = 6$) for 4 wk. The tumor size was measured with a linear caliper twice a week up to 4 wk, and the volume was estimated using the equation $V = (a \times b^2)/2$, where a is the large dimension and b the perpendicular diameter. After all the mice were sacrificed, part of the tissue was fixed in formalin and embedded in paraffin, and some parts were frozen in liquid nitrogen. Hematoxylin and eosin staining confirmed the presence of tumors. Total mRNA was prepared and RT-PCR analyses were performed as described previously.

Immunohistochemistry (IHC) of tumor xenografts

To assess EGFR expression and microvessel density (MVD) in xenograft tumors, rabbit polyclonal anti-EGFR antibody (diluted to 1:100, Boster, Wuhan) and rabbit polyclonal factor Ⅷ antibody (Boster, Wuhan) were used in IHC. Paraffin-embedded tissue sections (4 μm) were dried, deparaffinized, and rehydrated. Endogenous peroxidase was blocked with 3% hydrogenperoxide in ion free water for 30 min. After nonspecific binding sites were blocked with 10% goat serum, slides were incubated at 4℃ overnight with 1:100 dilution of primary antibody directed against EGFR and factor VIII followed by a 30-min incubation in a Horseradish peroxidase (HRP)-conjugated sheep anti-rabbit IgG secondary antibody. Sections were rinsed with PBS and developed with the DAB kit (Boster, Wuhan) and then counterstained with haematoxylin. Each slide was scanned at a low power (× 100) and the area with a higher number of new vessels was identified (hotspot). This region was then scanned at × 400 magnification. For individual tumors, microvessel count was scored by averaging the five field counts[7].

Statistical analysis

Quantitative results were expressed as mean ± SEM. Statistical analysis was performed using a two-tailed unpaired *t* test (between two groups) or a one way analysis of variance (ANOVA) (among three or more groups) with the computer software SAS 8.02. $P < 0.05$ was considered statistically significant.

RESULTS

Growth inhibition of BxPC-3 pancreatic cancer cells by erlotinib

Overall cell growth of BxPC-3 pancreatic cancer cells treated with erlotinib at different concentrations ranging from 1 to 100 μmol/L was determined by MTT assay. The results showed that erlotinib inhibited cell growth in a dose-dependent manner and significant growth inhibition was demonstrated at 72 h ($P < 0.01$). A similar result was obtained after erlotinib treatment for 96 h. We also treated BxPC-3 cells with erlotinib at 24 and 48 h. The results showed a trend toward growth inhibition, but it was not statistically significant among different concentrations ($P > 0.05$). Cell proliferation was again determined by MTT at 24, 48, 72 and 96 h. No significant difference in cell growth was noted between 24 h and 48 h, 72 h and 96 h at a concentration of 50 μmol/L. Percentage of survival cells is illustrated in Figure 1.

Effect of erlotinib treatment on cell cycle progression and apoptosis in BxPC-3 cells

To elucidate potential mechanisms of erlotinib-induced growth inhibition previously shown by MTT assay, we examined the distribution of cell cycle and apoptotic cells by flow cytometry and TUNEL. The concentration of the drug was 50 μmol/L which inhibited BxPC-3 cell growth by approximately 53.5% after 3 d of exposure. The results showed that erlotinib led to the accumulation of BxPC-3

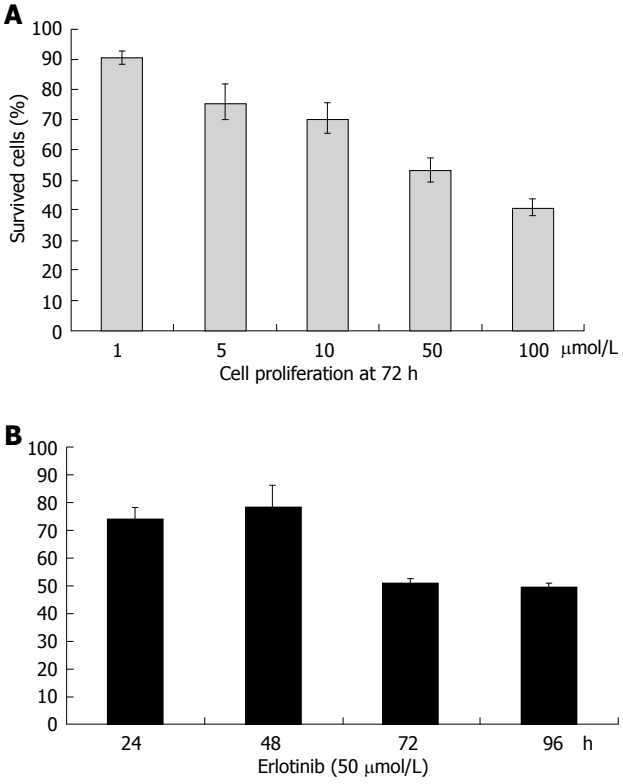


Figure 1 **A**: Growth percentage of BxPC-3 cell line after 72 h of exposure to erlotinib at varying concentrations ranging from 1-100 μmol/L. BxPC cell proliferation was significantly suppressed by erlotinib at different concentrations; **B**: Growth percentage of BxPC-3 cell line after exposure to erlotinib at a concentration of 50 μmol/L at different time points.

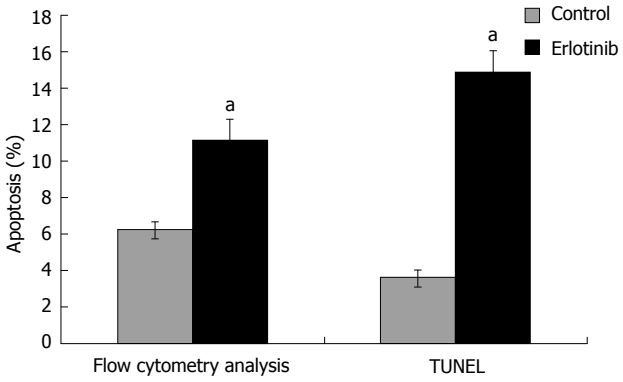


Figure 2 Induction of apoptosis in BxPC-3 cell line. There was a significant apoptosis in erlotinib treated group both in flow cytometry analysis and TUNEL assay after 48 h treatment ($^aP < 0.05$).

cells in G_0/G_1 phase, thereby decreasing the proportion of cells in the S phase (Table 2). To assess the effects of erlotinib on induction of cell apoptosis of pancreatic cancer cells, we performed a PI apoptosis and TUNEL assay after 48 h. Flow cytometric analysis showed an induction of apoptosis (11%) compared with the control (6%) ($P < 0.05$), which was further confirmed by TUNEL (AI 14.86 ± 1.20 to 3.60 ± 0.45) ($P < 0.05$) (Figure 2). The cell cycle alterations and cell apoptosis increase indicated that cell cycle arrest and increase in apoptosis are one of the mechanisms responsible for the antiproliferative action of erlotinib in BxPC-3 cells *in vitro*.

Table 2 Cell cycle analysis (%, mean ± SD)

Group	G_0/G_1	S	G_2/M
Control	63.31 ± 0.99	25.28 ± 0.88	11.40 ± 1.68
Erlotinib	73.40 ± 1.34[b]	14.15 ± 0.99[b]	12.44 ± 1.95

$^bP < 0.01$ *vs* control group.

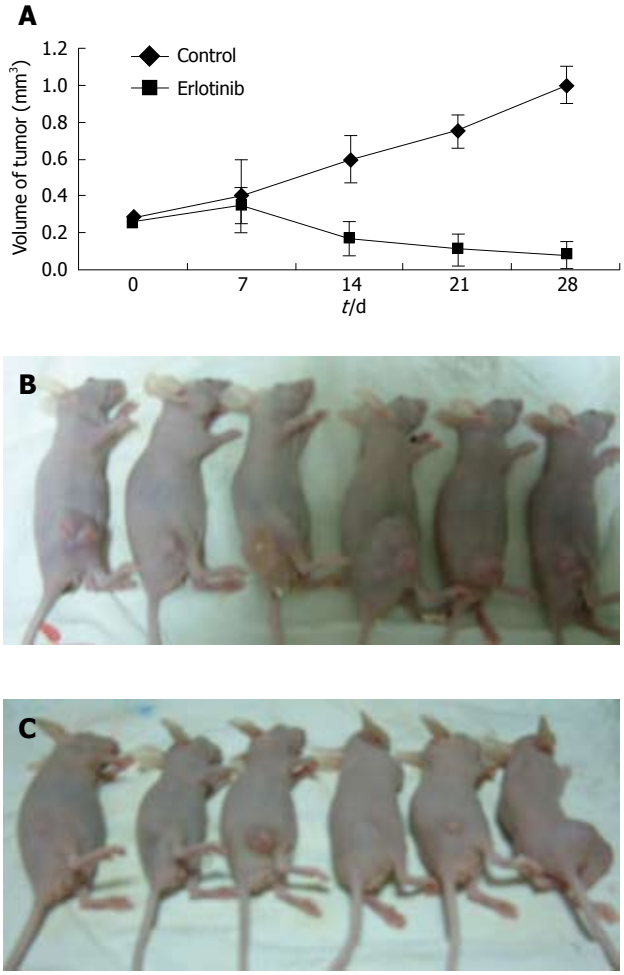


Figure 3 **A**: Effect of erlotinib on mean tumor volume in the BxPC-3 xenograft model. Mice were implanted with BxPC-3 cells. When palpable tumors were established, animals were randomly divided into two groups. The animals were continuously gavaged with the agents for 4 wk as described in Matericals and Methods. Tumor size was measured twice per week. Values are means, $n = 6$. **B**: Mice of control group; **C**: Mice of erlotinb group.

Effect of treatment with erlotinib on the growth of mouse xenografts

A nude mouse model of pancreatic cancer was used for the *in vivo* portion of the study to assess the *in vivo* antitumor activity of erlotinib. Heterotopic murine pancreatic carcinoma was successfully established in the flank of BALB/C nude mice. Erlotinib at a dose of 100 mg/kg per day was administered to mice bearing established BxPC-3 tumors. The results indicated that erlotinib significantly inhibited tumor growth. As shown in Figure 3, at day 14, the control group of mice showed an increased tumor volume by 114.3% of the initial tumor while the group treated with erlotinib showed a decreased tumor volume by 34.6%. At the end of the

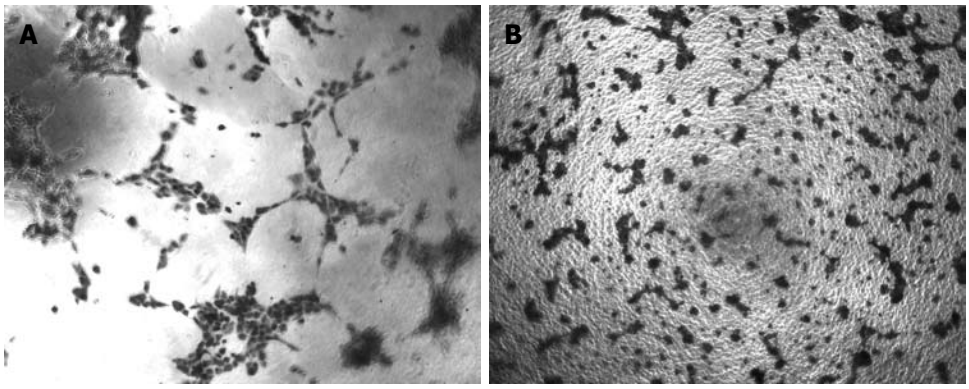


Figure 4 Effects of erlotinib on capillary formation by ECV304 were examined *in vitro*. **A**: When cultured on ECMatrix, ECV304 cells rapidly aligned with hollow tube-like structures; **B**: ECV304 cells were treated with erlotinib (100 μmol/L), and significant inhibition of tube formation was achieved compared with the control.

study in the BxPC-3 xenograft (day 28), the growth inhibition rate was 74.5% ($P < 0.05$) in the erlotinib treated group.

In vitro angiogenesis

EGFR TKIs have been reported to inhibit angiogenesis of pancreatic carcinoma. To determine whether erlotinib suppressed tumor vessel formation *in vitro*, we examined the effects of erlotinib on the ability of ECV304 to form capillary tube structures. ECMatrix is a solid gel of basement proteins prepared from the Engelbreth Holm-Swarm (EHS) mouse tumor and consists of laminin, collagen type Ⅳ, heparin sulphate proteoglycans, entactin, and nidogen. It also contains various growth and proteolytic enzymes that occur normally in EHS tumors. When cultured on ECMatrix, ECV304 cells rapidly aligned and formed tube-like structures (Figure 4A), however, the length of the tubes in the erlotinib treated cells was markedly reduced compared with the control which demonstrated that erlotinib significantly inhibited ECV304 tube formation (Figure 4B).

In vivo inhibition of EGFR and MVD after treatment with erlotinib

The EGFR expression pattern in the BxPC-3 tumors was examined by IHC staining. IHC analysis confirmed that the tumor xenograft tissues maintained both cell membrane and cytoplasmic EGFR expression. The expression of EGFR was decreased in the erlotinib treated groups (2.45 ± 0.81) compared with the control (10.65 ± 1.26) ($P < 0.05$). MVD was also assessed by immunostaining with factor Ⅷ-related antigen in the most intense areas of neovascularization. Representative images of the two groups are shown in Figure 5. MVD of erlotinib treated tumors (1.86 ± 0.43) was significantly lower than that of the control (5.98 ± 1.27) ($P < 0.05$).

Modulation of apoptosis-associated gene and VEGF expression in BxPC-3 cells and xenografts treated with erlotinib

The effect of erlotinib on the expression of EGFR, apoptosis-associated factors such as *bcl-2*, *bak*, *bax*, *bcl-xl* and *VEGF* mRNA was determined by RT-PCR. BxPC-3 cells were treated with erlotinib at different concentrations ranging from 5-200 μmol/L for 48 h. The results showed that the expression of *EGFR* and *VEGF* mRNA in the BxPC-3 cell line seemed to be down-regulated, the highest suppression was detected at the highest concentration used, 200 μmol/L. Densitometric analysis revealed the relative expression of VEGF at 200 μmol/L concentration of erlotinib (1.2% ± 0.68%) was significantly lower than that of the control (2.67% ± 0.13%) ($P < 0.05$). Apoptosis-associated gene including *bcl-xl* and *bcl-2* was suppressed by erlotinib in a concentration dependent manner; however, bax appeared to be up-regulated following erlotinib treatment at various concentrations; but it did not apparently affect bak expression. In the tumor xenograft tissues, the expression of *VEGF* mRNA was significantly lower (almost disappeared) in the erlotinib-treated group compared with that in the control ($P < 0.01$) (Figure 6).

DISCUSSION

Treatment options of pancreatic adenocarcinoma are unsatisfactory, and the prognosis of patients with pancreatic cancer is poor. Considering that the cure rate for these patients with surgery alone is low[8], development of potentially effective treatment is urgently needed. Many features of the pancreatic malignant phenotype are associated with the signaling networks that involve the EGFR. EGF and its receptor, EGFR, are over-expression in many human pancreatic cancers[9,10]. Recently, there is increasing evidence demonstrating the therapeutic potential of EGFR blockade in the management of pancreatic cancer and other malignancies[11-13]. EGFR inhibitors have shown activity in clinical trials in pancreatic, colorectal and non-small cell lung cancers[14-16]. Erlotinib is an orally available low-molecular-weight quinazolinamine that acts as a potent and reversible inhibitor of EGFR-TK activity. Single agent activity was observed in patients with non-small cell lung cancer, head and neck carcinoma and ovarian cancer[17-20]. A randomized phase Ⅲ placebo-controlled trial has shown that the combination of gemcitabine and erlotinib is associated with a modest but statistically significant survival benefit compared with gemcitabine and placebo and this represents an EGFR-targeted agent conferred benefit in addition to chemotherapy[21]. In this study, we evaluated the efficacy of erlotinib, as a single agent, on pancreatic cancer cells grown *in vitro* and *in vivo*

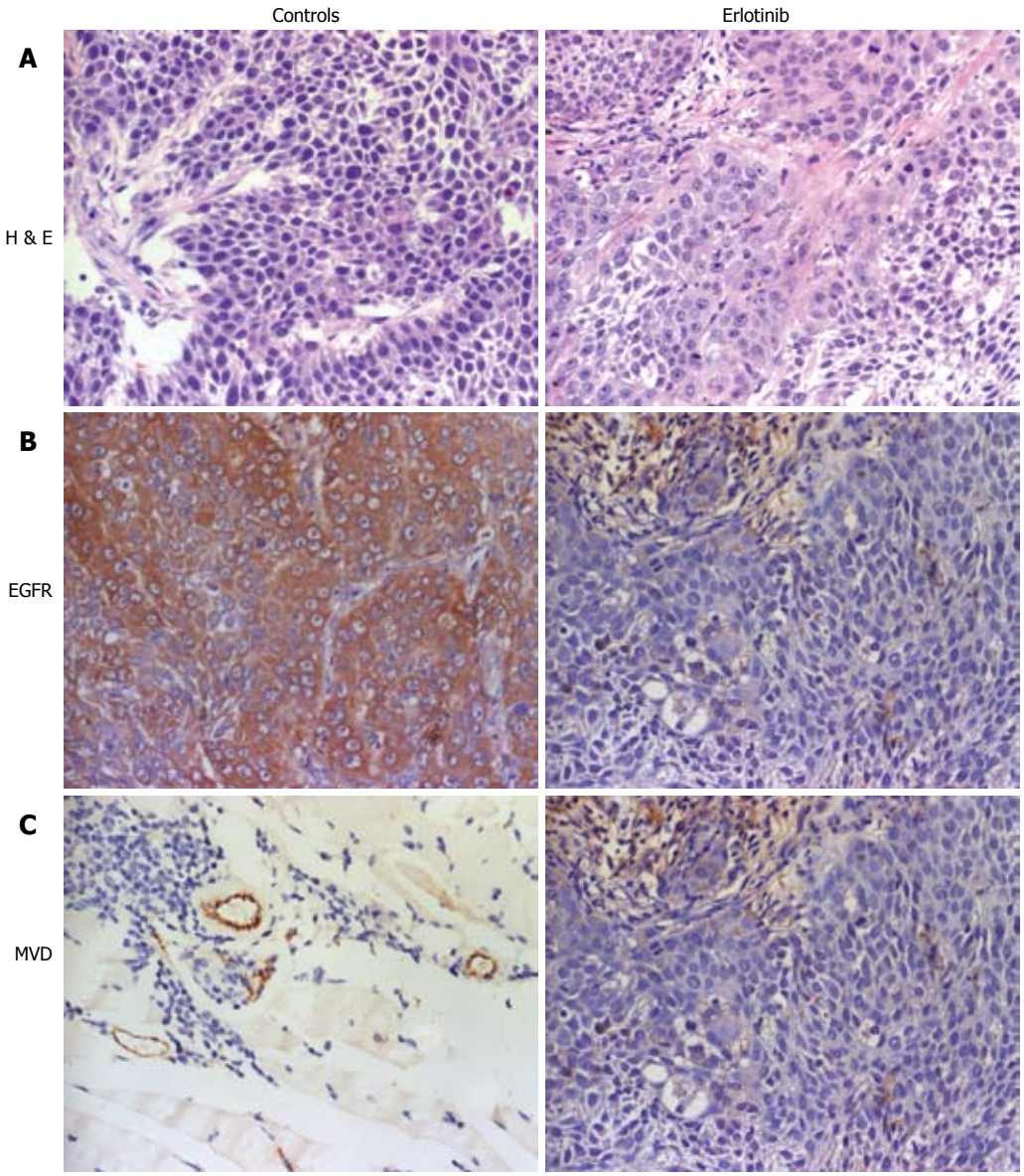


Figure 5 Expression of EGFR and the blood vessel endothelial cells in different treatment group in BxPC-3 mouse xenograft tissues. IHC was used to determine expression levels of EGFR and evaluate tumor microvessel density. **A**: HE staining for each sample (x 400); **B**: Expression of EGFR in treatment group was decreased compared with the control (x 400); **C**: Microvessel density of erlotinib treated group was lower than that of the control group.

using a nude mice xenograft model and explored the mechanisms involved as well. *In vitro* results showed an inhibition efficiency of 53.5% by erlotinib at the concentration of 50 μmol/L in the growth of cultured BxPC-3 pancreatic carcinoma cells as determined by MTT assay. The cell viability of BxPC-3 pancreatic cells was 53.5% with erlotinib treatment at a 50 μmol/L concentration by the MTT assay *in vitro*. There was no difference between 50 μmol/L and 100 μmol/L concentrations of erlotinib when cells were treated for 48 h (data not shown). We performed cell cycle analyses to characterize the underlying mechanisms of erlotinib's mode of action. Upon erlotinib treatment for 48 h, the proportion of cells in the G_0/G_1 phase increased to 73.4%, being significantly higher than that of the control. The antineoplastic effect of erlotinib is not solely due to cell cycle arrest. Induction of apoptosis by EGFR-TK inhibition has recently been reported[22,23]. In our study, we also observed cell apoptosis induced by erlotinib following cell cycle arrest. As we know, Bcl-2 members are crucial regulators of apoptotic cell death. We also checked the expression of anti-apoptotic factors such as bcl-2, bcl-xl as well as pro-apoptotic factors bax and bak. Erlotinib induced a moderate decrease in the expression of bcl-2 and bcl-xl at a concentration of $\geqslant$ 50 μmol/L. Our study *in vitro* provides evidence that the growth of BxPC-3 cells can be suppressed by erlotinib and at least two mechanisms are involved, i.e. cell cycle arrest and apoptosis. We used a nude mouse xenograft model to further evaluate the antitumor efficacy of erlotinib in pancreatic cancer *in vivo*. Erlotinib was very effective in suppressing the growth of BxPC-3 tumors in a subcutaneous tumor model. Tumor inhibition of approximately 74.5% was observed after 4 wk of treatment with erlotinib at a dose of 100 mg/kg per day. IHC staining of sections of subcutaneously implanted tumors showed constitutively high EGFR expression, however, the mice treated with 100 mg/kg erlotinib daily showed lower levels of EGFR. These results showed that erlotinib as monotherapy has strong antitumor activity in human BxPC-3 xenograft models expressing EGF receptors.

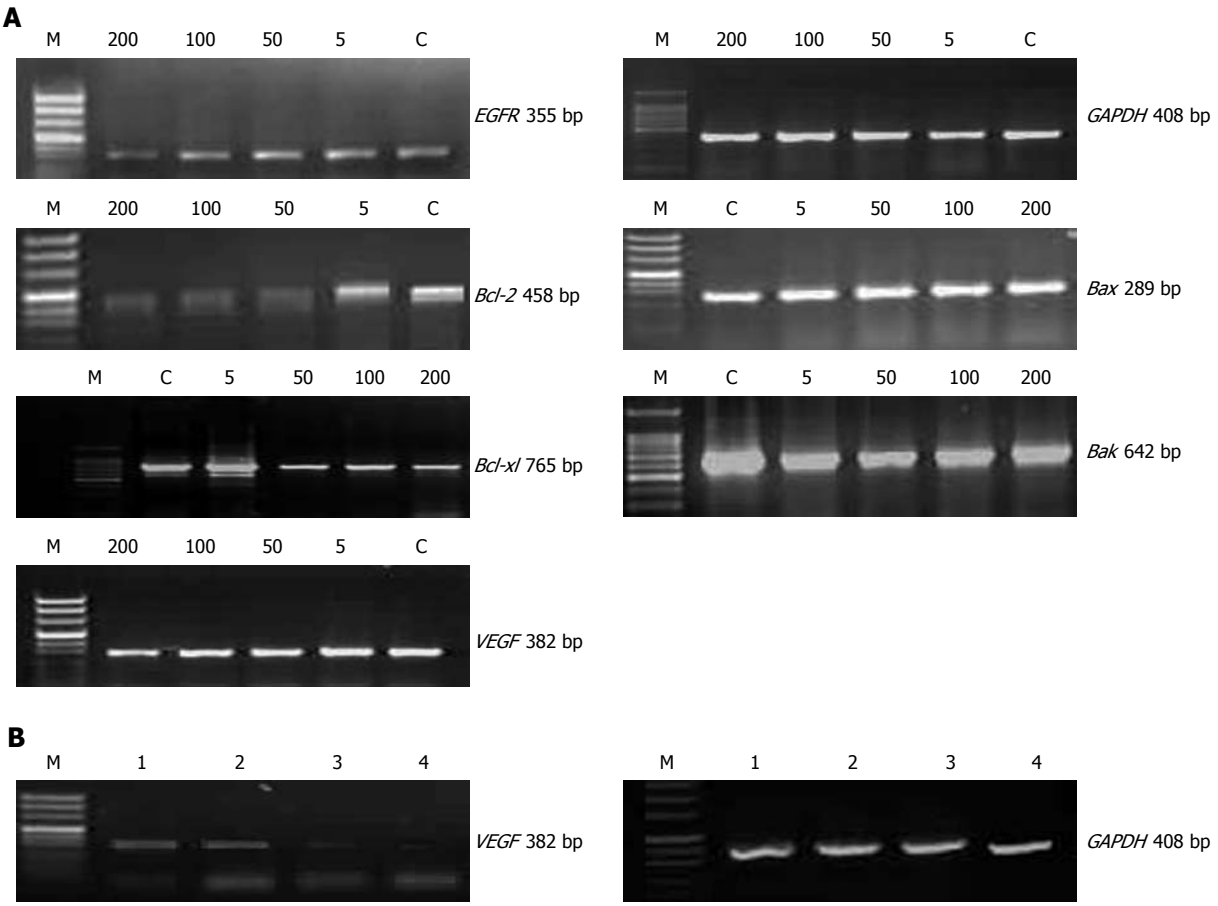


Figure 6 Expressions of *EGFR*, *Bcl-2*, *Bcl-xl*, *Bax*, *Bak* and *VEGF* mRNA in BxPC-3 cells treated with different concentrations (μmol/L) of erlotinib for 48 h and xenograft tissues were detected by RT-PCR. **A**: Effects of erlotinib on the expressions of EGFR, apoptosis-associated factors and *VEGF*. Lane M: DNA marker; Lane C: Control; other lanes: Different concentrations of erlotinib. **B**: Effects of erlotinib (100 mg/kg daily) on expression of VEGF in tumor tissues. Lane M: DNA marker; Lane 1, 2: Control group; Lane 3, 4: Treatment group. RT-PCR for *GAPDH* was performed in parallel and showed an equal amount of total RNA in the sample.

Numerous lines of evidence have shown that angiogenesis plays a significant clinicopathological role in tumors. Although pancreatic cancer is not a grossly vascular tumor, it often exhibits enhanced foli of endothelial cell proliferation. Moreover, several studies have reported a positive correlation between blood vessel density and disease progression in cases of pancreatic cancer, supporting the important role of angiogenesis in this disease[24-26]. EGFR signaling has previously been shown to play a role in angiogenesis[27]. In this study, we used the expression of VEGF, the MVD and tube formation assay to investigate the effects of erlotinib on angiogenesis both *in vitro* and *in vivo*. At the molecular level, VEGF is believed to be critical for pancreatic cancer angiogenesis[28]. Erlotinib exhibited a moderate decrease in VEGF expression *in vitro*, especially at a high concentration (200 μmol/L). Similarly, in xenograft tissues, there were marked reductions in the amounts of VEGF present in the treated group as compared with the control. As a unique tool, an *in vitro* capillary formation assay has been used to verify specific antiangiogenic activities of many agents with a good correlation to blood vessel formation *in vivo*[29]. Using this method, we observed that erlotinib as a single agent inhibited capillary tube formation by ECV304. Our immunohistochemical analysis of tumor specimens revealed that the treatment of mice with erlotinib produced a significant decrease in the number of tumor-associated blood vessels (MVD). The decrease in MVD could be attributable to a decrease in endothelial cell proliferation, as we proved *in vitro*.

In summary, our study showed that blockade of the EGFR signaling pathway by erlotinib has provided significant treatment in the BxPC-3 cell line *in vitro* and in nude mice and it may have a potential for the treatment of human pancreatic carcinoma. However, it is apparent that the use of a single signal transduction inhibitor cannot antagonize all the potentially relevant survival pathways in pancreatic cancers. As an adjuvant use in chemotherapy, targeting EGFR pathway seems to be a promising approach in the prevention and/or treatment of pancreatic cancer.

ACKNOWLEDGMENTS

We thank Shanghai Institute of Biochemistry and Cell Biology for providing technical assistance.

COMMENTS

Background

The activity of the epidermal growth factor receptor (EGFR), a tyrosine kinase

receptor of the ErbB family, is abnormally elevated in most human solid tumors, including pancreatic cancer, and is associated with progression and poor prognosis. There are two main categories of therapeutic strategy for targeting the EGFR pathway, specific anti-EGFR monoclonal antibodies and EGFR tyrosine kinase inhibitors (TKIs). Blockage of EGFR activity may lead to growth inhibition in pancreatic cancer.

Research frontiers

Molecularly targeted therapies have recently expanded the options available for patients with gastrointestinal tumors. Low weight EGFR TKIs such as erlotinib may play an important role in the treatment of human pancreatic carcinoma. Administration of erlotinib inhibites the BxPC-3 human pancreatic cancer cell line growth and induces antiangiogenic effect both *in vitro* and *in vivo*.

Innovations and breakthroughs

Small-molecule inhibitors of the EGFR tyrosine kinase, such as erlotinib, have shown promise in phase Ⅲ trials in non-small-cell lung cancer (NSCLC). But the anticancer mechanism has not been clearly elucidated especially in gastrointestinal tumors, including pancreatic cancer. The results indicate that the blockade of EGFR may provide a rationale for translating this therapeutic strategy into a clinical setting in some pancreatic cancer patients.

Applications

This study showed that blockade of the EGFR signaling pathway by erlotinib suppressed the BxPC-3 human pancreatic cancer cell line growth and induced antiangiogenic effects both *in vitro* and *in vivo*. As an adjuvant used in chemotherapy, targeting the EGFR pathway seems to be a promising approach in the prevention and or treatment of pancreatic cancer.

Terminology

The epidermal growth factor (EGF) receptor (or ErbB1) and the related ErbB4 are transmembrane receptor protein tyrosine kinases which bind extracellular ligands of the EGF family. ErbB2 and ErbB3 are "co-receptors" structurally related to ErbB1/ ErbB4, but ErbB2 is an "orphan" receptor and ErbB3 lacks tyrosine kinase activity. They transduced biological signals from the extracellular to the intracellular compartment. These families of ligands and receptors have been firmly linked to proliferative signaling and oncogenesis.

Peer review

In this paper, the effect of erlotinib on the proliferation and apoptosis of the human pancreatic cancer cell line BxPC-3 was studied. A dose-dependent inhibition of BxPC-3 cell growth *in vitro*, with a block of G_1-S transition, and rise in apoptosis were observed in erlotinib-treated cells. A decrease in tumor volume and microvessel density was also found in erlotinib-treated nude mice carrying BxPC-3 cell xerographs. This is a very interesting manuscript with some minor points needing further addressing.

REFERENCES

1 **Li D**, Xie K, Wolff R, Abbruzzese JL. Pancreatic cancer. *Lancet* 2004; **363**: 1049-1057

2 **MacKenzie MJ**. Molecular therapy in pancreatic adenocarcinoma. *Lancet Oncol* 2004; **5**: 541-549

3 **Matsuda K**, Idezawa T, You XJ, Kothari NH, Fan H, Korc M. Multiple mitogenic pathways in pancreatic cancer cells are blocked by a truncated epidermal growth factor receptor. *Cancer Res* 2002; **62**: 5611-5617

4 **Solorzano CC**, Baker CH, Tsan R, Traxler P, Cohen P, Buchdunger E, Killion JJ, Fidler IJ. Optimization for the blockade of epidermal growth factor receptor signaling for therapy of human pancreatic carcinoma. *Clin Cancer Res* 2001; **7**: 2563-2572

5 **Bruns CJ**, Solorzano CC, Harbison MT, Ozawa S, Tsan R, Fan D, Abbruzzese J, Traxler P, Buchdunger E, Radinsky R, Fidler IJ. Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma. *Cancer Res* 2000; **60**: 2926-2935

6 **Taraboletti G**, Giavazzi R. Modelling approaches for angiogenesis. *Eur J Cancer* 2004; **40**: 881-889

7 **Ciardiello F**, Caputo R, Damiano V, Caputo R, Troiani T, Vitagliano D, Carlomagno F, Veneziani BM, Fontanini G, Bianco AR, Tortora G. Antitumor effects of ZD6474, a small molecule vascular endothelial growth factor receptor tyrosine kinase inhibitor, with additional activity against epidermal growth factor receptor tyrosine kinase. *Clin Cancer Res* 2003; **9**: 1546-1556

8 **Smeenk HG**, Tran TC, Erdmann J, van Eijck CH, Jeekel J. Survival after surgical management of pancreatic adenocarcinoma: does curative and radical surgery truly exist? *Langenbecks Arch Surg* 2005; **390**: 94-103

9 **Poch B**, Gansauge F, Schwarz A, Seufferlein T, Schnelldorfer T, Ramadani M, Beger HG, Gansauge S. Epidermal growth factor induces cyclin D1 in human pancreatic carcinoma: evidence for a cyclin D1-dependent cell cycle progression. *Pancreas* 2001; **23**: 280-287

10 **Tobita K**, Kijima H, Dowaki S, Kashiwagi H, Ohtani Y, Oida Y, Yamazaki H, Nakamura M, Ueyama Y, Tanaka M, Inokuchi S, Makuuchi H. Epidermal growth factor receptor expression in human pancreatic cancer: Significance for liver metastasis. *Int J Mol Med* 2003; **11**: 305-309

11 **Giaccone G**, Gallegos Ruiz M, Le Chevalier T, Thatcher N, Smit E, Rodriguez JA, Janne P, Oulid-Aissa D, Soria JC. Erlotinib for frontline treatment of advanced non-small cell lung cancer: a phase II study. *Clin Cancer Res* 2006; **12**: 6049-6055

12 **Dragovich T**, McCoy S, Fenoglio-Preiser CM, Wang J, Benedetti JK, Baker AF, Hackett CB, Urba SG, Zaner KS, Blanke CD, Abbruzzese JL. Phase II trial of erlotinib in gastroesophageal junction and gastric adenocarcinomas: SWOG 0127. *J Clin Oncol* 2006; **24**: 4922-4927

13 **Philip PA**, Mahoney MR, Allmer C, Thomas J, Pitot HC, Kim G, Donehower RC, Fitch T, Picus J, Erlichman C. Phase II study of erlotinib in patients with advanced biliary cancer. *J Clin Oncol* 2006; **24**: 3069-3074

14 **Welch SA**, Moore MJ. Erlotinib: success of a molecularly targeted agent for the treatment of advanced pancreatic cancer. *Future Oncol* 2007; **3**: 247-254

15 **Cunningham D**, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, Bets D, Mueser M, Harstrick A, Verslype C, Chau I, Van Cutsem E. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med* 2004; **351**: 337-345

16 **Jackman DM**, Yeap BY, Lindeman NI, Fidias P, Rabin MS, Temel J, Skarin AT, Meyerson M, Holmes AJ, Borras AM, Freidlin B, Ostler PA, Lucca J, Lynch TJ, Johnson BE, Janne PA. Phase II clinical trial of chemotherapy-naive patients > or = 70 years of age treated with erlotinib for advanced non-small-cell lung cancer. *J Clin Oncol* 2007; **25**: 760-766

17 **Plestina S**, Samarzija M, Plestina S, Chalfe N, Zuljevic E. [Erlotinib in non-small cell lung cancer: experience of clinical hospital for lung diseases Jordanovac] *Lijec Vjesn* 2007; **129**: 387-390

18 **Thomas F**, Rochaix P, Benlyazid A, Sarini J, Rives M, Lefebvre JL, Allal BC, Courbon F, Chatelut E, Delord JP. Pilot study of neoadjuvant treatment with erlotinib in nonmetastatic head and neck squamous cell carcinoma. *Clin Cancer Res* 2007; **13**: 7086-7092

19 **Gordon AN**, Finkler N, Edwards RP, Garcia AA, Crozier M, Irwin DH, Barrett E. Efficacy and safety of erlotinib HCl, an epidermal growth factor receptor (HER1/EGFR) tyrosine kinase inhibitor, in patients with advanced ovarian carcinoma: results from a phase II multicenter study. *Int J Gynecol Cancer* 2005; **15**: 785-792

20 **Soulieres D**, Senzer NN, Vokes EE, Hidalgo M, Agarwala SS, Siu LL. Multicenter phase II study of erlotinib, an oral epidermal growth factor receptor tyrosine kinase inhibitor, in patients with recurrent or metastatic squamous cell cancer of the head and neck. *J Clin Oncol* 2004; **22**: 77-85

21 **Moore MJ**, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, Au HJ, Murawa P, Walde D, Wolff RA, Campos D, Lim R, Ding K, Clark G, Voskoglou-Nomikos T, Ptasynski M, Parulekar W. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol* 2007; **25**: 1960-1966

22 **Ng SS**, Tsao MS, Nicklee T, Hedley DW. Effects of the epidermal growth factor receptor inhibitor OSI-774, Tarceva, on downstream signaling pathways and apoptosis in human pancreatic adenocarcinoma. *Mol Cancer Ther* 2002; **1**: 777-783

23 **Huang S**, Armstrong EA, Benavente S, Chinnaiyan P, Harari PM. Dual-agent molecular targeting of the epidermal growth factor receptor (EGFR): combining anti-EGFR antibody with tyrosine kinase inhibitor. *Cancer Res* 2004; **64**: 5355-5362

24 **Seo Y**, Baba H, Fukuda T, Takashima M, Sugimachi K. High expression of vascular endothelial growth factor is associated with liver metastasis and a poor prognosis for patients with ductal pancreatic adenocarcinoma. *Cancer* 2000; **88**: 2239-2245

25 **Fujimoto K**, Hosotani R, Wada M, Lee JU, Koshiba T, Miyamoto Y, Tsuji S, Nakajima S, Doi R, Imamura M. Expression of two angiogenic factors, vascular endothelial growth factor and platelet-derived endothelial cell growth factor in human pancreatic cancer, and its relationship to angiogenesis. *Eur J Cancer* 1998; **34**: 1439-1447

26 **Khorana AA**, Hu YC, Ryan CK, Komorowski RA, Hostetter G, Ahrendt SA. Vascular endothelial growth factor and DPC4 predict adjuvant therapy outcomes in resected pancreatic cancer. *J Gastrointest Surg* 2005; **9**: 903-911

27 **Azuma M**, Tamatani T, Fukui K, Yuki T, Yoshida H, Bando T, Hoque MO, Kamogashira T, Ogino K, Nishino N. Identification of EGF as an angiogenic factor present in conditioned medium from human salivary gland adenocarcinoma cell clones with varying degrees of metastatic potential. *Cancer Lett* 1994; **84**: 189-198

28 **Xie K**, Wei D, Shi Q, Huang S. Constitutive and inducible expression and regulation of vascular endothelial growth factor. *Cytokine Growth Factor Rev* 2004; **15**: 297-324

29 **Weiss RH**, Marshall D, Howard L, Corbacho AM, Cheung AT, Sawai ET. Suppression of breast cancer growth and angiogenesis by an antisense oligodeoxynucleotide to p21(Waf1/Cip1). *Cancer Lett* 2003; **189**: 39-48

S- Editor Li DL **L- Editor** Ma JY **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5412

World J Gastroenterol 2008 September 21; 14(35): 5412-5418
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Biological impact of hepatitis B virus X-hepatitis C virus core fusion gene on human hepatocytes

Zhen Ma, Qin-Hai Shen, Guo-Min Chen, Da-Zhi Zhang

Ma Zhen, Qin-Hai Shen, Guo-Min Chen, Da-Zhi Zhang, Institute for Viral Hepatitis, Chongqing Medical University, Chongqing 400010, China
Author contributions: Chen GM, Ma Z designed the research; Shen QH, Ma Z performed the research; Shen QH, Zhang DZ and Ma Z analyzed the data; Ma Z wrote the paper.
Correspondence to: Guo-Min Chen, Institute for Viral Hepatitis, Chongqing Medical University, Chongqing 400010, China. mazhen1972@163.com
Telephone: +86-23-63712639 Fax: +86-23-63712639
Received: May 9, 2006 Revised: May 25, 2008
Accepted: June 1, 2008
Published online: September 21, 2008

Abstract

AIM: To investigate the biological impact of hepatitis B virus X- hepatitis C virus core (HBV X-HCV C) fusion gene on hepatoma cells.

METHODS: The recombinant adenoviruses Ad-XC, Ad-X and Ad-C expressing HBV X-HCV C fusion gene, *HBV X* gene and *HCV C* gene were constructed, respectively. Hepatoma cells were infected with different recombinant adenoviruses. MTT, colony-forming experiment, FCM, TUNEL assay were performed to observe the biological impact of the HBV X-HCV C fusion gene on liver cells.

RESULTS: MTT showed that the Ad-XC group cells grew faster than the other group cells. Colony-forming experiment showed that the colony-forming rate for the Ad-XC group cells was significantly higher than that for the other group cells. FCM analysis showed that Ad-XC/Ad-X/Ad-C infection enhanced the progression of G1→S phase in the HepG2 cell cycle. The apoptosis index of the Ad-XC, Ad-X, Ad-C group cells was significantly lower than that of the Ad0 and control group cells. Semi-quantitative RT-PCR showed that the expression level of c-myc was the highest in Ad-XC infected cells. Tumor formation was found at the injected site of mice inoculated with Ad-XC-infected LO2 cells, but not in control mice.

CONCLUSION: Ad-XC, Ad-X and Ad-C facilitate the proliferation activity of HepG2 cells and inhibit their apoptosis *in vitro*. The effect of Ad-XC is significantly stronger than that of Ad-X and Ad-C. Up-regulation of c-myc may be one of the mechanisms underlying the synergism of *HBV X* and *HCV C* genes on hepatocarcinogenesis in athymic nude mice.

Key words: Hepatitis B virus X gene; Hepatitis C virus core gene; Hepatocellular carcinoma; Proliferation; Apoptosis

Peer reviewer: Peter L Lakatos, MD, PhD, Assistant Professor, 1st Department of Medicine, Semmelweis University, Koranyi S 2A, Budapest H1083, Hungary

Ma Z, Shen QH, Chen GM, Zhang DZ. Biological impact of hepatitis B virus X-hepatitis C virus core fusion gene on human hepatocytes. *World J Gastroenterol* 2008; 14(35): 5412-5418 Available from: URL: http://www.wjgnet.com/1007-9327/14/5412.asp DOI: http://dx.doi.org/10.3748/wjg.14.5412

INTRODUCTION

Hepatocellular carcinoma (HCC) is the second most common cancer in China. Many etiological factors are related with HCC development. Chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) and prolonged dietary exposure to aflatoxin are responsible for about 80% of all HCCs in human beings. Chronic HBV and HCV infection often results in cirrhosis and enhances the probability of developing HCC. The underlying mechanisms leading to malignant transformation of infected cells, however, remain unclear. Based on epidemic data, super-infection with HBV and HCV is associated with the increased frequency in the development of HCC, but the relative mechanism remains elusive. It has been reported that both *HBV X* gene and *HCV core* (*HCV C*) gene play an important role in hepatocarcinogenesis. The fact that *HBV X* and *HCV C* genes induce HCC in transgenic mice offers more evidence for the relationship between these genes and HCC.

Imbalance between proliferation and apoptosis may contribute to hepatocarcinogenesis. HBV X and HCV C proteins are multiple-functional proteins, and can deregulate cell cycle check points, transactivate or activate cellular and viral genes, which are involved in transcription regulation, signal transduction pathway, cell cycle regulation, *etc*. Therefore, they may deregulate cell cycle and apoptosis and may have a common target

point. However, if there is synergism of HBV X and HCV C proteins on hepatocarcinogenesis still remains unclear. In the present study, recombinant adenoviruses expressing HBV X-HCV C fusion protein, HBV X protein and HCV C protein were constructed, and their effect on the biological behavior and expression of c-myc in hepatocytes were investigated.

MATERIALS AND METHODS

Materials

Pyrobest™ DNA polymerase, S*al* I , E*co*RV, H*ind*Ⅲ and T4 DNA ligase were purchased from TaKaRa Company (Dalian, China). Plasmid pecob6 was constructed by professor Ren Hong in our institute, plasmid pcDNA3.1/HCV-C was constructed by Dr. Wei-xian Chen in our institute. Lipofectamine was purchased from Invitrogen Company (California, USA). P*ac* I and P*me* I were from New England Biolabs (Beijing, China). Primers were synthesized by Shanghai Sangon Company (Shanghai, China). AdEasy system was a gift from professor Tong-Chuan He, University of Chicago Medical Center. DH5α was kept in our laboratory. Fetal bovine serum and calf serum were purchased from Hyclone (Utah, USA). RPMI 1640 was provided by GIBCO (New York, USA). 293 cells, LO2 and HepG2 cells were purchased from Shanghai Cytobiology Research institute of Chinese Academy of Science (Shanghai, China). BALB/c nude mice were from Shanghai Experimental Animal Centre of Chinese Academy of Science. Mouse anti-human hepatitis B virus X-protein monoclonal antibody was purchased from CHEMICON (California, USA). Monoclonal antibody to hepatitis C virus core protein was purchased from BIODESIGN (Maine, USA).

Construction, identification and amplification of recombinant adenoviruses

The recombinant adenoviruses Ad-XC, Ad-X and Ad-C containing *HBV X-HCV C* fusion gene, *HBV X* gene and *HCV C* gene, were constructed using the AdEasy system[1]. *HBV X* and *HCV C* genes were amplified by PCR from pecob6 or pcDNA3.1-HCV C. Using gene SOEing method[2], *HBV X* and *HCV C* genes were fused through a linker coding for a sequence rich in glycine. The sequences of gene primers used in this study are listed in Table 1. The fragment was bi-mold-cut with S*al* I and E*co*RV and inserted into two spots of S*al* I and E*co*RV of the pAdTrack-CMV. The recombinant shuttle plasmid was confirmed by PCR, double restriction nuclease digestion and sequencing. Mini preparations were performed using the conventional alkaline lysis method. The linearized shuttle plasmid was co-transformed with adenoviral backbone plasmid pAdEasy-1 to *E. coli* BJ5183 by electroporation. The cloned candidate was further tested by restriction nuclease digestion with P*ac* I and PCR. After digested with P*ac* I , the recombinant adenoviral plasmid was transfected into 293 cells for package. Generation of recombinant adenoviruses was monitored by GFP expression. Transfected cells were collected 12-15 d after transfection by scraping cells off flasks and pelleting them with 1 mL PBS. After three cycles of freezing and rapid thawing at 37℃, 10 μL proteinase K was added into 5 μL of viral lysate at 55℃ for 1 h and boiled for 5 min and 2 μL of which was used as a model to identify the correct recombinant adenovirues (*HBV X*, *HCV C* and *HBV X-HCV C* fusion gene). The upstream sequence of adenovirus primer is 5'-CTGTGGACCGTGAGGATA-3', the downstream sequence of adenovirus primer of adenovirus primer is 5'-TGTTGGGCATAGATTGTT-3' (Table 1). PCR system contained 2 μL viral DNA, 2 μL 10 μmol/L primer, 5 μL 10 × PCR buffer, 3 μL $MgCl_2$, 4 μL 2 mmol/L dNTP and 1 μL Taq enzyme. Water was added until the final volume of PCR reached 50 μL. Thirty-five cycles of PCR were carried out, each consisting of 94℃ for 30 s, 51℃ for 40 s, 72℃ for 40 s, and a final extension at 72℃ for 10 min. Electrophoresis on 1.0% agarose gel was performed. The identified positive recombinant adenoviruses were amplified in 293 cells for further experiment. The virus titer was tested with GFP expression and limited dilution method.

Table 1 Sequences of gene primers

Target gene	Primer sequences	Product (bp)
HBV X gene	P1: 5′-ATCTGTCGACATGGCTG CTAGGCTGTGCT G-3′	465
	P2: 5′-CGCGGATATCTTAGGC AGAGGTGAAAAAGT TG-3′	
HCV C gene	P3: 5′-ACTGGTCGACATGAGC ACGAATCCTAAACCT C-3′	576
	P4: 5′-ACTGGATATCTTAGGC TGAAGCGGGCAC AG-3′	
HBV X-Linker fragment	P1: 5′-ATCTGTCGACATGGCT GCTAGGCTGTGCT G-3′	510
	P2′: 5′-GCTGCCGCCACCGCCG CTTCCGCCACCGCCGCTTGCC ACCGGCAGAGGTGAAAAAGTTGCA-3′	
Linker-HCV C fragment	P3′: 5′-GGTGGCGGTGGAAGCG GCGGTGGCGGCGGAAGCGGC GGTGGCGGCAGCATGAGCACG AATCCTAAACCTC -3′	621
	P4: 5′-ACTGGATATCTTAGGCT GAAGCGGGCAC AG-3′	
HBV X-HCV C fusion gene	P1: 5′-ATCTGTCGACATGGCTG CTAGGCTGTGCT G-3′	1086
	P4: 5′-ACTGGATATCTTAGGCT GAAGCGGGCAC AG-3′	
c-myc	5′-TTCGGGTAGTGGAAAACCAG-3′ 5′-CAGCAGCTCGAATTTCTTCC-3′	203
β-actin	5′-GTGGGGCGCCCCAGGCACCA-3′ 5′CTTCCTTAATGTCACGCACGATTTC-3′	540

Infective efficiency of recombinant adenoviruses

Two hundred and ninety three, NIH3T3 and HepG2 cells were seeded in six-well plates in the appropriate medium at a density of 2×10^5 cells/well, infected with various adenoviruses at a multiplicity of infection (MOI) from 5 to 100, and incubated at 37℃ for 48 h. The number of cells expressing GFP was recorded under an inversion difference fluorescence microscope.

The percentage of cells with GFP expression was calculated.

Infection with recombinant adenoviruses and expression of fusion protein

The human liver cancer cell line HepG2 was incubated in an atmosphere containing 50 mL/L CO_2 at 37℃. When the density reached 90%, the experimental groups were infected with Ad-XC, Ad-X, Ad-C or Ad0, respectively, at different MOI. During the process, the culture fluid was shaken every 30 min, 4 h later. Another 6 mL 10% NCS RPMI 1640 was added. The control group was a non-virus group. After HepG2 cells were incubated for 48 h, total protein was extracted using RIPA. The expression of HBV X-HCV C fusion protein, HBV X and HCV C protein was detected by Western blot.

MTT assay

HepG2 cells (1.5×10^3 cells/well) were plated in 96-well plates (16 wells for each group) and infected with various MOI of Ad0, Ad-C, Ad-X or Ad-XC, respectively. Cell proliferation was determined by MTT assay. After 1-7 d, 20 μL of MTT solution (5 mg/mL) was added to each well. After incubation for 4 h at 37℃, MTT was removed and 200 μL dimethyl sulfoxide (Sigma) was added. The mixture was shaken and the crystals were fully dissolved for about 10 min. The A value of each well was detected at a test wavelength of 490 nm. Cell growth curve was plotted according to the A values.

Colony-forming experiment

HepG2 cells of five groups were digested into a single-cell suspension and inoculated into a six-well plate. Each well was inoculated with 1.0×10^3 cells. The cells were incubated for 12 d and fixed with methanol, stained with Giemsa stain fluid. Then the number of colonies with more than 50 cells was recorded. The experiment was repeated five times.

Flow cytometry

When the density of HepG2 cells reached 90%, the experimental groups were infected with Ad-XC, Ad-X, Ad-C and Ad0, respectively. Forty-eight hours after infection, the cells were collected (using trypsin digestion) and centrifuged at 1000 r/min for 5 min. The upper clear fluid was discarded, PBS was added to adjust the cell density to 10^6/mL. One hundred microliters of cell suspension was put into a tube, into which 200 μL DNA-PREPTMLPR was added and mixed. Two microliters of DNA-PREPTM stain reagent (PI stain) was added and mixed after 30 s. After 30 min, the cell cycle was detected by flow cytometry (FCM).

Cell apoptosis assay

Cell apoptosis was estimated by TUNEL staining. HepG2 cells were planted into 24-well plates at a density of 1×10^5 cells/well and infected with Ad-XC, Ad-X, Ad-C and Ad0 respectively. At the same time, 1 mL TNF-α (100 ng/mL) was added into each well. After incubation at 37℃ for 48 h, the cells were fixed with 4% paraformaldehyde and chilled in ice bath for 4 min with permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate). Then, 50 μL of TUNEL mixture was added, incubated in a humidified chamber at 37℃ for 90 min. The TUNEL mixture was removed, 50 μL POD was added and incubated for another 40 min. The cells were rinsed with PBS, stained with DAB, and detected by optic microscopy.

Effect of Ad-XC infection on c-myc mRNA expression in HepG2 cells

Expression of c-myc mRNA in each group was assayed by semi-quantitative RT-PCR. β-actin was used as an internal control. Total RNA was extracted with TRIZOL reagent. RT-PCR was performed using an access RT-PCR system (Promega). The reaction volume was 50 μL containing 10 μL AMV/Tf1 buffer, 2 μL $MgSO_4$, 1 μL dNTP, 1 μL target gene sense and anti-sense primers, 1 μL β-actin primer pair, 1 μL AMV reverse transcriptase, 1 μL Tf1 DNA polymerase, 2 μg RNA and nuclease-free water. The sequences of gene primers are listed in Table 1. Thirty cycles of amplification were performed, each consisting of denaturation at 94℃ for 45 s, annealing at 58℃ for 30 s, extension at 37℃ for 1 min, an initial denaturation at 45℃ 45 min and at 94℃ for 2 min, and a final extension at 72℃ for 10 min. About 5 μL PCR products were separated by electrophoresis on 10 g/L agarose gel and detected by ultraviolet radiography. The densities of bands were analyzed using a Bio imaging system, the ratio of c-myc density to β-actin density was represented as the relative expression level of mRNA. The semi-quantitative detection was analyzed five times.

Nude mice experiment

LO2 cells were infected with Ad0 or Ad-XC. The infected cells were collected and resuspended in 200 μL PBS after 48 h. Three BALB/c nude mice were subcutaneously inoculated with the infected LO2 cells randomly (Ad0, $n = 1$; Ad-XC, $n = 2$). Tumors were observed every 2 d for 6 wk.

Statistical analysis

All data were expressed as mean ± SE. The significance for the difference between groups was assessed with SPSS 12.0 by one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Identification of recombinant adenoviruses by PCR co-amplification method

The PCR product depending on the viral DNA model was evaluated by 1.0% agarose electrophoresis. The recombinant adenoviruses that could amplify 465 bp HBV X cDNA fragment/576 bp HCV C cDNA fragment/HBV X-HCV C fusion gene fragment and a 759 bp virus gene frame fragment at the same time were obtained (Figure 1). Recombinant adenoviruses

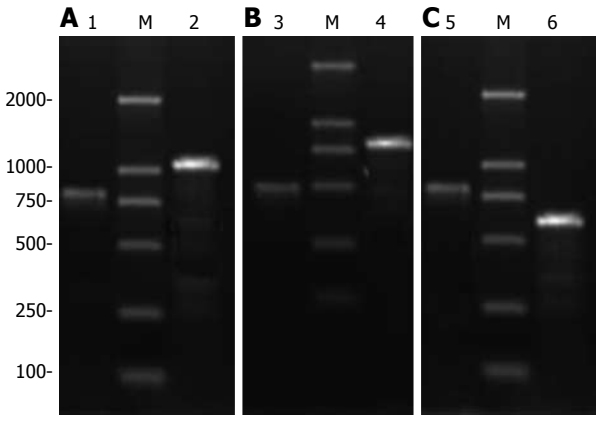


Figure 1 PCR verifying recombinant adenoviruses of Ad-XC (**A**), Ad-X (**B**), and Ad-C (**C**). M: DL2000; lanes 1, 4 and 5: PAdEasy-1; lane 2: HBV X-HCV C fusion gene; lane 3: X gene; lane 6: C gene.

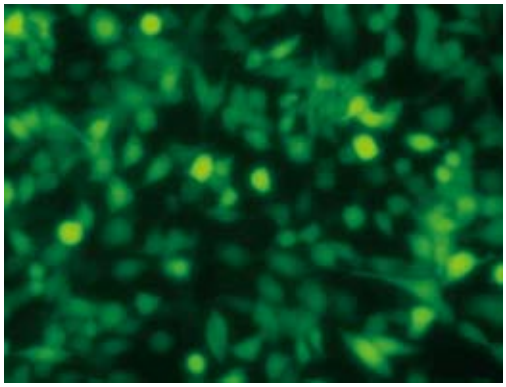


Figure 2 HepG2 cells infected with recombinant adenoviruses (x 200).

containing HBV X cDNA fragment, HCV C cDNA fragment and HBV X-HCV C fusion gene fragment, respectively, were produced.

Titer and transfection rate of recombinant adenoviruses

The titer of amplified recombinant adenoviruses Ad-XC, Ad-X, Ad-C and Ad0 was 1.9×10^9 pfu/mL, 2.0×10^9 pfu/mL, 2.2×10^9 pfu/mL, 1.7×10^9 pfu/mL, respectively. When the MOI was 20 or greater, the infection rate of HepG2 cells reached 100% (Figure 2).

Expression of different proteins in HepG2 cells

Forty-eight hours after infection, Western blot revealed the expression of HBV X-HCV C fusion protein, HBV X and HCV C proteins in HepG2 cells infected with recombinant adenovirus (Figure 3).

Effect of recombinant adenoviruses on growth curve of HepG2 cells

The expression of HBV X-HCV C fusion gene, *HBV X* and *HCV C* genes improved cell proliferation significantly compared with that of control HepG2 cells. The A values were higher in HepG2 cells infected with Ad-XC, Ad-X and Ad-C than in control HepG2 cells (Figure 4). These results indicate that HBV X-HCV C fusion gene, HBV X and HCV C genes, especially Ad-XC, stimulated the metabolic activity and the viability of HepG2 cells.

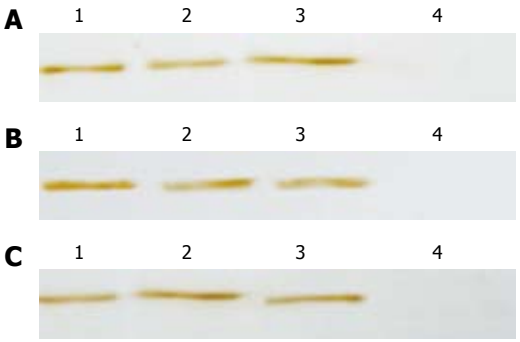


Figure 3 Western blotting displaying expression of fusion protein (**A**), HBV X protein (**B**), and HCV C protein (**C**). Lanes 1-3: Infected HepG2 cells; lane 4: Uninfected HepG2 cells.

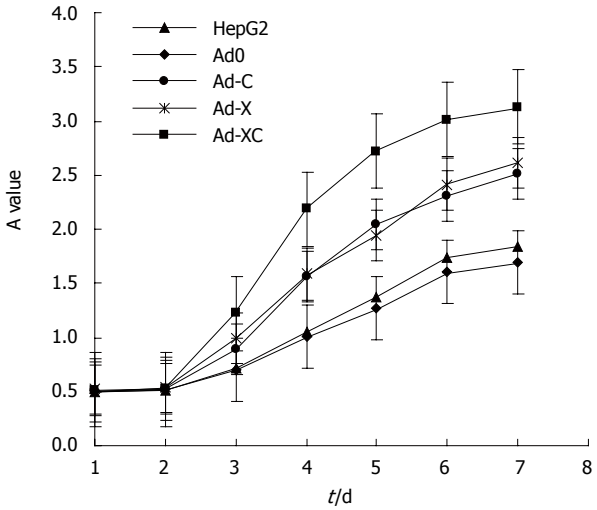


Figure 4 Growth status change in HepG2 cells after infection.

Table 2 Cell cycle of HepG2 cells infected with recombinant adenovirus (%, mean ± SE)

	G_0/G_1	S	G_2/M
HepG2	71.57 ± 0.79	19.18 ± 0.77	9.25 ± 0.76
Ad0	72.94 ± 1.84	18.38 ± 0.9	8.68 ± 1.12
Ad-C	50.21 ± 1.37	32.15 ± 0.15[b]	17.65 ± 1.22[b]
Ad-X	48.17 ± 1.13	33.19 ± 1.47[b]	18.64 ± 0.56[b]
Ad-XC	36.49 ± 0.84	42.06 ± 0.24[a,b]	21.45 ± 0.89[a,b]

[a]$P < 0.005$ *vs* Ad-C and Ad-X cells; [b]$P < 0.001$ *vs* HepG2 and Ad0 cells.

Effect of recombinant adenoviruses on colony-forming ability of HepG2 cells

The colony-forming rate of Ad-XC infected HepG2 cells was 82.2% ± 6.1%, significantly higher than that of the Ad-X, Ad-C, Ad0 infected HepG2 cells and control cells (53% ± 4.1%, 49% ± 7.1%, 27.6% ± 5.1%, 30.2% ± 4.4%, respectively, $P < 0.0001$, $n = 5$).

Effect of recombinant adenoviruses on cell cycle

Cell cycles from FCM are listed in Table 2. Compared

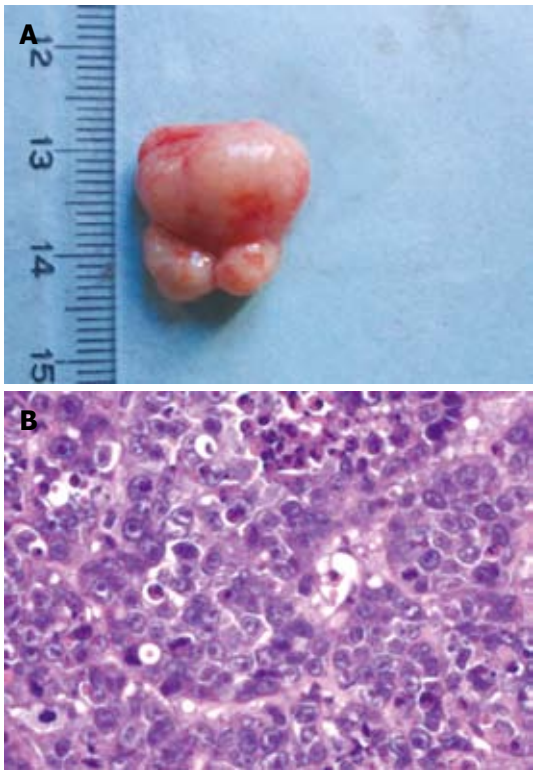


Figure 5 Surgical specimens of tumor tissue (**A**) and histological examination (**B**) showing a 1.5 cm tumor (HE staining, × 400).

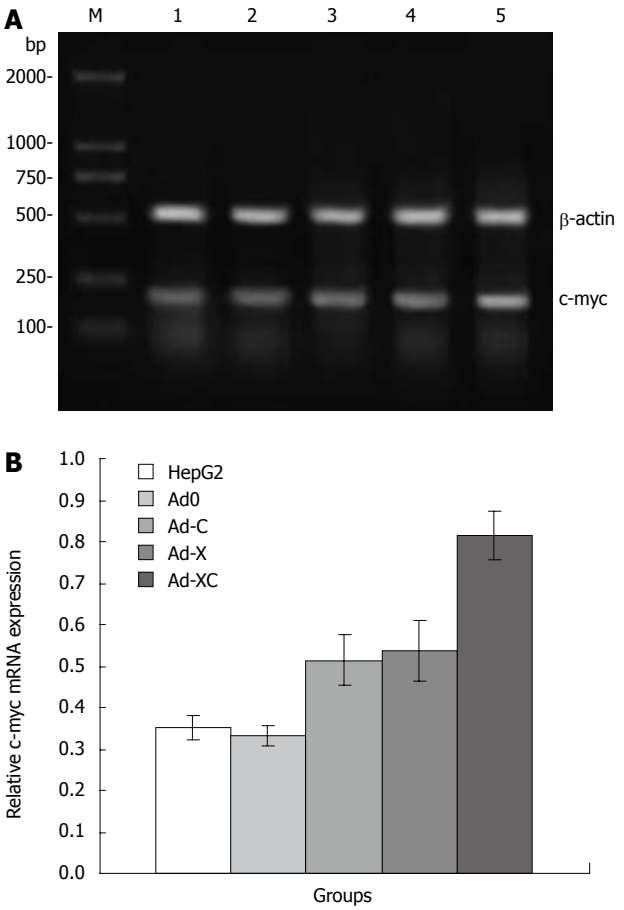


Figure 6 RT-PCR revealing mRNA expression of c-myc (**A**) and relative mRNA expression level of c-myc (**B**) in HepG2 cells. M: DL2000; lanes 1-5: HepG2, Ad0, Ad-C, Ad-X and Ad-XC cells ($P < 0.05$, Ad-XC group *vs* other groups).

with Ad0 and non-virus group cells, the number of cells in G_0/G_1 phase was decreased, but increased in Ad-XC, Ad-X, Ad-C groups at S phase, indicating that proliferation of the cells was active. A significant difference was observed in cell proliferation between the Ad-XC group and other groups ($P < 0.0001$, $n = 3$).

Cell apoptosis assay

TUNEL showed that the apoptosis rate of HepG2, Ad0, Ad-C, Ad-X and Ad-XC cells was 20.7% ± 0.6%, 21.8% ± 0.9%, 12.6% ± 0.8%, 11.7% ± 0.9% and 5.1% ± 0.8%, respectively. The apoptosis rate of the experimental group decreased obviously in comparison to the control group. The apoptosis rate of Ad-XC was the lowest. The apoptosis rate of these five groups of cells differed sharply when compared to each other ($P < 0.0001$, $n = 3$).

Nude mice experiment

Tumor formation was observed at the injection site of mice inoculated with Ad-XC infected LO2 cells (Figure 5), but not in control mice.

Effect of Ad-XC infection on c-myc mRNA expression in HepG2 cells

The mRNA level of c-myc in Ad-XC cells was the highest (Figure 6), indicating that transient expression of *HBV X-HCV C* fusion gene obviously induced expression of c-myc in HepG2 cells.

DISCUSSION

HCC is one of the most common malignant tumors in the world. Chronic hepatitis B and C are responsible for the great majority of cases of HCC worldwide. Both HBV and HCV are parenterally transmitted and superinfection is not uncommon in intravenous drug users and in countries with a high prevalence of HBV[3]. The risk of developing HCC in subjects with both HBV and HCV infections has been investigated in two meta-analyses[4,5], showing that there is a synergistic hepatocarcinogenic interaction between HBV and HCV infections and that the increased risk is super-additive but not multiplicative.

It was reported that transgenic mice with hepatitis B and C have the oncogenic potential of *HBV X* and *HCV C* genes in the liver[6,7]. It was also reported that HBV X and HCV C proteins have an oncogenic potential[8-14], but the involvement of their synergisms in hepatocarcinogenesis remains unclear. HBV X and HCV C proteins additively repress the universal cyclin-dependent kinase inhibitor p21 gene at the transcription level and additively stimulate cell growth, suggesting that additive repression of p21 is important to understand the cooperative development of HCC due to these two proteins[15]. When HBV X and HCV C proteins transform mouse fibroblast NIH3T3 cells in cooperation, they additively stimulate cell growth, especially in the absence of serum growth factors. Cells expressing these two viral proteins exhibit a higher tumorigenicity, as demonstrated in athymic nude mice[16]. HBV X protein

increases liver pathogenesis in HCV transgenic mice by a mechanism involving an imbalance between hepatocyte death and regeneration[17]. In the present study, Ad-XC, Ad-X and Ad-C could facilitate the proliferation activity of HepG2 cells and inhibit their apoptosis *in vitro*. The effect of Ad-XC was significantly stronger than that of Ad-X and Ad-C, suggesting a more than additive but less than multiplicative effect of *HBV X* and *HCV C* genes on hepatocarcinogenesis as demonstrated in athymic nude mice[16].

The increased expression of oncogene is thought to be a major cause for tumor formation/progression. C-myc, an oncogene located on 8q24, may be important in hepatocarcinogenesis. The expression level of c-myc in the cells transiently transfected with the HBV X gene was much higher than that in the control cells[18]. c-myc protein expression above its basal level significantly increased c-myc stability, as revealed by its prolonged intracellular half-life in HepG2 expressing HCV core protein, suggesting that HCV core protein may promote cell cycle progression in HepG2 cells by increasing the stability of c-myc oncoprotein[19]. The present study aimed to evaluate the expression level of c-myc in cells infected with different recombinant adenoviruses by RT-PCR. The highest expression level of c-myc was observed in Ad-XC infected cells, suggesting that up-regulation of c-myc expression may be one of the mechanisms underlying the synergism of *HBV X* and *HCV C* genes in hepatocarcinogenesis.

In conclusion, HBV X and HCV C genes have a synergism in hepatocarcinogenesis. The reasons for the interaction are uncertain, although the increased c-myc expression in the presence of both genes with tumor promoting effects, including the enhanced up-regulation of c-myc expression, may play a role in hepatocarcinogenesis. Interaction between hepatocarcinogenic effects of the two genes remains to be investigated.

ACKNOWLEDGMENTS

The authors thank Professor Tong-Chuan He at the Molecular Oncology Laboratory, The University of Chicago Medical Center for Technical Support.

COMMENTS

Background

Chronic hepatic B virus (HBV) and hepatic C virus (HCV) infection often results in cirrhosis and enhances the risk of developing HCC. The underlying mechanism leading to malignant transformation of infected cells, however, remains unclear. Based on epidemic data, Super-infection with HBV and HCV is associated with an increased frequency in the development of HCC, but the relative mechanism remains to be elucidated. It was reported that both HBV X and HCV core (HCV C) genes play an important role in hepatocarcinogenesis.

Research frontiers

The fact that HBV X and HCV C genes induce HCC in transgenic mice offers more evidence for the relationship between these genes and HCC. However, whether there is a synergism of HBV X and HCV C proteins on hepatocarcinogenesis is still unclear.

Innovations and breakthroughs

In the present study, recombinant adenoviruses expressing HBV X-HCV C fusion protein were constructed, and their effects on biological behavior and c-myc expression level in hepatocytes were investigated.

Applications

HBV X and HCV C genes may have a synergism in hepatocarcinogenesis. The reasons for the interaction are uncertain, although increased c-myc expression in the presence of both genes with tumor promoting effects, including the enhanced up-regulation of c-myc expression, may play a role in hepatocarcinogenesis. Interaction between hepatocarcinogenic effects of the two genes remains to be investigated.

Peer review

This is an elegant, well designed study investigating the combined effect of adenoviruses expressing HBV-X and HCV-C genes on the proliferation and apoptosis of HepG2 cells. The major finding of the study was that the combined effect of the two genes on cell proliferation and apoptosis was superior over that of HBV-X or HCV-C gene alone. The authors also stressed that the underlying mechanism may be, at least, partly explained by the increased c-myc expression.

REFERENCES

1 **He TC**, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci USA* 1998; **95**: 2509-2514
2 **Horton RM**, Hunt HD, Ho SN, Pullen JK, Pease LR. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* 1989; **77**: 61-68
3 **Zarski JP**, Bohn B, Bastie A, Pawlotsky JM, Baud M, Bost-Bezeaux F, Tran van Nhieu J, Seigneurin JM, Buffet C, Dhumeaux D. Characteristics of patients with dual infection by hepatitis B and C viruses. *J Hepatol* 1998; **28**: 27-33
4 **Donato F**, Boffetta P, Puoti M. A meta-analysis of epidemiological studies on the combined effect of hepatitis B and C virus infections in causing hepatocellular carcinoma. *Int J Cancer* 1998; **75**: 347-354
5 **Shi J**, Zhu L, Liu S, Xie WF. A meta-analysis of case-control studies on the combined effect of hepatitis B and C virus infections in causing hepatocellular carcinoma in China. *Br J Cancer* 2005; **92**: 607-612
6 **Kim CM**, Koike K, Saito I, Miyamura T, Jay G. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. *Nature* 1991; **351**: 317-320
7 **Moriya K**, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, Matsuura Y, Kimura S, Miyamura T, Koike K. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med* 1998; **4**: 1065-1067
8 **Zhang JL**, Zhao WG, Wu KL, Wang K, Zhang X, Gu CF, Li Y, Zhu Y, Wu JG. Human hepatitis B virus X protein promotes cell proliferation and inhibits cell apoptosis through interacting with a serine protease Hepsin. *Arch Virol* 2005; **150**: 721-741
9 **Murakami S**. Hepatitis B virus X protein: a multifunctional viral regulator. *J Gastroenterol* 2001; **36**: 651-660
10 **Chan DW**, Ng IO. Knock-down of hepatitis B virus X protein reduces the tumorigenicity of hepatocellular carcinoma cells. *J Pathol* 2006; **208**: 372-380
11 **Lai MM**, Ware CF. Hepatitis C virus core protein: possible roles in viral pathogenesis. *Curr Top Microbiol Immunol* 2000; **242**: 117-134
12 **Shan Y**, Chen XG, Huang B, Hu AB, Xiao D, Guo ZM. Malignant transformation of the cultured human hepatocytes induced by hepatitis C virus core protein. *Liver Int* 2005; **25**: 141-147
13 **Chen RF**, Li ZH, Zou SQ, Chen JS. Effect of hepatitis C virus core protein on modulation of cellular proliferation and apoptosis in hilar cholangiocarcinoma. *Hepatobiliary Pancreat Dis Int* 2005; **4**: 71-74
14 **Koike K**. Molecular basis of hepatitis C virus-associated hepatocarcinogenesis: lessons from animal model studies. *Clin Gastroenterol Hepatol* 2005; **3**: S132-S135
15 **Han HJ**, Jung EY, Lee WJ, Jang KL. Cooperative repression

of cyclin-dependent kinase inhibitor p21 gene expression by hepatitis B virus X protein and hepatitis C virus core protein. *FEBS Lett* 2002; **518**: 169-172

16 **Jung EY**, Kang HK, Chang J, Yu DY, Jang KL. Cooperative transformation of murine fibroblast NIH3T3 cells by hepatitis C virus core protein and hepatitis B virus X protein. *Virus Res* 2003; **94**: 79-84

17 **Keasler VV**, Lerat H, Madden CR, Finegold MJ, McGarvey MJ, Mohammed EM, Forbes SJ, Lemon SM, Hadsell DL, Grona SJ, Hollinger FB, Slagle BL. Increased liver pathology in hepatitis C virus transgenic mice expressing the hepatitis B virus X protein. *Virology* 2006; **347**: 466-475

18 **Zhang X**, Dong N, Zhang H, You J, Wang H, Ye L. Effects of hepatitis B virus X protein on human telomerase reverse transcriptase expression and activity in hepatoma cells. *J Lab Clin Med* 2005; **145**: 98-104

19 **Ruggieri A**, Murdolo M, Harada T, Miyamura T, Rapicetta M. Cell cycle perturbation in a human hepatoblastoma cell line constitutively expressing Hepatitis C virus core protein. *Arch Virol* 2004; **149**: 61-74

S- Editor Zhong XY **L- Editor** Wang XL **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5419

World J Gastroenterol 2008 September 21; 14(35): 5419-5427
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Liver *insulin-like growth factor 2* methylation in hepatitis C virus cirrhosis and further occurrence of hepatocellular carcinoma

Philippe Couvert, Alain Carrié, Jacques Pariès, Jenny Vaysse, Audrey Miroglio, Antoine Kerjean, Pierre Nahon, Jamel Chelly, Jean-Claude Trinchet, Michel Beaugrand, Nathalie Ganne-Carrié

Philippe Couvert, Alain Carrié, INSERM U551, Dyslipoproteinemia and Atherosclerosis Research Unit, Paris F-75013, France; Université Paris6, UMR S551, Paris F-75013, France; AP-HP, Groupe hospitalier Pitié Salpétrière, Service de Biochimie endocrinienne et Oncologique, Paris F-75013, France
Jacques Pariès, Department of Public health, Jean Verdier Hospital, AP-HP, Bondy 93140, France
Jenny Vaysse, Department of Biochemistry, Jean Verdier Hospital, AP-HP, Bondy 93140, France
Pierre Nahon, Jean-Claude Trinchet, Michel Beaugrand, Nathalie Ganne-Carrié, Department of Hepato-Gastroenterology, Jean Verdier Hospital, AP-HP, Bondy 93140, France; UPRES EA3409, University Paris 13, Paris F-75651, France
Audrey Miroglio, Antoine Kerjean, Jamel Chelly, Cochin Institut-INSERM-CNRS-University Paris 5-CHU Cochin, Paris 75014, France
Author contributions: Ganne-Carrié N, Trinchet JC, Carrié A and Beaugrand M designed research; Nahon P selected patients; Pariès J analyzed data; Vaysse J tested IGF2 serum level; Couvert P, Kerjean A, Miroglio A and Chelly J performed molecular analyses; Couvert P and Ganne-Carrié N wrote the paper.
Correspondence to: Dr. Nathalie Ganne-Carrié, Department of Hepato-Gastroenterology, Jean Verdier Hospital, AP-HP, Bondy 93140, France. nathalie.ganne@jvr.aphp.fr
Telephone: +33-1-48026296 Fax: +33-1-48026202
Received: May 23, 2008 Revised: July 14, 2008
Accepted: July 21, 2008
Published online: September 21, 2008

Abstract

AIM: To assess the predictive value of the insulin-like growth factor 2 (*Igf2*) methylation profile for the occurrence of Hepatocellular Carcinoma (HCC) in hepatitis C (HCV) cirrhosis.

METHODS: Patients with: (1) biopsy-proven compensated HCV cirrhosis; (2) available baseline frozen liver sample; (3) absence of detectable HCC; (4) regular screening for HCC; (5) informed consent for genetic analysis were studied. After DNA extraction from liver samples and bisulfite treatment, unbiased PCR and DHPLC analysis were performed for methylation analysis at the *Igf2* locus. The predictive value of the *Igf2* methylation profile for HCC was assessed by Kaplan-Meier and Cox methods.

RESULTS: Among 94 included patients, 20 developed an HCC during follow-up (6.9 ± 3.2 years). The methylation profile was hypomethylated, intermediate and hypermethylated in 13, 64 and 17 cases, respectively. In univariate analysis, two baseline parameters were associated with the occurrence of HCC: age (P = 0.01) and prothrombin (P = 0.04). The test of linear tendency between the three ordered levels of *Igf2* methylation and probability of HCC occurrence was significant (Log Rank, P = 0.043; Breslow, P = 0.037; Tarone-Ware, P = 0.039).

CONCLUSION: These results suggest that hypomethylation at the *Igf2* locus in the liver could be predictive for HCC occurrence in HCV cirrhosis.

Key words: Liver cancer; Cirrhosis; Insulin-growth factor 2, DNA methylation

Peer reviewer: Dr. Maribel Rodriguez-Torres, Fundacion De Investigacion De Diego, Ave. De Diego 359 Suite 302, Santurce 00909, Puerto Rico

Couvert P, Carrié A, Paries J, Vaysse J, Miroglio A, Kerjean A, Nahon P, Chelly J, Trinchet JC, Beaugrand M, Ganne-Carrié N. Liver *insulin-like growth factor 2* methylation in hepatitis C virus cirrhosis and further occurrence of hepatocellular carcinoma. *World J Gastroenterol* 2008; 14(35): 5419-5427 Available from: URL: http://www.wjgnet.com/1007-9327/14/5419.asp DOI: http://dx.doi.org/10.3748/wjg.14.5419

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent malignant neoplasms worldwide[1], and its incidence has increased in the past decade in Europe and the USA[2-5]. In Western countries, HCC mostly develops in the presence of cirrhosis. Chronic Hepatitis C virus (HCV) infection plays an important role in the increased incidence of HCC in the western world[6] where HCC is presently the leading cause of death

of patients with HCV related cirrhosis[7]. In these patients, the annual incidence of HCC varies from 2% to 6%[8-13]. The main predictive factors of HCC in patients with HCV-cirrhosis are age over 50[14-17], male gender[12,14,15,17], increased serum alpha-fetoprotein (AFP) baseline levels[12,18,19], symptoms of portal hypertension, such as thrombopenia[17,18] or esophageal varices[17,19], obesity[20,21] and diabetes[22,23]. Identification of molecular abnormalities associated with an increased risk of HCC is particularly important to improve knowledge of both the pathways of liver carcinogenesis and the outcomes.

Insulin-like growth factor 2 (IGF2) is a fetal growth peptide produced by the liver which is structurally and functionally closely related to insulin[24]. It is over expressed in a wide variety of neoplasms[25,26] and is involved in experimental liver carcinogenesis. *In vitro*, a pathophysiological link between IGF2 over expression and hepatocyte proliferation was demonstrated by Lin *et al*[27], who found high concentrations of IGF2 in human hepatoma cell lines HuH7 and HepG2 and showed that antisense oligonucleotides complementary to *Igf2* mRNA reduced both *Igf2* mRNA and protein, in association with decreased cell proliferative activity. *In vivo*, Rogler *et al*[28] reported an increased frequency of HCC in *Igf2* transgenic mice and serum IGF2 has been recently proposed as a marker for human HCC to improve the diagnostic accuracy and sensitivity in patients with low serum AFP level[29].

Various epigenetic alterations have been reported in human cancers, including global DNA hypomethylation, gene hypomethylation and promoter hypermethylation, and *Igf2* loss of imprinting[30]. The *Igf2* gene is controlled by genomic imprinting, a non-Mendelian inherited epigenetic process that leads to the silencing of either a maternal or paternal allele[31,32]. In the liver, unlike in other tissues, its expression is monoallelic (maternally imprinted) during the fetal period and becomes biallelic thereafter. Early observations showed over expression of the *Igf2* gene in liver tumors and preneoplastic hepatic foci in different animal models as well as in human HCC[33,34]. This over expression is associated with re-expression of the fetal pattern of *Igf2* transcripts and restoration of monoallelic *Igf2* expression in preneoplastic hepatic foci[35] as well as in HCC[36], and with re-expression of monoallelic fetal promoters P2-P4[37] and loss of activity of the adult biallelic promoter P1[38]. One key factor of these epigenetic changes is the alteration of the genomic methylation pattern within regulatory Differentially Methylated Regions (DMRs) of imprinted genes, which inappropriately leads to loss of imprinting in the *Igf2* gene[39] and to transcriptional activation of the normally silent maternal allele. Hypomethylation at the *Igf2* locus has been found in many type of cancers, including ovarian, lung and colon[40]. In a previous study analyzing the methylation status of *Igf2* DMR2 in 71 liver samples from mostly viral HCC compared to 6 normal liver samples, we observed a hypomethylated profile at the *Igf2* locus in 89% of cases of HCC in contrast with the pattern observed in normal livers[41]. In addition, Cui *et al*[42] showed that hypomethylation of the *Igf2* gene in peripheral blood lymphocytes (PBL) is associated with a predisposition to colorectal cancer, suggesting that the epigenetic alteration of *Igf2* could be an early event in colorectal carcinogenesis.

The aim of the present study was to investigate whether hypomethylation at the *Igf2* locus in the liver is a predisposing factor for HCC in patients with HCV-related cirrhosis. Thus, we analyzed the methylation status of the *Igf2* gene spanning the 11p15 imprinted domain in patients with compensated HCV-related cirrhosis who were prospectively followed-up with periodic HCC screening.

MATERIALS AND METHODS

Patients

Ninety-four patients were retrospectively selected for this study among all patients hospitalized for liver biopsy between January, 1989 and December, 2000 in our department, based on the following criteria: (1) compensated (Child-Pugh A) HCV-related cirrhosis with presence of serum HCV RNA; (2) absence of viral co-infection by hepatitis B virus or human immunodeficiency virus; (3) regular follow-up until death with periodic HCC screening by liver ultrasonography and test of serum AFP levels every 6 mo at least; (4) absence of detectable HCC at enrollment; (5) available baseline frozen (-80℃) liver biopsy specimen for genetic study; (6) informed consent for genetic analysis obtained from the patient according to French guidelines.

Baseline demographic, clinical, biological and histological data (at time of liver biopsy) were recorded. All patients were prospectively followed-up. Complete physical examination, standard biochemical tests, serum AFP determination and abdominal ultrasonography were repeated every 6 mo. When a focal liver lesion or increased AFP levels were detected, tomodensitometry and, whenever possible, fine needle guided liver biopsy were performed. Diagnostic criteria for HCC were: (1) histological and (2) clinical, in patients with AFP value greater than 400 ng/mL and evidence of focal liver lesion at imaging techniques. After 2002, the HCC diagnosis was based on the guidelines of the European Association for the Study of the Liver[43].

Twenty-five histopathologically normal liver samples were also studied as control cases.

DNA extraction, bisulfite treatment of DNA and methylation analysis

DNA from frozen liver biopsies was extracted and treated with sodium bisulfite. Unbiased PCR amplification and Denaturing High Performance Liquid Chromatography (DHPLC) analysis were

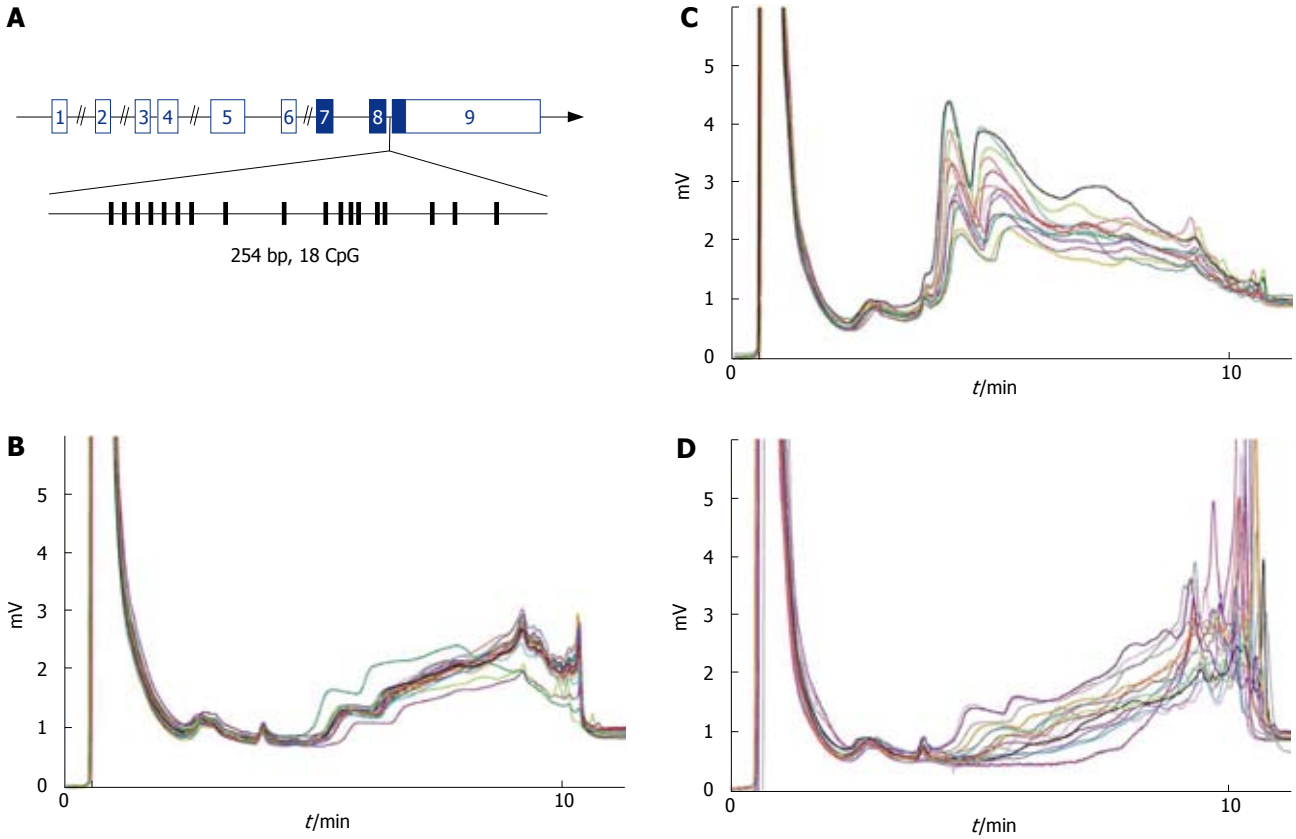


Figure 1 Methylation analysis of *Igf2* DMR2 in normal and HCV related cirrhosis livers. **A**: Exon-intron structure of *Igf2* gene. Exons are shown as numbered boxes (plain are coding). The 254bp fragment of *Igf2* DMR2 amplified for methylation analysis is enlarged below. Vertical lines indicate CpG positions. **B**: DHLPLC chromatograms of PCR products from normal liver samples. Twenty-two out of 25 are superimposable, and this major profile was used to assess hypermethylated profiles (ie more methylated than normal liver). **C** and **D**: DHPLC chromatograms of PCR products from HCV-related cirrhosis. Among 94 samples, 13 (**C**) and 17 (**D**) samples show respectively hypomethylated and hypermethylated profiles.

used for methylation analysis at the DMR of exon 8 and 9 of the *Igf2* imprinted gene (Figure 1A), as previously reported[42]. We amplified a DNA fragment encompassing 18 CpGs of the *Igf2* gene (Accession number AC005809; nt 43058-43312; 254 bp) by nested-PCR using the following primers: forward external 5'-GTTAAGAGGTTTTAGAGGTTATAGG-3', reverse external 5'-CCTTCCAAAACCTAACCTAAAAACA-3', forward internal 5'-GGGAAAGGGGTTTAGGAT-TTTTAT-3', reverse internal 5'-ATAATTTACTCCCCC-TTCAACCTC-3'. PCRs were performed in 3 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, 0.5 μmol/L of each primer and 1.25 U of AmpliTaq Gold® DNA polymerase (Perkin Elmer, Norwalk, CT) under the following conditions: 94℃ for 10 min followed by 40 cycles of 94℃ for 45 s, 62℃ for 45 s, 72℃ for 1 min and a final extension step of 10 min at 72℃. After the first round of DNA amplification, a 1 μL aliquot of the PCR solution was used for the nested PCR.

Methylation profiles were studied by a newly developed DHPLC-based method, as previously described[44]. Briefly, DHPLC scanning was performed on an automated DHPLC instrument (WAVE®, Hitachi model D-7000. Chromatography Data Station Software, Transgenomic LTD Cheshire, UK); the column used was a DNAsep® Cartridge (Transgenomic, Santa Clara, CA) and the running temperature experimentally evaluated was 57℃. In a second step, methylation profiles of PCR products from liver biopsies were analyzed in comparison with PCR products from reference 100% methylated and 0% methylated control alleles, and with PCR product from normal liver biopsies. Fifteen microliter aliquots of the PCR products were eluted within a linear acetonitrile gradient. Because of the difference in retention times, the methylation patterns could be assessed by DHPLC independently of sequencing information by overlaying the DHPLC profiles with those of reference fragments. Methylation profiles were objectively classified in three categories as follows: samples displaying a higher proportion of methylated alleles than normal liver were considered as hypermethylated (M); samples which showed less methylation than normal liver were sorted according to the maximal absorbances of their first (4 min < retention time < 5 min, demethylated alleles) and last (10 min < retention time < 11 min, methylated alleles) elution specific peaks, by calculating R = [Abs (first peak) - Abs (baseline)]/[Abs (last peaks) - Abs (baseline)]; samples with R>2 (high proportion of demethylated alleles) were considered hypomethylated (U), the others being intermediate (UM). Reproducibility of the method was checked by double testing of randomly chosen

Table 1 Baseline characteristics in 94 patients with Child-Pugh A hepatitis C-related cirrhosis and defined methylation profile at the *insulin growth factor 2* gene: distribution and prognostic value for the occurrence of hepatocellular carcinoma at 10 years in univariate analysis

		Patients (n = 94)	No HCC (n = 74)	HCC (n = 20)	HR (95% CI)	P
Gender	Female	39	32	7	1	
	Male	55	42	13	1.38 (0.55-3.48)	0.49
Age (yr)		57.7 ± 13.7	56.0 ± 14.4	63.9 ± 8.0	1.05 (1.01-1.09)	0.01
BMI (kg/m^2)		25.0 ± 4.8	25.1 ± 4.8	24.7 ± 4.8	1.00 (0.90-1.11)	0.96
BMI ⩾ 30 kg/m^2	No	80	64	16	1	
	Yes	14	10	4	2.16 (0.72-6.51)	0.17
Diabetes	No	73	57	16	1	
	Yes	21	17	4	1.00 (0.33-2.98)	0.99
Oesophageal	0 or Ⅰ	78	63	15	1	
Varices grade	Ⅱ or Ⅲ	16	11	5	2.47 (0.89-6.83)	0.08
Bilirubin (μmol/L)		15.1 ± 8.8	14.6 ± 8.3	17.2 ± 10.4	1.05 (1.00-1.10)	0.05
Albumin (g/L)		41.1 ± 5.7	41.1 ± 5.6	41.0 ± 6.3	0.98 (0.90-1.06)	0.59
Prothrombin (%)		84.1 ± 16.2	85.2 ± 15.9	79.7 ± 16.9	0.97 (0.95-1.00)	0.04
Platelets (× 10^3/mm^3)		151 ± 61	155 ± 64	138 ± 51	1.00 (0.99-1.00)	0.29
ALAT (× ULN)		3.2 ± 2.5	3.2 ± 2.6	3.2 ± 1.8	0.97 (0.80-1.19)	0.80
ASAT (× ULN)		2.9 ± 2.1	2.8 ± 2.0	3.2 ± 2.5	1.10 (0.91-1.33)	0.33
GGT (× ULN)		2.8 ± 2.7	2.8 ± 2.9	2.7 ± 1.7	0.99 (0.84-1.16)	0.86
AFP (ng/mL)		16.4 ± 26.9	17.2 ± 29.2	13.3 ± 15.7	1.01 (0.99-1.02)	0.54
Knodell score		10.8 ± 2.2	10.6 ± 2.4	11.7 ± 2.4	1.23 (1.00-1.51)	0.06
Serum IGF2 (ng/mL)[1]		279.6 ± 114.3	291.4 ± 117.3	234.1 ± 92.1	0.99 (0.98-1.01)	0.08
Liver Igf2	U	13	9	4	7.64 (0.85-68.62)	0.07
Methylation profile	UM	64	49	15	3.98 (0.53-30.14)	0.18
(3 classes)	M	17	16	1	1	

Continuous values are used for quantitative parameters. HR: Hazard ratio; CI: Confidence interval; HCC: Hepatocellular carcinoma; BMI: Body mass index; ALAT: Alanine amino-transferase; GGT: Gamma glutamyl transferase; ULN: Upper limit of normal; AFP: Alpha-foetoprotein; IGF2: Insulin-growth factor 2. [1]Performed in 63 patients.

samples. To rule out interpretation bias, clinical database including outcome of the patients, especially in relation to the occurrence of HCC, was kept by clinicians (NG, PN, JCT, MB) and not available for molecular biologists (PC, AK, AM, JC).

IGF2 serum quantification

Frozen serum collected at enrollment and stored at -25℃ was available in 63 (67%) of the 94 included patients. In these patients, serum IGF2 was quantified using an enzymatic amplified "two step" sandwich-type immunoassay (active IGF2 ELISA, Diagnostic Systems Laboratories, Webster, USA). Each sample was duplicated and tested blindly.

Statistical analysis

Data were expressed as mean ± SEM and percentages. All means were compared using the Mann-Whitney rank-sum test or the Kruskal-Wallis nonparametric analysis of variance. Furthermore, continuous variables were transformed into binary information according to median and cut-off points. Associations were tested in 2 × 2 cross tabulations using the Fisher's exact test. In case of larger cross tabulations, and as appropriate according to the validity conditions, liaisons were tested by the Pearson's Chi-square, or by computing either the exact probability value or the Monte Carlo estimate of the exact probability value. The basic non parametric Kaplan-Meier method[45] was used to search for heterogeneity of time-dependent cumulative probabilities of HCC according to levels of methylation and a linear trend between HCC probability and ordered methylation levels. From then on and practically, we used a series of tests, the Log Rank (Mantel-Cox) test, the Breslow test (Generalized Wilcoxon), and Tarone-Ware test in the two situations in which we attempted to test the heterogeneity of HCC occurrence or a linear trend between HCC probability and ordered methylation levels[46]. As regards to heterogeneity of risk according to *Igf2* levels, the Cox regression[47] was used for the estimation of the Hazard Ratios and 95% CI intervals. The 0.05 probability level was used for all statistical significance. Statistical analyses were performed using SPSS software (SPSS 10.05, SPSS Inc., Chicago, IL) and STATXACT (StatXact, CYTEL Software Corporation, Cambridge, MA).

In Table 1, the expression HR = 1.05 (1.01-1.09) is linguistically awkward, henceforth, 1.05 indicates that with each extra year in age the estimated hazard is 1.05 times that for subjects one year younger. Another way to express this variation is to convert it into a percentage difference in hazard by using the expression 100 × (HR - 1). Then, 100 × (1.05 - 1) = 5% tells us that the HR of HCC is 5% higher for each additional year of age.

RESULTS

Characterization and interpretation of methylation profiles

Methylation profiles of normal liver samples were highly

Table 2 Baseline characteristics according to the methylation profile at the *Igf2* locus (U, UM, and M, respectively, for hypomethylated, normal and hypermethylated patterns) in patients with Child-Pugh A hepatitis C-related cirrhosis

	U (*n* = 13)	UM (*n* = 64)	M (*n* = 17)	Asymptotic global *P*-value
Male gender (%)	7 (53.8%)	38 (59.4%)	10 (58.8%)	0.934
Age (yr)	58.15 (16.71)	57.55 (13.00)	57.89 (14.41)	0.985
Alcohol (g/d)	43.08 (64.21)	22.28 (45.97)	38.24 (77.48)	0.551
Tobacco (Pack, yr)	3.8 (7.1)	6.1 (13.1)	5.7 (11.5)	0.981
BMI (kg/m^2)	26.32 (5.18)	25.41 (4.97)	23.86 (4.04)	0.244
Diabetes (%)	2 (15.4%)	14 (21.9%)	4 (23.5%)	0.883
Platelets (× 10^3/mm^3)	147.28 (64.46)	152.32 (63.71)	177.30 (127.43)	0.958
Prothrombin (%)	79.46 (17.81)	84.58 (16.59)	85.59 (13.28)	0.433
Albumin (g/L)	40.45 (4.96)	40.77 (6.27)	42.76 (3.40)	0.437
Bilirubin (μmol/L)	14.58 (9.66)	15.16 (8.85)	15.50 (8.45)	0.731
ALAT (× ULN)	3.23 (2.13)	3.23 (2.61)	3.14 (2.16)	0.961
AFP (ng/mL)	13.58 (11.24)	13.88 (18.57)	28.21 (51.52)	0.530
Serum IGF2 (ng/mL)[1]	249.64 (81.23)	276.32 (116.97)	321.55 (129.24)	0.393
OV grade Ⅱ or Ⅲ (%)	5 (38.5%)	9 (14.1%)	2 (11.8%)	0.084
Knodell score (mean, SD)	11.6 (1.8)	10.6 (2.3)	10.7 (2.5)	0.414
HCV genotype 1[2] (%)	7 (63.6%)	41 (78.8%)	8 (53.3%)	0.125

Quantitative variables are expressed as means (SD). BMI: Body mass index; ALAT: Alanine amino-transferase; ULN: Upper limit of normal; AFP: Alpha-foetoprotein; IGF2: Insulin growth factor 2; OV: Esophageal varices; HCV: hepatitis C virus. [1]Performed in 63 patients, [2]known in 78 patients (11, 52, 15 patients respectively in U, UM and M groups).

similar (Figure 1B) and 22 of 25 were superimposable. Among 94 tested patients, 13 (14%) were considered as hypomethylated (U), 64 (68%) as intermediate (UM) and 17 (18%) as hypermethylated (M) (Figure 1C). All double tested samples showed similar results in both experiments (data not shown).

Baseline patient characteristics

The main characteristics of patients at enrollment were not significantly different according to the methylation profile at the *Igf2* locus as shown in Table 2. All patients but 2 were Caucasians (1 from Africa with M profile and 1 from Asia with UM profile). In addition, the proportion of patients who received antiviral treatment during the study (72.7% in U, 58.8% in UM and 61.7% in M; $P = 0.738$), the proportion of sustained responders (22.2% in U, 31.4% in UM and 40.0% in M; $P = 0.707$), the mean follow-up (5.20 ± 3.63 years in U, 7.21 ± 3.09 years in UM, 7.22 ± 3.04 years in M; $P = 0.198$), were not statistically different between the three groups.

IGF2 serum quantification

Each IGF2 serum level measurement was duplicated and results were reproducible in 98% of cases. The mean serum value was 279.6 ng/mL (range, 36-640) without any significant difference between patients with U, UM or M methylation profiles at the *Igf2* locus (Table 2).

Predictive value for HCC

During a mean follow-up of 6.9 ± 3.2 years, 20 patients developed an HCC (4, 15 and 1 cases, in patients with U, UM and M methylation profile, respectively). The cumulative incidence of HCC at 10 years reaches 30.8% in patients with a U profile, 23.4% in patients with a UM profile, and 24.7% in patients with either a U or a UM profile in contrast with 5.9% only in patients with a M profile.

In the Cox analysis testing successively each of the 17 baseline studied variables, two were predictive for the occurrence of HCC: age at liver biopsy ($P = 0.01$; HR, 1.05; 95% CI, 1.01-1.09) and prothrombin time ($P = 0.04$, HR, 0.97; 95% CI, 0.95-1.00; Table 1). Moreover, a clear trend ($0.05 < P < 0.1$) was observed for 5 baseline variables: bilirubin, esophageal varices, Knodell score, serum IGF2 level and liver *Igf2* hypomethylation.

When patients with a U profile were compared in a paired way to those with M profile, the Log-Rank test was significant ($P = 0.047$). Moreover, the test for linear tendency between the 3 ordered levels of *Igf2* methylation and cumulative probability of HCC occurrence was significant (Log Rank, $P = 0.043$; Breslow, $P = 0.037$; Tarone-Ware, $P = 0.039$; Figure 2).

DISCUSSION

A growing body of evidence underlines that both DNA hypomethylation, leading to genomic instability, and regional CpG hypermethylation, leading to silence tumor suppressor gene, are dominant events during HCC development[48]. Calvisi *et al* recently showed that the extent of genome-wide hypomethylation progressively increased from non-neoplastic surrounding liver to fully malignant HCC[49], indicating that genomic hypomethylation is an important prognostic factor in HCC and opens the possibility of using molecular targets for chemoprevention or

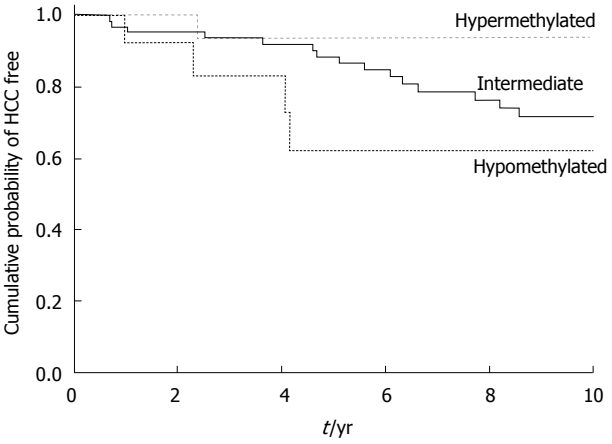


Figure 2 Occurrence of hepatocellular carcinoma at 10 years according to the methylation profile at the *Igf2* gene in 94 patients with Child-Pugh A hepatitis C-related cirrhosis (Kaplan-Meier method). Test of heterogeneity of HCC distributions: (log-rank test) $P = 0.13$. Test of the linear trend between levels of *Igf2* methylation and corresponding survival functions: Breslow (Generalized Wilcoxon) $P = 0.037$.

treatment of HCC. Regarding *Igf2* locus, we have observed hypomethylation at *Igf2* exon 8-9 in 90% (28/31) of HCV associated HCC, in contrast to the normal methylation pattern of two other genes located in the same area, the 11p15 locus[41]. This indicates that alterations in the IGF2 pathway are a pivotal event in hepatocarcinogenesis, at least in patients with HCV-related cirrhosis.

Our results suggest a possible link between *Igf2* hypomethylation in the liver of caucasians with uncomplicated and compensated HCV cirrhosis and the further occurrence of HCC. We observed a significant increased cumulative incidence of HCC at 10 years in patients with a hypomethylated pattern compared with those with a hypermethylated profile (30.8% *versus* 5.9%, $P = 0.047$) and a significant linear tendency between the ordered levels of *Igf2* methylation and the probability of HCC occurrence. The other variables identified in our patients were 2 well-known predictive factors for the occurrence of HCC: age[14-17] and prothrombin time related to liver failure[12,16-18] (Table 1). Due to a low number of patients, we could not show significant link with other known predictive factors (esophageal varices and bilirubin serum levels $0.05 < P < 0.08$). Conversely, as previous studies in patients with HCV-cirrhosis, we did not identify male gender[16,19], high AFP serum level[14,17], low platelet count[14,16,19] and diabetes[21] as predictive factors for the occurrence of HCC. These results, observed in Caucasians, may be different in other ethnic groups. However, IGF2 overexpression in HCC, which is mainly due to aberrant activation of the epigenetically regulated *Igf2* promoters, seems to be independent of ethnic origins. In addition to hypermethylation of promoters of several tumor suppressing genes found even in premalignant conditions, *Igf2* hypomethylation could thus contribute to the multistep process leading to malignant transformation[50].

This link between *Igf2* methylation and HCC occurrence should be validated in an external independent cohort. It may be underestimated in this study for several reasons. First, although the cumulative incidence was as high as previously reported in HCV-cirrhosis, the relative number of patients who further developed HCC ($n = 20$) is low. Secondly, being given cirrhotic liver heterogeneity and the small size of liver samples obtained by fine needle percutaneous biopsy, the extent of *Igf2* hypomethylation could have been underestimated. In addition, as samples were not microdissected, we analyzed not only hepatocytes, but also a variable amount of other minority hepatic cell types, such as sinusoidal cells and Kupffer cells, which may not share the same methylation pattern. Lastly, *Igf2* hypomethylation could be a late event in hepatocarcinogenesis, present to a low extent in uncomplicated cirrhosis and occurring later with the onset of liver failure and/or portal hypertension.

If there is a true link, whether these altered methylation patterns at *Igf2* locus lead to significant changes in expression profile and the function of genetic networks, or whether these changes just indicate severe epigenetic disturbances, remains to be investigated. A link between increased IGF2 expression and HCV infection has already been reported, showing that IGF2 over expression is significantly associated with HCV replication in patients with HCV-related cirrhosis[51]. The persistent process of hepatocyte damage and regeneration in HCV chronic hepatitis could provoke uncontrolled growth of hepatocytes and lead to malignant transformations due to disruption of growth regulation or mitogenic factors. However, whether HCV plays a direct or indirect role in IGF2 deregulation remains unknown. One could wonder if the link between Igf2 and HCC could be mediated by diabetes and metabolic syndrome. Indeed, epidemiological association between diabetes mellitus and HCC has been corroborated by molecular studies related to IGF1 or Igf Binding Protein 3. However, conversely to IGF1, IGF2 is mainly a fetal protein and its insulin-like metabolic effects in the post-natal period remains uncertain[52].

In these experiments, epigenetic changes in *Igf2*, potentially leading to re-expression of its fetal pattern, could be considered in parallel with AFP over expression as the hallmark of some fetal characteristics in the cirrhotic liver. The lack of correlation between the *Igf2* intron 8-9 methylation profile and IGF2 serum levels may be explained by an IGF2 local over expression leading to an autocrine effect, as previously suggested by Cariani *et al*[53].

Our observation that hypomethylation at the *Igf2* exon 8-9 is present in 14% of patients (13/94) with uncomplicated HCV-related cirrhosis and associated with a trend of overrisk of cancer are comparable to recent studies in the field of colorectal cancer[42,54]. Cui

et al[42] observed *Igf2* hypomethylation in normal colonic mucosa in 30% of patients with colorectal cancer in contrast to 10% in healthy patients. Moreover, *Igf2* hypomethylation is present in mesoderm-derived PBL and abnormal methylation profiles in this tissue are also highly correlated with both familial and personal histories of colorectal cancer. The prevalence of abnormal methylation patterns in PBL increases from 6.5% in patients with no personal history of colorectal cancer to 23% and 28%, respectively in patients with a personal history of adenoma or a family history of colorectal cancer, and to 56% in patients with colorectal cancer. These facts support a possible role of epigenetic changes of *Igf2* in the early steps of colorectal carcinogenesis. The most obvious unanswered question is whether the *Igf2* hypomethylation profile in PBL could be constitutive, resulting from inherited genetic mutations, or due to environmental events leading to epigenetic alterations. To try to answer this question in the field of HCC, further studies are ongoing in the PBL of a large cohort of patients with HCV-related cirrhosis screened for HCC and in healthy controls. If the results are comparable to those in patients with colorectal cancer, this may have clinical implications for defining high risk patients for HCC eligible for intensive screening and/or to reduce their risk with the use of dietary and/or therapeutic agents developed to reverse the epigenetic alterations such as methylated-oligonucleotides[55-57].

COMMENTS

Background

Hepatocellular carcinoma (HCC) is considered the fifth most frequent malignant neoplasm worldwide. In high incidence areas, it is strongly associated with viral hepatitis B and C and liver cirrhosis. Identification of molecular abnormalities associated with an increased risk of HCC is particularly important to improve knowledge of both the pathways of liver carcinogenesis and the outcomes.

Research frontiers

Insulin-like growth factor 2 (IGF2) is a fetal growth peptide produced by the liver, which is over expressed in a wide variety of neoplasms including HCC and is involved in experimental liver carcinogenesis. In a previous work analyzing the methylation status of *Igf2* in 71 HCC liver samples, we observed an hypomethylated profile in 89% of HCC.

Innovation and breakthroughs

Not only can *Igf2* hypomethylation be observed in HCC liver samples, but also in premalignant hepatitis C cirrhotic livers. In this case, *Igf2* hypomethylation is associated with a higher risk of HCC occurrence than *Igf2* hypermethylation.

Applications

Studies examining the Igf2 methylation status in hepatitis C cirrhotic liver could help identify patients with a high risk and patients with a low risk of HCC occurrence.

Peer review

This is a well designed study with interesting results. These results suggest that hypomethylation at the *Igf2* locus in the liver could be predictive for HCC occurrence in HCV cirrhosis.

REFERENCES

1 **Bosch FX**, Ribes J, Borras J. Epidemiology of primary liver cancer. *Semin Liver Dis* 1999; **19**: 271-285
2 **De Vos Irvine H**, Goldberg D, Hole DJ, McMenamin J. Trends in primary liver cancer. *Lancet* 1998; **351**: 215-216
3 **Deuffic S**, Poynard T, Buffat L, Valleron AJ. Trends in primary liver cancer. *Lancet* 1998; **351**: 214-215
4 **El-Serag HB**, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. *N Engl J Med* 1999; **340**: 745-750
5 **Taylor-Robinson SD**, Foster GR, Arora S, Hargreaves S, Thomas HC. Increase in primary liver cancer in the UK, 1979-94. *Lancet* 1997; **350**: 1142-1143
6 **El-Serag HB**, Mason AC. Risk factors for the rising rates of primary liver cancer in the United States. *Arch Intern Med* 2000; **160**: 3227-3230
7 **Benvegnu L**, Gios M, Boccato S, Alberti A. Natural history of compensated viral cirrhosis: a prospective study on the incidence and hierarchy of major complications. *Gut* 2004; **53**: 744-749
8 **Bolondi L**, Sofia S, Siringo S, Gaiani S, Casali A, Zironi G, Piscaglia F, Gramantieri L, Zanetti M, Sherman M. Surveillance programme of cirrhotic patients for early diagnosis and treatment of hepatocellular carcinoma: a cost effectiveness analysis. *Gut* 2001; **48**: 251-259
9 **Colombo M**, de Franchis R, Del Ninno E, Sangiovanni A, De Fazio C, Tommasini M, Donato MF, Piva A, Di Carlo V, Dioguardi N. Hepatocellular carcinoma in Italian patients with cirrhosis. *N Engl J Med* 1991; **325**: 675-680
10 **Kobayashi K**, Sugimoto T, Makino H, Kumagai M, Unoura M, Tanaka N, Kato Y, Hattori N. Screening methods for early detection of hepatocellular carcinoma. *Hepatology* 1985; **5**: 1100-1105
11 **Cottone M**, Turri M, Caltagirone M, Parisi P, Orlando A, Fiorentino G, Virdone R, Fusco G, Grasso R, Simonetti RG. Screening for hepatocellular carcinoma in patients with Child's A cirrhosis: an 8-year prospective study by ultrasound and alphafetoprotein. *J Hepatol* 1994; **21**: 1029-1034
12 **Zoli M**, Magalotti D, Bianchi G, Gueli C, Marchesini G, Pisi E. Efficacy of a surveillance program for early detection of hepatocellular carcinoma. *Cancer* 1996; **78**: 977-985
13 **Oka H**, Kurioka N, Kim K, Kanno T, Kuroki T, Mizoguchi Y, Kobayashi K. Prospective study of early detection of hepatocellular carcinoma in patients with cirrhosis. *Hepatology* 1990; **12**: 680-687
14 **Bruno S**, Silini E, Crosignani A, Borzio F, Leandro G, Bono F, Asti M, Rossi S, Larghi A, Cerino A, Podda M, Mondelli MU. Hepatitis C virus genotypes and risk of hepatocellular carcinoma in cirrhosis: a prospective study. *Hepatology* 1997; **25**: 754-758
15 **Chiba T**, Matsuzaki Y, Abei M, Shoda J, Aikawa T, Tanaka N, Osuga T. Multivariate analysis of risk factors for hepatocellular carcinoma in patients with hepatitis C virus-related liver cirrhosis. *J Gastroenterol* 1996; **31**: 552-558
16 **Fattovich G**, Giustina G, Degos F, Diodati G, Tremolada F, Nevens F, Almasio P, Solinas A, Brouwer JT, Thomas H, Realdi G, Corrocher R, Schalm SW. Effectiveness of interferon alfa on incidence of hepatocellular carcinoma and decompensation in cirrhosis type C. European Concerted Action on Viral Hepatitis (EUROHEP). *J Hepatol* 1997; **27**: 201-205
17 **Degos F**, Christidis C, Ganne-Carrie N, Farmachidi JP, Degott C, Guettier C, Trinchet JC, Beaugrand M, Chevret S. Hepatitis C virus related cirrhosis: time to occurrence of hepatocellular carcinoma and death. *Gut* 2000; **47**: 131-136
18 **Ikeda K**, Saitoh S, Kobayashi M, Suzuki Y, Suzuki F, Tsubota A, Arase Y, Murashima N, Chayama K, Kumada H. Long-term interferon therapy for 1 year or longer reduces

the hepatocellular carcinogenesis rate in patients with liver cirrhosis caused by hepatitis C virus: a pilot study. *J Gastroenterol Hepatol* 2001; **16**: 406-415

19 **Gramenzi A**, Andreone P, Fiorino S, Camma C, Giunta M, Magalotti D, Cursaro C, Calabrese C, Arienti V, Rossi C, Di Febo G, Zoli M, Craxi A, Gasbarrini G, Bernardi M. Impact of interferon therapy on the natural history of hepatitis C virus related cirrhosis. *Gut* 2001; **48**: 843-848

20 **N'Kontchou G**, Paries J, Htar MT, Ganne-Carrie N, Costentin L, Grando-Lemaire V, Trinchet JC, Beaugrand M. Risk factors for hepatocellular carcinoma in patients with alcoholic or viral C cirrhosis. *Clin Gastroenterol Hepatol* 2006; **4**: 1062-1068

21 **Ioannou GN**, Splan MF, Weiss NS, McDonald GB, Beretta L, Lee SP. Incidence and predictors of hepatocellular carcinoma in patients with cirrhosis. *Clin Gastroenterol Hepatol* 2007; **5**: 938-945, 945.e1-945.e4

22 **Lai MS**, Hsieh MS, Chiu YH, Chen TH. Type 2 diabetes and hepatocellular carcinoma: A cohort study in high prevalence area of hepatitis virus infection. *Hepatology* 2006; **43**: 1295-1302

23 **Veldt BJ**, Chen W, Heathcote EJ, Wedemeyer H, Reichen J, Hofmann WP, de Knegt RJ, Zeuzem S, Manns MP, Hansen BE, Schalm SW, Janssen HL. Increased risk of hepatocellular carcinoma among patients with hepatitis C cirrhosis and diabetes mellitus. *Hepatology* 2008; **47**: 1856-1862

24 **Le Roith D**. Seminars in medicine of the Beth Israel Deaconess Medical Center. Insulin-like growth factors. *N Engl J Med* 1997; **336**: 633-640

25 **LeRoith D**, Roberts CT Jr. The insulin-like growth factor system and cancer. *Cancer Lett* 2003; **195**: 127-137

26 **Scharf JG**, Braulke T. The role of the IGF axis in hepatocarcinogenesis. *Horm Metab Res* 2003; **35**: 685-693

27 **Lin SB**, Hsieh SH, Hsu HL, Lai MY, Kan LS, Au LC. Antisense oligodeoxynucleotides of IGF-II selectively inhibit growth of human hepatoma cells overproducing IGF-II. *J Biochem* 1997; **122**: 717-722

28 **Rogler CE**, Yang D, Rossetti L, Donohoe J, Alt E, Chang CJ, Rosenfeld R, Neely K, Hintz R. Altered body composition and increased frequency of diverse malignancies in insulin-like growth factor-II transgenic mice. *J Biol Chem* 1994; **269**: 13779-13784

29 **Tsai JF**, Jeng JE, Chuang LY, You HL, Ho MS, Lai CS, Wang LY, Hsieh MY, Chen SC, Chuang WL, Lin ZY, Yu ML, Dai CY. Serum insulin-like growth factor-II and alpha-fetoprotein as tumor markers of hepatocellular carcinoma. *Tumour Biol* 2003; **24**: 291-298

30 **Thorgeirsson SS**, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. *Nat Genet* 2002; **31**: 339-346

31 **Falls JG**, Pulford DJ, Wylie AA, Jirtle RL. Genomic imprinting: implications for human disease. *Am J Pathol* 1999; **154**: 635-647

32 **Reik W**, Walter J. Genomic imprinting: parental influence on the genome. *Nat Rev Genet* 2001; **2**: 21-32

33 **Sohda T**, Yun K, Iwata K, Soejima H, Okumura M. Increased expression of insulin-like growth factor 2 in hepatocellular carcinoma is primarily regulated at the transcriptional level. *Lab Invest* 1996; **75**: 307-311

34 **Cariani E**, Lasserre C, Seurin D, Hamelin B, Kemeny F, Franco D, Czech MP, Ullrich A, Brechot C. Differential expression of insulin-like growth factor II mRNA in human primary liver cancers, benign liver tumors, and liver cirrhosis. *Cancer Res* 1988; **48**: 6844-6849

35 **Aihara T**, Noguchi S, Miyoshi Y, Nakano H, Sasaki Y, Nakamura Y, Monden M, Imaoka S. Allelic imbalance of insulin-like growth factor II gene expression in cancerous and precancerous lesions of the liver. *Hepatology* 1998; **28**: 86-89

36 **Takeda S**, Kondo M, Kumada T, Koshikawa T, Ueda R, Nishio M, Osada H, Suzuki H, Nagatake M, Washimi O, Takagi K, Takahashi T, Nakao A, Takahashi T. Allelic-expression imbalance of the insulin-like growth factor 2 gene in hepatocellular carcinoma and underlying disease. *Oncogene* 1996; **12**: 1589-1592

37 **Uchida K**, Kondo M, Takeda S, Osada H, Takahashi T, Nakao A, Takahashi T. Altered transcriptional regulation of the insulin-like growth factor 2 gene in human hepatocellular carcinoma. *Mol Carcinog* 1997; **18**: 193-198

38 **Li X**, Nong Z, Ekstrom C, Larsson E, Nordlinder H, Hofmann WJ, Trautwein C, Odenthal M, Dienes HP, Ekstrom TJ, Schirmacher P. Disrupted IGF2 promoter control by silencing of promoter P1 in human hepatocellular carcinoma. *Cancer Res* 1997; **57**: 2048-2054

39 **Cui H**, Onyango P, Brandenburg S, Wu Y, Hsieh CL, Feinberg AP. Loss of imprinting in colorectal cancer linked to hypomethylation of H19 and IGF2. *Cancer Res* 2002; **62**: 6442-6446

40 **Feinberg AP**. Imprinting of a genomic domain of 11p15 and loss of imprinting in cancer: an introduction. *Cancer Res* 1999; **59**: 1743s-1746s

41 **Poirier K**, Chalas C, Tissier F, Couvert P, Mallet V, Carrie A, Marchio A, Sarli D, Gicquel C, Chaussade S, Beljord C, Chelly J, Kerjean A, Terris B. Loss of parental-specific methylation at the IGF2 locus in human hepatocellular carcinoma. *J Pathol* 2003; **201**: 473-479

42 **Cui H**, Cruz-Correa M, Giardiello FM, Hutcheon DF, Kafonek DR, Brandenburg S, Wu Y, He X, Powe NR, Feinberg AP. Loss of IGF2 imprinting: a potential marker of colorectal cancer risk. *Science* 2003; **299**: 1753-1755

43 **Bruix J**, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, Christensen E, Pagliaro L, Colombo M, Rodes J. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. *J Hepatol* 2001; **35**: 421-430

44 **Couvert P**, Poirier K, Carrie A, Chalas C, Jouannet P, Beldjord C, Bienvenu T, Chelly J, Kerjean A. DHPLC-based method for DNA methylation analysis of differential methylated regions from imprinted genes. *Biotechniques* 2003; **34**: 356-362

45 **Kaplan E**, Meier P. Nonparametric Estimation from Incomplete Observations. *J Am Stat Assoc* 1958; **53**: 457-481

46 **Tarone RE**. Tests for trend in life table analysis. *Biometrika* 1975; **62**: 679-682

47 **Cox DR**. Regression models and life tables (with discussion). *J R Stat Soc* 1972; **34**: 187-220

48 **Teufel A**, Staib F, Kanzler S, Weinmann A, Schulze-Bergkamen H, Galle PR. Genetics of hepatocellular carcinoma. *World J Gastroenterol* 2007; **13**: 2271-2282

49 **Calvisi DF**, Ladu S, Gorden A, Farina M, Lee JS, Conner EA, Schroeder I, Factor VM, Thorgeirsson SS. Mechanistic and prognostic significance of aberrant methylation in the molecular pathogenesis of human hepatocellular carcinoma. *J Clin Invest* 2007; **117**: 2713-2722

50 **Tischoff I**, Tannapfe A. DNA methylation in hepatocellular carcinoma. *World J Gastroenterol* 2008; **14**: 1741-1748

51 **Tanaka S**, Takenaka K, Matsumata T, Mori R, Sugimachi K. Hepatitis C virus replication is associated with expression of transforming growth factor-alpha and insulin-like growth factor-II in cirrhotic livers. *Dig Dis Sci* 1996; **41**: 208-215

52 **Bach LA**, Rechler MM. Insulin-like growth factors and diabetes. *Diabetes Metab Rev* 1992; **8**: 229-257

53 **Cariani E**, Seurin D, Lasserre C, Franco D, Binoux M, Brechot C. Expression of insulin-like growth factor II (IGF-II) in human primary liver cancer: mRNA and protein analysis. *J Hepatol* 1990; **11**: 226-231

54 **Cruz-Correa M**, Cui H, Giardiello FM, Powe NR, Hylind L, Robinson A, Hutcheon DF, Kafonek DR, Brandenburg S, Wu Y, He X, Feinberg AP. Loss of imprinting of insulin growth factor II gene: a potential heritable biomarker for colon neoplasia predisposition. *Gastroenterology* 2004; **126**: 964-970

55 **Yao X**, Hu JF, Daniels M, Shiran H, Zhou X, Yan H, Lu H, Zeng Z, Wang Q, Li T, Hoffman AR. A methylated oligonucleotide inhibits IGF2 expression and enhances survival in a model of hepatocellular carcinoma. *J Clin Invest* 2003; **111**: 265-273

56 **Santini V**, Kantarjian HM, Issa JP. Changes in DNA methylation in neoplasia: pathophysiology and therapeutic implications. *Ann Intern Med* 2001; **134**: 573-586

57 **Esteller M**. Relevance of DNA methylation in the management of cancer. *Lancet Oncol* 2003; **4**: 351-358

S- Editor Zhong XY **L- Editor** Rippe RA **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5428

World J Gastroenterol 2008 September 21; 14(35): 5428-5431
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Distribution of secretory inhibitor of platelet microbicidal protein among anaerobic bacteria isolated from stool of children with diarrhea

Iuri B Ivanov, Viktor A Gritsenko

Iuri B Ivanov, Viktor A Gritsenko, Department of Human Microbiology, Institute of Cellular and Intracellular Symbiosis, Russian Academy of Sciences, Pionerskaya st., 11, Orenburg 460000, Russia

Author contributions: Ivanov IB and Gritsenko VA contributed equally to this work.

Supported by The Russian Foundation of Basic Research and Government of Orenburg region, No. 07-04-97624 and No. 08-04-99105

Correspondence to: Dr. Iuri B Ivanov, Department of Human Microbiology, Institute of Cellular and Intracellular Symbiosis, Russian Academy of Sciences, Volgogradskaya st., 36/3, of .93, Orenburg 460056, Russia. mil-for@rambler.ru
Telephone: +7-35-32775417 Fax: +7-35-32774463

Received: January 31, 2008 Revised: July 29, 2008
Accepted: August 5, 2008
Published online: September 21, 2008

Abstract

AIM: To study the secretory inhibitor of platelet microbicidal protein (SIPMP) phenotypes of faecal anaerobic isolates from patients with diarrhea.

METHODS: Faecal isolates of anaerobic bacteria (*B. fragilis*, n = 42; *B. longum*, n = 70; *A. israelii*, n = 21; *E. lentum*, n = 12) from children with diarrhea were tested. SIPMP production was tested by inhibition of platelet microbicidal protein (PMP) bioactivity against *B. subtilis* and was expressed as percentage of inhibition of PMP bactericidal activity.

RESULTS: Among anaerobic isolates 80% of *B. longum* strains, 85.7% of *A. israelii* strains, 50% of *E. lentum* strains and 92.86% of *B. fragilis* strains were SIPMP-positive. The isolated anaerobic organisms demonstrated SIPMP production at a mean level of 13.8% ± 0.7%, 14.7% ± 1.8%, 3.9% ± 0.9% (P < 0.05) and 26.8% ± 7.5% (P < 0.05) for bifidobacteria, *A. israelii*, *E. lentum* and *B. fragilis,* respectively.

CONCLUSION: Data from the present study may have significant implications in understanding the pathogenesis of microecological disorders in the intestine, as well as for future improvement in the prevention and therapy of anaerobe-associated infections.

Key words: Platelet microbicidal protein; Secretory inhibitor; Anaerobic bacteria; Intestine

Peer reviewer: Tomohiko Shimatani, Assistant Professor, Department of General Medicine, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 7348551, Japan

Ivanov IB, Gritsenko VA. Distribution of secretory inhibitor of platelet microbicidal protein among anaerobic bacteria isolated from stool of children with diarrhea. *World J Gastroenterol* 2008; 14(35): 5428-5431 Available from: URL: http://www.wjgnet.com/1007-9327/14/5428.asp DOI: http://dx.doi.org/10.3748/wjg.14.5428

INTRODUCTION

Anaerobic microorganisms are important constituents of human intestinal microbiota[1]. Enzymes produced by these bacteria provide nutrients for growth, participate in the pathogenesis of infections involving these bacteria, *etc.* Infections caused by anaerobic bacteria are increasingly being recognized as a major problem in clinical medicine[2,3]. The commensal anaerobic bacterial flora of the colon may undergo changes during diarrhea, owing to colonization of the intestine by pathogens and to rapid intestinal transit[4]. As it is difficult to establish exactly the significance of various anaerobic microorganisms in the pathogenesis of infections, it is imperative to delineate both microbial and host factors that contribute to its development. Identifying such a factor(s) produced by anaerobes is important for understanding and possibly modulating interactions between these bacteria and the host.

The intestinal mucosa forms a primary barrier providing both barrier function and immediate effective recognition of bacterial products invading the mucosa. This is of great importance for the prevention of permanent and chronic inflammation as a reaction to the commensal intestinal flora and the multitude of antigens present in the intestinal lumen[5].

The major role of endogenous cationic antimicrobial peptides in preventing the onset of infection has been emphasized recently[6,7]. In mammals, these peptides have evolved to have a central function in the host defense properties of granulocytic leukocytes, mucosal surfaces, skin and other epithelia[7]. Antibacterial protection of intestinal mucosa is provided in part by Paneth cell-derived antibacterial peptides[8-10]. Such peptides have also been found by several authors in human platelets

and are designated platelet microbicidal proteins (PMPs)[11,12]. These peptides are secreted at sites of infection and exert microbicidal activity against many pathogens[11]. Bukharin *et al* showed that an enhanced level of PMP in coprofiltrates of patients is associated with *Salmonella* gastroenteritis[13]. However, we suspect that successful pathogens (especially anaerobes) would have involved mechanisms to resist or degrade the inhibitory and microbicidal activities presented by the host. For example, in a recent publication[14], we reported the detection of an extracellular staphylococcal product, designated secretory inhibitor of platelet microbicidal protein (SIPMP), which causes local inhibition of the bactericidal action of PMP in the fluid phase. We also demonstrated that SIPMP represents a hitherto unrecognized determinant of staphylococcal pathogenicity and SIPMP production is associated with a prostatitis source.

At the same time, it is surprising that no extracellular product of anaerobic microorganisms with remarkable anti-PMP potential has been described. Thus, in this communication we report on *in vitro* detection of SIPMP phenotypes of faecal anaerobic isolates from patients with diarrhea.

MATERIALS AND METHODS

Clinical isolates were obtained from diarrhea stool samples collected from April to December 2000 in Orenburg Regional Child Hospital. A total of 145 strains of anaerobic bacteria (*B. fragilis*, $n = 42$; *B. longum*, $n = 70$; *A. israelii*, $n = 21$; *E. lentum*, $n = 12$) were kindly provided by Natalia Elagina (Department of Dysbiosis, Institute of Cellular and Intracellular Symbiosis). Bacteria were isolated from children with diarrhea (ranging from 10 mo old to 6 year old) and identified at the Anaerobe Laboratory, Department of Dysbiosis, Institute of Cellular and Intracellular Symbiosis, Russian Academy of Sciences, Orenburg.

PMP was prepared and standardized as described previsiously[12].

SIPMP production was performed by viable counting according to the recently proposed procedures[14]. Strains were grown in brain heart infusion broth (BHI, Oxoid), supplemented with yeast extract (0.5%) under anaerobiosis conditions (90% N_2/10% CO_2), at 37℃, for 48 h and cell-free supernatants were obtained by centrifugation. Bacterial supernatants were sterilized by filtration *via* 0.45 μm pore-size membranes (Millipore). Each culture supernatant (0.6 mL) (an equal volume of BHI was loaded in the control tubes) was combined with 0.3 mL of PMP at 3.0 μg/mL and incubated at 37℃. After 1 h, 100 μL *B. subtilis* suspension at 10^4 CFU/mL was added to each of the tubes. The tubes were incubated on a rotary shaker (300 r/min) at 37℃. After 1 h, aliquots of 200 μL were plated on blood agar plates. Colonies were counted after incubating overnight at 37℃ and numbers of surviving microorganisms were calculated. The SIPMP production as expressed in percentage of inhibition of PMP bactericidal activity and calculated by using the formula: % inhibition = (No. - Nk1) × 100/(Nk2 - Nk1), where No. was the number of surviving *B. subtilis* cells in the presence of bacterial supernatant and PMP, Nk1 was the number of surviving *B. subtilis* cells in the presence of PMP alone, and Nk2 was the number of surviving *B. subtilis* cells in BHI.

All of the experiments were carried out in triplicate and mean values and SEM were calculated. The differences between groups of microorganisms were assessed by using Student's *t*-test. $P \leqslant 0.05$ was considered significant.

Table 1 SIPMP production of fecal isolates of anaerobic bacteria *n* (%)

Organism	No. of SIPMP-producing strains (total/%) with different levels of SIPMP[1]			
	0	0.1-10.0	10.1-20.0	> 20
B. longum ($n = 70$)	14 (20)	0 (0)	56 (80)	0 (0)
A. israelii ($n = 21$)	3 (14.3)	0 (0)	18 (85.7)	0 (0)
E. lentum ($n = 12$)	6 (50)	6 (50)	0 (0)	0 (0)
B. fragilis ($n = 42$)	3 (7.14)	0 (0)	2 (4.76)	37 (88.1)

[1]SIPMP was expressed in percentage of inhibition of PMP bactericidal activity.

RESULTS

For exclusion the cooperative inhibitory effect of PMP and culture supernatants on *B. subtilis*, each culture supernatant was combined with *B. subtilis* suspension. After coincubation for 1 h, aliquots were plated on blood agar plates. Colonies were counted after incubating overnight at 37℃ and numbers of surviving microorganisms were calculated. None of the supernatants tested inhibited growth of *B. subtilis* cells. The stability of SIPMP was tested by subjecting culture supernatants to boiling for 30 min. This treatment completely destroyed the biological activity of SIPMP. Among anaerobic isolates 80% of *B. longum* strains, 85.7% of *A. israelii* strains, 50% of *E. lentum* strains and 92.86% of *B. fragilis* strains were SIPMP-positive (Table 1). The extracellular products of bacteria reduced the PMP-induced killing of *B. subtilis*. The isolated anaerobic organisms demonstrated SIPMP production at a mean level of 13.8% ± 0.7%, 14.7% ± 1.8%, 3.9% ± 0.9% ($P < 0.05$) and 26.8% ± 7.5% ($P < 0.05$) for bifidobacteria, *A. israelii*, *E. lentum* and *B. fragilis* respectively.

DISCUSSION

At local sites of microbial infections, epithelial cells, platelets, neutrophils, or macrophages release large amounts of different bactericidal peptides[7]. However, most infections are the result of contamination of host tissues with anaerobic flora from the gut[15] despite the presence of multiple antibacterial peptides in intestinal cells and mucus[7,16,17]. There is an urgent need to understand the virulence properties of anaerobic

organisms that may take part in their resistance to cationic antimicrobial peptides; identifying such a factor(s) would be helpful in devising effective treatment strategies.

In the present work, we detected an extracellular bacterial product of anaerobic microorganisms with remarkable anti-PMP potential which, to our knowledge, has not been described before. We anticipate that SIPMP serves to protect invading bacteria by inducing local consumption of PMP in the fluid phase. The strategy underlying this process would be straightforward and effective. We believe that SIPMP represents a widespread and hitherto unrecognized determinant of bacterial pathogenicity. Similarly, in a study of distribution of streptococcal inhibitor of complement variants in pharyngitis and invasive isolates by Hoe *et al*[18], 62% of group A streptococci from patients with pharyngitis produced this extracellular protein. Collectively, our study and the results of several studies[19-21] suggest that the inactivation of components of innate immunity may be important for bacterial pathogens to induce and perpetuate infections of different localization by surviving or avoiding microbicidal proteins mediated clearance. Bacteria-derived proteases may contribute to mucosal surface destruction, and are likely to impair host defense by degrading antimicrobial peptides[22]. It was confirmed by the fact that the lowest level of SIPMP production was observed with the non-protease producing species *E. lentum*. On the other hand, proteases of anaerobic microorganisms caused platelet aggregation with followed by release of a number of antibacterial proteins[11,23].

In contrast to *B. fragilis*, normal microflora have low levels of SIPMP. Hypothetically, the constituents of normal flora must have basal levels of resistance to the antimicrobial host defense factors. It is possible that low levels of inactivation of PMP activity by normal organisms are sufficient to protect them from PMP-dependent killing, thus providing stability of intestinal microflora. We believe that SIPMP is the stable characteristic and the same strains express more SIPMP in case of infection. On the other hand, our results suggest that the normal microflora was replaced by other organisms with pronounced pathogenic properties in patients with persistent infection[24].

At the same time, in the presence of infections, properties of normal microflora probably could change. The constituents of normal microflora, receiving signs of pathogenicity, are capable of causing diseases, as has been shown for lactobacilli and staphylococci[24,25].

The predominantly anaerobic microbiota of the distal ileum and colon contain an extraordinarily complex variety of metabolically active bacteria that intimately interact with the host's epithelial cells and mucosal immune system[26]. Crohn's disease, ulcerative colitis, and pouchitis are the result of continuous microbial antigenic stimulation of pathogenic immune responses as a consequence of host genetic defects in mucosal barrier function, innate bacterial killing, or immunoregulation. Identification of these host and microbial alterations in individual patients should lead to selective targeted interventions that correct underlying abnormalities and induce sustained and predictable therapeutic responses[27]. New treatment strategies aim at neutralization of such pathogenic properties of microorganisms as pronounced resistance to the cationic antimicrobial peptides and/or ability to inhibit the antimicrobial host defense factors and thereby improve the quality of life in patients[28-30].

Data from the present study may have significant implications in understanding the pathogenesis of microecological disorders in intestine, as well as for future improvement in the prevention of and therapy for anaerobe-associated infections. However, the exact mechanism of PMP inhibition in anaerobic bacteria remains to be determined, as does its molecular characteristics, occurrence and possible significance *in vivo*.

COMMENTS

Background

Anaerobic microorganisms are important constituents of human intestinal microbiota. Infections caused by anaerobic bacteria are increasingly being recognized as a major problem in clinical medicine. The commensal anaerobic bacterial flora of the colon may undergo changes during diarrhea, owing to colonization of the intestine by pathogens and to rapid intestinal transit. The major role of endogenous cationic antimicrobial peptides in preventing the onset of infection has been emphasized recently. Such peptides have been found in platelets and are designated platelet microbicidal proteins (PMPs). It is shown that an enhanced level of PMP in coprofiltrates of patients is associated with *Salmonella* gastroenteritis. Here we made an attempt to *in vitro* detection of secretory inhibitor of platelet microbicidal protein (SIPMP) phenotypes of faecal anaerobic isolates from patients with diarrhea.

Research frontiers

The article focuses on inhibition of PMP by extracellular bacterial products of faecal anaerobic microorganisms isolated from stool of children with diarrhea. Among anaerobic isolates 80% of *B. longum* strains, 85.7% of *A. israelii* strains, 50% of *E. lentum* strains and 92.86% of *B. fragilis* strains were SIPMP-positive. The isolated anaerobic organisms demonstrated SIPMP production at a mean level of 13.8% ± 0.7%, 14.7% ± 1.8%, 3.9% ± 0.9% ($P < 0.05$) and 26.8% ± 7.5% ($P < 0.05$) for bifidobacteria, *A. israelii*, *E. lentum* and *B. fragilis*, respectively.

Innovations and breakthroughs

In the present work, the authors detected an extracellular bacterial product of anaerobic microorganisms with remarkable anti-PMP potential that has not been described before. SIPMP represents a widespread and hitherto unrecognized determinant of bacterial pathogenicity.

Applications

Data from the present study may have significant implications in understanding the pathogenesis of microecological disorders in intestine, as well as for future improvement in the prevention and therapy of anaerobe-associated infections.

Terminology

PMP is a group of small cationic peptides isolated from rabbit and human platelets after stimulation by acid or thrombin; the secretory inhibitor of PMP is an extracellular bacterial product with anti-PMP activity.

Peer review

In this manuscript, the authors reported the detection of SIPMP phenotypes of faecal anaerobic isolates from patients with diarrhea. The study was well performed and interesting.

REFERENCES

1 **Finegold SM**. Normal human intestinal flora. *Ann Ist Super Sanita* 1986; **22**: 731-737

2 **Finegold SM**. The role of anaerobes in human infections.

Scand J Infect Dis Suppl 1981; **26**: 9-13
3 **Briedigkeit H**, Gobel U. [Anaerobic bacteria as the cause of endogenous infections] *Z Arztl Fortbild Qualitatssich* 1997; **91**: 165-170
4 **Balamurugan R**, Janardhan HP, George S, Raghava MV, Muliyil J, Ramakrishna BS. Molecular studies of fecal anaerobic commensal bacteria in acute diarrhea in children. *J Pediatr Gastroenterol Nutr* 2008; **46**: 514-519
5 **Hausmann M**, Rogler G. Immune-non immune networks in intestinal inflammation. *Curr Drug Targets* 2008; **9**: 388-394
6 **Durr M**, Peschel A. Chemokines meet defensins: the merging concepts of chemoattractants and antimicrobial peptides in host defense. *Infect Immun* 2002; **70**: 6515-6517
7 **Ganz T**. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol* 2003; **3**: 710-720
8 **Meyer-Hoffert U**, Hornef MW, Henriques-Normark B, Axelsson LG, Midtvedt T, Putsep K, Andersson M. Secreted enteric antimicrobial activity localises to the mucus surface layer. *Gut* 2008; **57**: 764-771
9 **Wershil BK**, Furuta GT. 4. Gastrointestinal mucosal immunity. *J Allergy Clin Immunol* 2008; **121**: S380-S383; quiz S415
10 **Dann SM**, Eckmann L. Innate immune defenses in the intestinal tract. *Curr Opin Gastroenterol* 2007; **23**: 115-120
11 **Yeaman MR**. The role of platelets in antimicrobial host defense. *Clin Infect Dis* 1997; **25**: 951-968; quiz 969-970
12 **Ivanov IB**. In vitro resistance to human platelet microbicidal protein among urethral staphylococcal and enterococcal isolates with its correlation with prostatitis. *Indian J Med Microbiol* 2005; **23**: 253-255
13 **Bukharin OV**, Kagan YD, Burmistrova AL. Salmonellae and salmonelloses. Ekaterinburg: Russian Academy of Sciences, 2000; 96-102
14 **Ivanov IB**, Gritsenko VA, Kuzmin MD. Staphylococcal secretory inhibitor of platelet microbicidal protein is associated with prostatitis source. *J Med Microbiol* 2006; **55**: 1645-1648
15 **MacFie J**, O'Boyle C, Mitchell CJ, Buckley PM, Johnstone D, Sudworth P. Gut origin of sepsis: a prospective study investigating associations between bacterial translocation, gastric microflora, and septic morbidity. *Gut* 1999; **45**: 223-228
16 **Selsted ME**, Ouellette AJ. Mammalian defensins in the antimicrobial immune response. *Nat Immunol* 2005; **6**: 551-557
17 **Vaishnava S**, Behrendt CL, Hooper LV. Innate immune responses to commensal bacteria in the gut epithelium. *J Pediatr Gastroenterol Nutr* 2008; **46** Suppl 1: E10-E11
18 **Hoe NP**, Vuopio-Varkila J, Vaara M, Grigsby D, De Lorenzo D, Fu YX, Dou SJ, Pan X, Nakashima K, Musser JM. Distribution of streptococcal inhibitor of complement variants in pharyngitis and invasive isolates in an epidemic of serotype M1 group A Streptococcus infection. *J Infect Dis* 2001; **183**: 633-639
19 **Ganz T**. Fatal attraction evaded. How pathogenic bacteria resist cationic polypeptides. *J Exp Med* 2001; **193**: F31-F34
20 **Ulvatne H**, Haukland HH, Samuelsen O, Kramer M, Vorland LH. Proteases in Escherichia coli and Staphylococcus aureus confer reduced susceptibility to lactoferricin B. *J Antimicrob Chemother* 2002; **50**: 461-467
21 **Shelburne CE**, Coulter WA, Olguin D, Lantz MS, Lopatin DE. Induction of {beta}-defensin resistance in the oral anaerobe Porphyromonas gingivalis. *Antimicrob Agents Chemother* 2005; **49**: 183-187
22 **Potempa J**, Banbula A, Travis J. Role of bacterial proteinases in matrix destruction and modulation of host responses. *Periodontol 2000* 2000; **24**: 153-192
23 **McRedmond JP**, Fitzgerald DJ. A growing set of platelet-activating bacterial proteins. *Blood* 2002; **99**: 387-388
24 **Ivanov IB**, Gritsenko VA, Kuzmin MD. Distribution of secretory inhibitor of platelet microbicidal protein among urethral isolates with its correlation with prostatitis. *Asian J Androl* 2008; **10**: 189-192
25 **Hillier SL**, Krohn MA, Klebanoff SJ, Eschenbach DA. The relationship of hydrogen peroxide-producing lactobacilli to bacterial vaginosis and genital microflora in pregnant women. *Obstet Gynecol* 1992; **79**: 369-373
26 **Bukharin OV**, Valyshev AV, Elagina NN. [Antilysozyme activity of anaerobic bacteria from fecal microflora in man] *Zh Mikrobiol Epidemiol Immunobiol* 2000; 20-22
27 **Sartor RB**. Microbial influences in inflammatory bowel diseases. *Gastroenterology* 2008; **134**: 577-594
28 **Abramzon OM**, Kirillov DA, Pan'kov AS, Perunova NB, Elagina NN, Valyshev AV, Bukharin OV. [Modifying action of oxytocin on the biological properties of the causative agents of anaerobic non-clostridial infection] *Zh Mikrobiol Epidemiol Immunobiol* 2003; (**4**): 71-74
29 **Kirillov DA**, Chainikova IN, Perunova NB, Chelpachenko OE, Pan'kov AS, Smoliagin AI, Valyshev AV. [Effect of a polyoxydonium immunoregulator on the biological properties of microorganisms] *Zh Mikrobiol Epidemiol Immunobiol* 2003; (**4**): 74-78
30 **Alekseenko SA**, Krapivnaia OV, Kamalova OK, Vasiaev VIu, Pyrkh AV. [Dynamics of clinical symptoms, indices of quality of life, and the state of motor function of the esophagus and rectum in patients with functional dyspepsia and irritable bowel syndrome after Helicobacter pylori eradication] *Eksp Klin Gastroenterol* 2003; (**4**): 54-58, 115

S- Editor Li DL **L- Editor** Li M **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5432

World J Gastroenterol 2008 September 21; 14(35): 5432-5435
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Is there a role for Tc-99m (V) DMSA scintigraphy in ischemic colitis?

Maria I Stathaki, Ioannis E Koutroubakis, Sophia I Koukouraki, Elias A Kouroumalis, Nikolaos S Karkavitsas

Maria I Stathaki, Sophia I Koukouraki, Nikolaos S Karkavitsas, Department of Nuclear Medicine, University Hospital of Heraklion, Heraklion 71110, Crete, Greece
Ioannis E Koutroubakis, Elias A Kouroumalis, Department of Gastroenterology, University Hospital of Heraklion, Heraklion 71110, Crete, Greece
Author contributions: Stathaki MI, Koukouraki SI, Karkavitsas NS performed and evaluated the scintigraphic images; Koutroubakis IE, Kouroumalis EA collected all clinical data; Stathaki MI, Koutroubakis IE analyzed the data and wrote the paper.
Correspondence to: Ioannis E Koutroubakis, MD, PhD, Assistant Professor of Medicine, University Hospital Heraklion, Department of Gastroenterology, PO Box 1352, Heraklion 71110, Crete, Greece. ktjohn@her.forthnet.gr
Telephone: +30-28-10392253 Fax: +30-28-10542085
Received: May 14, 2008 Revised: August 26, 2008
Accepted: September 2, 2008
Published online: September 21, 2008

Abstract

AIM: To evaluate the role of pentavalent Tc-99m dimercaptosuccinic acid [Tc-99m (V) DMSA] in the diagnosis of ischemic colitis.

METHODS: Fourteen patients with endoscopically and histologically confirmed ischemic colitis were included in the study. Tc-99m (V) DMSA scintigraphy was performed within 2 d after colonoscopy. Images were considered positive when an area of increased activity was observed in the region of interest and negative when no abnormal tracer uptake was detected.

RESULTS: In 3 out of the 14 patients, Tc-99m (V) DMSA images showed moderate activity in the bowel. The scintigraphic results corresponded with the endoscopic findings. In the other 11 patients, no abnormal tracer uptake was detected in the abdomen.

CONCLUSION: Besides the limited number of patients, Tc-99m (V) DMSA could not be considered as a useful imaging modality for the evaluation of ischemic colitis.

Key words: Scintigraphy; Technetium-99m pentavalent dimercaptosuccinic acid; Ischemic colitis; Intestinal ischemia; Diagnosis

Peer reviewers: Rami Eliakim, Professor, Department of Gastroenterology, Rambam Medical Center, PO Box 9602, Haifa 31096, Israel; Wallace F Berman, MD, Professor, PO Box 3009 DUMC, Durham, NC 27710, United States

Stathaki MI, Koutroubakis IE, Koukouraki SI, Kouroumalis EA, Karkavitsas NS. Is there a role for Tc-99m (V) DMSA scintigraphy in ischemic colitis? *World J Gastroenterol* 2008; 14(35): 5432-5435 Available from: URL: http://www.wjgnet.com/1007-9327/14/5432.asp DOI: http://dx.doi.org/10.3748/wjg.14.5432

INTRODUCTION

Ischemic colitis (IC) initially was described by Boley *et al* in 1963[1] and represents the most common form of gastrointestinal ischemia[2]. It is presented either as an occlusive or a nonocclusive form, usually seen in the elderly population with associated co-morbid factors[2-4]. Its pathophysiologic characteristic is the sudden loss of blood flow and the extent of damage is proportional to the degree and the duration of tissue hypoxia[5,6]. Moreover the disruption of the mucosal barrier may lead to inflow of intraluminal bacteria and toxins from the gut[3]. The clinical spectrum ranges from transient self-limited ischemia with brief episodes of abdominal pain and rectal bleeding to fulminant transmural necrosis, perforation and death[5,6]. The histological findings include mucosal necrosis and ulcerations, submucosal edema and haemorrhage or transmural infarction[2,3,5].

The identification of colonic ischemia is highly dependent upon clinical suspicion[2,7]. Although invasive, colonoscopy and colonic biopsies have become the standard for diagnosing ischemic colitis[6,8].

The radionuclide imaging in IC is an area under investigation. Since mucosal inflammatory changes often coexist with bowel ischemia, radiotracers used to localise inflammation could probably play an important role in the diagnosis of IC. A few reports of the scintigraphic findings using radionuclide labelled leukocytes have been published[9-12]. Moreover, recently pentavalent Tc-99m dimercaptosuccinic acid [Tc-99m (V) DMSA] has been successfully used in the identification of intestinal inflammation[13-15]. To our knowledge, its role in the diagnosis of intestinal ischemia has not been yet reported in the literature.

The aim of the present study was to determine

whether Tc-99m (V) DMSA scintigraphy could provide an alternative non-invasive imaging modality in the diagnosis of IC.

MATERIALS AND METHODS

Patients

We examined fourteen patients, (5 males and 9 females, mean age 70.6 years) with clinically, endoscopically and histologically confirmed IC. All patients included were admitted at the Department of Gastroenterology of the University Hospital Heraklion, Crete, Greece, within two days after the onset of symptoms. The patients were non-surgically treated and non-received medical therapy that would interfere with scintigraphic results. Half of the patients reported daily tobacco use. Endoscopic assessment was performed the day following the hospital admission in all cases.

Concerning the disease type, transient IC was the most frequent presenting in 9 patients (62.4%) followed by reversible ischemic colopathy in 3 patients (21.4%), chronic ulcerative IC in 1 patient (7.1%) and ischemic colonic stricture in 1 patient (7.1%).

The lesions were distributed depending on their locations: splenic flexure in 8 patients (57.1%), rectosigmoid in 3 patients (21.4%), right colon in 2 patients (14.2% and extensive IC in 1 patient (7.1%)

All patients were subjected to standard laboratory tests such as red and white blood cell counts, haemoglobin and hematocrit level, platelet count, albumin level, erythrocyte sedimentation rate and C-reactive protein level. Colonoscopy with biopsies was performed in all patients. The endoscopic findings for each bowel segment were evaluated by blinded specialists.

The study was approved by the ethics committee and patients were provided with an informed consent.

Scintigraphic imaging

Tc-99m (V) DMSA scintigraphy was performed in fourteen patients with IC. In all patients scintigraphy was performed within 2 d after colonoscopy, in order to avoid any variation in disease activity. Imaging was performed after intravenous administration of 555 MBq (15 mCi) of Tc-99m (V) DMSA. A gamma-camera (Millenium; GE Medical Systems, Milwaukee, Wis) equipped with a low energy all purpose collimator was used. With the patient in the supine position, planar views of the abdomen were obtained 4 hours after radiotracer injection. Before scanning patients were asked to void their bladders to avoid false results.

The bowel was divided to five segments: small bowel (A), ascending colon (B), transverse colon (C), descending colon (D) and rectosigmoid (E). Images were considered positive when an area of increased uptake was observed and negative when no abnormal tracer uptake was detected in any of the five segments. In the event of a positive result, semi quantitative measurements were included with reference to the uptake in the iliac crest and was graded as: 0: No uptake in the region of interest, 1: Faint uptake less than the iliac crest bone marrow, 2: Moderate uptake similar to that of the iliac crest, 3: Severe uptake greater than the iliac crest bone marrow.

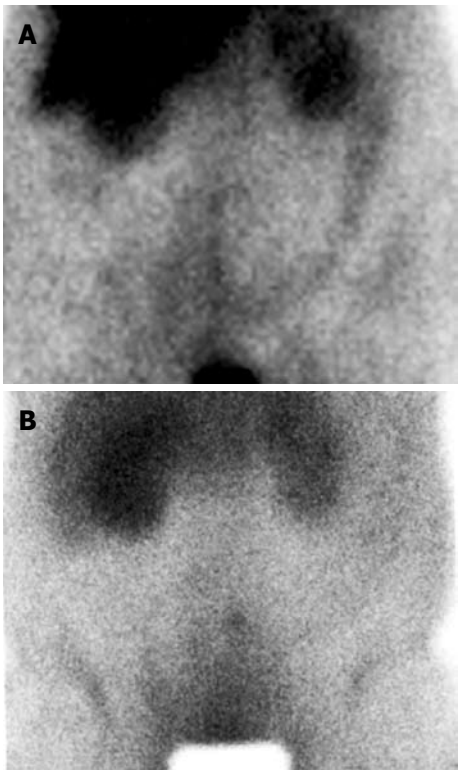


Figure 1 Anterior Tc-99m (V) DMSA scintigram in a patient with ischemic colitis: Moderate uptake of the radiotracer in the splenic flexure and the descending colon (**A**) and no uptake of the radiotracer in the bowel (**B**).

Scintigrams were blindly evaluated by three nuclear medicine physicians (MIS, SIK, NSK) and the results were compared with endoscopic and clinical data.

RESULTS

No patients showed adverse events during or after scintigraphy. A total of fourteen patients were included in this study. In all patients, endoscopy revealed characteristic findings suggestive of IC, which was histologically confirmed. In three out of the fourteen patients, the Tc-99m (V) DMSA scintigraphy demonstrated moderate uptake in the bowel (Figure 1A). Compared to endoscopic findings, radionuclide images were in agreement with the segments concerned. In the other eleven patients, Tc-99m (V) DMSA revealed no increased uptake in the bowel at all (Figure 1B).

Positive cases included one case with localization in the splenic flexure, one with ischemic rectosigmoiditis and one with extensive IC. Concerning the disease type among positive cases there were two cases with transient IC and one with chronic ulcerative IC. The small number of cases does not permit further statistical analysis.

The calculated sensitivity was only 21.4%. Owing to the low sensitivity and the false-negative results the study was stopped.

DISCUSSION

The most important finding in our study was that the majority of the patients with IC, who underwent Tc-99m (V) DMSA scintigraphy, yielded false- negative results. Only three of them showed increased tracer uptake in the regions of interest which corresponded with the findings at colonoscopy.

The pathophysiologic basis of IC is the sudden loss of blood supply. Localized low flow states involve mostly the splenic flexure and the rectosigmoid junction while systemic low flow states involve mostly the right colon, follow a relatively benign course and may affect younger patients[4,5,7,8].

Colonic ischemia may be precipitated by several conditions, such as shock, colon cancer, surgical intervention on the aorta or the mesenteric vessels, autoimmune disease, coagulopathies, long-distance running, constipation, illicit drug use and medications[2,4,6]. Recent studies have suggested a role of prothrombotic disorders in the development of IC[16,17].

No test specific for IC has yet been developed. The diagnosis of IC depends on the clinical evaluation of the patient in association with the biochemical, radiological, endoscopic and histologic assessment[18]. Most laboratory tests will be normal usually, yet in the event of abnormal results they have been found nonspecific[3,5,8].

Radiological evidence includes a wide spectrum of findings, which are frequently nonspecific, insensitive, and often they cannot easily differentiate ischemic from other forms of colitis[5,7,8].

Endoscopic assessment is the most sensitive and specific method of evaluating the colon for ischemic injury. The visual inspection and the ability to biopsy the mucosa allow the clinician in the majority of cases to make a firm diagnosis[6,8].

CT imaging has been employed in the evaluation of patients with abdominal pain of unknown etiology. Besides its limitations, it may be used to detect abnormalities and suggest the diagnosis, exclude other serious medical conditions and narrow the differential diagnosis[19-21].

Non-invasive Doppler sonography has been used as well. Although a high specificity has been reported, it is limited by overlying bowel gas, operator dependent quality and poor sensitivity for low flow vessel disease[3,5,8].

More recently, scintigraphic methods have been used in the diagnosis of IC. In-111 or Tc-99m labeled leukocyte scintigraphy has been studied and demonstrated successful imaging of bowel infarction, yet the localization mechanism still remains unclear. It is suggested that the presence of polymorphonuclear leukocytes in the inflammatory response to tissue ischemia, as a result of reperfusion injury may play the primary role[9-12]. However, the time-consuming preparation procedure, the handling and the reinjection of blood constitute shortcomings of radiotracer labeled leukocyte imaging.

Tc-99m (V) DMSA is a low-molecular weight complex that has been used successfully in the scintigraphic diagnosis of inflammation[22,23]. The suggested mechanisms of uptake by inflammatory lesions is either the infiltration into the interstitial space caused by increased capillary permeability or its similar behavior to phosphate ion since it seems to accumulate in areas where calcification is present[22,23].

Its role in the evaluation of intestinal inflammation has been already reported[13,14], moreover when compared to Tc-99m HMPAO labeled leukocytes, it seems to provide a useful, non-invasive, practical, easy to prepare and accurate alternative method for the assessment of disease activity in patients with IBD[15].

Based on the simultaneous presence of inflammatory response to tissue ischemia, we assumed that Tc-99m (V) DMSA could localize successfully the ischemic bowel and assist in the detection and diagnosis of IC.

The results of our study stand in contrast with the aforesaid assumption. The expected abnormally increased uptake was detected only in three out of the fourteen patients with IC while all other cases yielded false-negative scintigraphic results which were probably due to the presence of a milder degree of inflammatory response compared to the positive ones. It is possible that in the false negative cases of our study there was mainly a transient mucosal congestion and the chronic inflammation was not sufficient to provide successful bowel uptake of the radiotracer.

In conclusion, our data suggest that Tc-99m (V) DMSA has no possible role in the detection and diagnosis of IC.

COMMENTS

Background

Pentavalent Tc-99m dimercaptosuccinic acid [Tc-99m (V) DMSA] has been proved advantageous in the imaging of various inflammatory lesions, intestinal inflammation included.

Research frontiers

Tc-99m (V) DMSA could successfully localize the ischemic bowel and assist in the diagnosis of IC due to the simultaneous presence of inflammatory response to tissue ischemia.

Innovations and breakthroughs

We examined fourteen patients, (5 males and 9 females, mean age 70.6 years) with clinically, endoscopically and histologically confirmed IC. In all patients scintigraphy was performed within 2 d after colonoscopy, in order to avoid any variation in disease activity. In three out of the fourteen patients, the Tc-99m (V) DMSA scintigraphy demonstrated moderate uptake in the bowel however in the other eleven patients, Tc-99m (V) DMSA revealed no increased uptake in the bowel at all (false negative results).

Applications

Despite the fact that Tc-99m (V) DMSA scintigraphy has been used successfully in the evaluation of intestinal inflammation it seems to have no role in the diagnosis of ischemic colitis.

Peer review

The present study is relevant as it emphasizes the role of radionuclide imaging in IC and the focus of interest in future studies.

REFERENCES

1 **Boley SJ**, Schwartz S, Lash J, Sternhill V .Reversible vascular occlusion of the colon. *Surg Gynecol Obstet* 1963; **116**: 53-60

2 **Scharff JR**, Longo WE, Vartanian SM, Jacobs DL, Bahadursingh AN, Kaminski DL. Ischemic colitis: spectrum of disease and outcome. *Surgery* 2003; **134**: 624-629;

discussion 629-630
3 **MacDonald PH**. Ischaemic colitis. *Best Pract Res Clin Gastroenterol* 2002; **16**: 51-61
4 **Arnott ID**, Ghosh S, Ferguson A. The spectrum of ischaemic colitis. *Eur J Gastroenterol Hepatol* 1999; **11**: 295-303
5 **Sreenarasimhaiah J**. Diagnosis and management of ischemic colitis. *Curr Gastroenterol Rep* 2005; **7**: 421-426
6 **Anon R**, Bosca MM, Sanchiz V, Tosca J, Almela P, Amoros C, Benages A. Factors predicting poor prognosis in ischemic colitis. *World J Gastroenterol* 2006; **12**: 4875-4878
7 **Sreenarasimhaiah J**. Diagnosis and management of intestinal ischaemic disorders. *BMJ* 2003; **326**: 1372-1376
8 **Baixauli J**, Kiran RP, Delaney CP. Investigation and management of ischemic colitis. *Cleve Clin J Med* 2003; **70**: 920-921, 925-926, 928-930 passim
9 **Bell D**, Jackson M, Connaughton JJ. Indium-111 neutrophil imaging in ischemic colitis. *J Nucl Med* 1986; **27**: 1782-1783
10 **Moallem AG**, Gerard PS, Japanwalla M. Positive In-111 WBC scan in a patient with ischemic ileocolitis and negative colonoscopies. *Clin Nucl Med* 1995; **20**: 483-485
11 **Vijayakumar V**, Bekerman C, Blend MJ. Preoperative prediction of extent and severity of ischemic colitis by imaging with in-111 labeled leukocytes. *Clin Nucl Med* 1991; **16**: 98-102
12 **Hyun H**, Pai E, Blend MJ. Ischemic colitis: Tc-99m HMPAO leukocyte scintigraphy and correlative imaging. *Clin Nucl Med* 1998; **23**: 165-167
13 **Lee BF**, Chiu NT, Wu DC, Tsai KB, Liu GC, Yu HS, Wang ST. Use of 99mTc (V) DMSA scintigraphy in the detection and localization of intestinal inflammation: comparison of findings and colonoscopy and biopsy. *Radiology* 2001; **220**: 381-385
14 **Koutroubakis IE**, Koukouraki SI, Dimoulios PD, Velidaki AA, Karkavitsas NS, Kouroumalis EA. Active inflammatory bowel disease: evaluation with 99mTc (V) DMSA scintigraphy. *Radiology* 2003; **229**: 70-74
15 **Stathaki MI**, Koutroubakis IE, Koukouraki SI, Karmiris KP, Moschandreas JA, Kouroumalis EA, Karkavitsas NS. Active inflammatory bowel disease: head-to-head comparison between 99mTc-hexamethylpropylene amine oxime white blood cells and 99mTc(V)-dimercaptosuccinic acid scintigraphy. *Nucl Med Commun* 2008; **29**: 27-32
16 **Koutroubakis IE**, Sfiridaki A, Theodoropoulou A, Kouroumalis EA. Role of acquired and hereditary thrombotic risk factors in colon ischemia of ambulatory patients. *Gastroenterology* 2001; **121**: 561-565
17 **Koutroubakis IE**, Theodoropoulou A, Sfiridaki A, Kouroumalis EA. Low plasma protein Z levels in patients with ischemic colitis. *Dig Dis Sci* 2003; **48**: 1673-1676
18 **Robert JH**, Mentha G, Rohner A. Ischaemic colitis: two distinct patterns of severity. *Gut* 1993; **34**: 4-6
19 **Balthazar EJ**, Yen BC, Gordon RB. Ischemic colitis: CT evaluation of 54 cases. *Radiology* 1999; **211**: 381-388
20 **Thoeni RF**, Cello JP. CT imaging of colitis. *Radiology* 2006; **240**: 623-638
21 **Wiesner W**, Mortele KJ, Glickman JN, Ji H, Khurana B, Ros PR. CT findings in isolated ischemic proctosigmoiditis. *Eur Radiol* 2002; **12**: 1762-1767
22 **Lee BF**, Chiu NT, Chang JK, Liu GC, Yu HS. Technetium-99m(V)-DMSA and gallium-67 in the assessment of bone and joint infection. *J Nucl Med* 1998; **39**: 2128-2131
23 **Ercan MT**, Gulaldi NC, Unsal IS, Aydin M, Peksoy I, Hascelik Z. Evaluation of Tc-99m(V) DMSA for imaging inflammatory lesions: an experimental study. *Ann Nucl Med* 1996; **10**: 419-423

S- Editor Zhong XY **L- Editor** Alpini GD **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5436

World J Gastroenterol 2008 September 21; 14(35): 5436-5441
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Direct hemoperfusion with a polymyxin B-immobilized cartridge in intestinal warm ischemia reperfusion

Hiroaki Sato, Kiyohiro Oshima, Kazuhisa Arakawa, Katsumi Kobayashi, Hodaka Yamazaki, Yujin Suto, Izumi Takeyoshi

Hiroaki Sato, Kazuhisa Arakawa, Katsumi Kobayashi, Hodaka Yamazaki, Yujin Suto, Izumi Takeyoshi, Department of Thoracic and Visceral Organ Surgery, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan
Kiyohiro Oshima, Intensive Care Unit, Gunma University Hospital, Maebashi, Gunma 371-8511, Japan
Author contributions: Sato H, Oshima K and Takeyoshi I contributed equally to this work; Sato H and Takeyoshi I designed research; Sato H, Arakawa K, Kobayashi K, Yamazaki H, and Suto Y performed research; Sato H and Oshima K analyzed data; and Sato H and Oshima K wrote the paper.
Correspondence to: Izumi Takeyoshi, MD, Professor, Chairman, Department of Thoracic and Visceral Organ Surgery, Gunma University Graduate School of Medicine, 3-39-15 Showa-machi, Maebashi, Gunma 371-8511, Japan. takeyosi@showa.gunma-u.ac.jp
Telephone: +81-27-2208245 Fax: +81-27-2208255
Received: June 30, 2008 Revised: July 28, 2008
Accepted: August 4, 2008
Published online: September 21, 2008

Abstract

AIM: To investigate the effectiveness of direct hemoperfusion with polymyxin B-immobilized fibers (DHP-PMX therapy) on warm ischemia-reperfusion (I/R) injury of the small intestine.

METHODS: The proximal jejunum and distal ileum of mongrel dogs were resected. Warm ischemia was performed by clamping the superior mesenteric artery (SMA) and vein (SMV) for 2 h. Blood flow to the proximal small intestine was restored 1 h after reperfusion, and the distal small intestine was used as a stoma. The experiment was discontinued 6 h after reperfusion. The dogs were divided into two groups: the DHP-PMX group (n = 6, DHP-PMX was performed for 180 min; from 10 min prior to reperfusion to 170 min after reperfusion) and the control group (n = 5). The rate pressure product (RPP), SMA blood flow, mucosal tissue blood flow, and intramucosal pH (pHi) were compared between the two groups. The serum interleukin (IL)-10 levels measured 170 min after reperfusion were also compared.

RESULTS: The RPP at 6 h after reperfusion was significantly higher in the PMX group than in the control group (12 174 ± 1832 mmHg/min *vs* 8929 ± 1797 mmHg/min, $P < 0.05$). The recovery rates of the SMA blood flow at 1 and 6 h after reperfusion were significantly better in the PMX group than in the control group (61% ± 7% *vs* 44% ± 4%, $P < 0.05$, and 59% ± 5% *vs* 35% ± 5%, $P < 0.05$, respectively). The recovery rate of the mucosal tissue blood flow and the pHi levels at 6 h after reperfusion were significantly higher in the PMX group (61% ± 8% *vs* 31% ± 3%, $P < 0.05$ and 7.91 ± 0.06 *vs* 7.69 ± 0.08, $P < 0.05$, respectively). In addition, the serum IL-10 levels just before DHP-PMX removal were significantly higher in the PMX group than in the control group (1 569 ± 253 pg/mL *vs* 211 ± 40 pg/mL, $P < 0.05$).

CONCLUSION: DHP-PMX therapy reduced warm I/R injury of the small intestine. IL-10 may play a role in inhibiting I/R injury during DHP-PMX therapy.

Key words: Ischemia-reperfusion injury; Interleukin-10; Polymyxin B-immobilized hemoperfusion cartridge; PMX

Peer reviewer: Yuji Naito, Professor, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-8566, Japan

Sato H, Oshima K, Arakawa K, Kobayashi K, Yamazaki H, Suto Y, Takeyoshi I. Direct hemoperfusion with a polymyxin B-immobilized cartridge in intestinal warm ischemia reperfusion. *World J Gastroenterol* 2008; 14(35): 5436-5441 Available from: URL: http://www.wjgnet.com/1007-9327/14/5436.asp DOI: http://dx.doi.org/10.3748/wjg.14.5436

INTRODUCTION

The small intestinal villi are extremely sensitive to ischemia-reperfusion (I/R) injury, and many microcirculatory disturbances contribute to structural and functional changes[1]. I/R injury of the small intestine is consequently a critical problem that is important in situations such as the interruption of blood flow to the intestines due to abdominal aortic aneurysm surgery, small intestinal transplantation, surgery involving cardiopulmonary bypass, strangulated hernias, and neonatal necrotizing enterocolitis[2]. Intestinal I/R injury produces injury in both the intestines and distant organs including the lungs, kidneys, and liver[3]; therefore, it is associated with

high rates of morbidity and mortality in both surgical and trauma patients[4].

A polymyxin B-immobilized fiber column (PMX cartridge, Toraymyxin; Toray Industries, Tokyo, Japan), which was developed in Japan in 1994, is an extracorporeal hemoperfusion device that uses polymyxin-B fixed to α-chloroacetamide-methyl polystyrene-derived fibers packed in the cartridge. Direct hemoperfusion with PMX (DHP-PMX) therapy can remove circulating endotoxins and reduce various cytokines, even in patients with high levels of plasma cytokines[5]. DHP-PMX has been used for the treatment of endotoxemia[6] and reported to lower inflammatory cytokine and plasminogen activator inhibitor-1 (PAI-1) levels immediately[7]. DHP-PMX therapy has also been attempted for severe sepsis secondary to intra-abdominal infection[8], acute lung injury, and acute respiratory distress syndrome caused by sepsis[9], and its effectiveness has been reported. Recently, we hypothesized that DHP-PMX therapy could reduce I/R injury and demonstrated the usefulness of this therapy on pulmonary warm I/R injury in a canine model[10].

In this study, we evaluated the effectiveness of DHP-PMX on warm I/R injury of the small intestine using a canine model.

MATERIALS AND METHODS

Animals

Eleven adult mongrel dogs of both sexes, weighing 7.5-15.5 kg, were used in this study. The dogs were fasted but had free access to water for 24 h prior to the experiment. All of the animals were cared for in accordance with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 85-23; revised 1985). This study was also approved by the Animal Care and Experimentation Committee, Gunma University, Showa Campus, Japan.

Surgical procedures

After intramuscular administration of ketamine hydrochloride (2 mg/kg), the animals were anesthetized. Endotracheal intubation was performed, and the animals were ventilated with a respirator (Servo Ventilator 900C; Siemens-Elema, Solna, Sweden). The inspired O_2 concentration (FIO_2) was set at 1.0 during the experiment. Mechanical ventilation was performed with a tidal volume of 20 mL/kg and a rate of 10 breaths/min. General anesthesia was maintained with the inhalation of 1% to 2% isoflurane, and muscular relaxation was obtained with additional pancuronium bromide (0.1 mg/kg) every 20 min. The surgery was performed under sterile conditions. A polyethylene catheter was positioned in the abdominal aorta through the right femoral artery and connected to a pressure transducer to record arterial pressure. Another polyethylene catheter was inserted into the right femoral vein to use as a venous infusion line. The infusion rate for the lactated Ringer's solution was set at 30 mL/kg per h for 6 h after reperfusion. Laparotomy was performed *via* a midline incision after the blood pressure and respiration parameters stabilized. The small intestine was isolated with the vascular pedicle, and both the superior mesenteric artery (SMA) and the superior mesenteric vein (SMV) were dissected from the surrounding lymph nodes, plexuses, and tissues. The proximal jejunum and distal ileum were resected to interrupt the intramural blood flow. Warm ischemia was induced by clamping the SMA and SMV for 2 h. Proximal intestinal continuity was restored with an end-to-end anastomosis 1 h after the reperfusion. The resected distal intestine was used as a stoma to measure the tissue blood flow and pHi. During the 6-h experimental period, the abdominal cavity was closed and opened temporarily for each measurement. The experiment was discontinued 6 h after the reperfusion. Catecholamines were not used at any point during the experiment.

Experimental groups

The experimental study was composed of two groups: the DHP-PMX group (n = 6) and the control group (n = 5). The animals were randomly assigned to either the DHP-PMX group or the control group. In the DHP-PMX group, a double-lumen catheter was inserted into the portal vein through the left gastric vein, and DHP with PMX was performed with a flow rate of 80 mL/min for 180 min (from 10 min prior to reperfusion to 170 min after reperfusion) using that catheter. DHP was not performed in the control group.

Arterial blood pressure and heart rate (HR)

The arterial blood pressure and HR were directly monitored through a catheter connected to a transducer (Spectramed TA 1017; San-ei Co., Tokyo, Japan). The rate pressure product (HR × systolic pressure, RPP) was also calculated.

SMA blood flow

The SMA blood flow was measured prior to ischemia and at 1, 3, and 6 h after reperfusion using an electromagnetic blood flow meter (Model MFV-3100; Nihonkohden Co., Ltd., Tokyo, Japan). The SMA blood flow was expressed as the percentage of the level that was determined prior to ischemia.

Tissue blood flow measurements

The tissue blood flow was measured in the small bowel mucosa using a laser Doppler flow meter (ALF 21; Advance Co., Ltd., Tokyo, Japan) prior to ischemia and at 1, 3, and 6 h after reperfusion. Each measurement was made at three points by inserting a probe through the stoma and placing it against the antimesenteric side of the bowel lumen. The laser probe reading reflects tissue blood flow within about 1.0 mm of the surface of the bowel wall. Tissue blood flow was calculated as the mean of the three measurements and expressed as the percentage of the level determined prior to ischemia.

Intramucosal pH (pHi) measurements

pHi was measured prior to ischemia and at 1, 3, and 6 h

after reperfusion. This method has been described previously[11]. In brief, a tonometer (Trip; Tonometrics, Helsinki, Finland) was inserted into the small bowel lumen through the stoma. Within 40 min, the PCO_2 of the saline in the balloon placed at the tip of the tonometer had equilibrated with the intraluminal PCO_2, which reflects the mucosal PCO_2 of the bowel. The HCO_3^- concentration in the bowel wall was assumed to be the same as the HCO_3^- concentration in the arterial blood. The saline PCO_2 and HCO_3^- concentration in the artery were determined with a blood gas analyzer (Stat Profile M; Nova Biomedical Co., Waltham, MA) and were used to calculate the pHi using the Henderson-Hasselbach equation: pHi = 6.1 + log[arterial HCO_3^-/(0.03 × saline PCO_2)].

Measuring serum interleukin-10 (IL-10) levels

Arterial blood samples were collected 170 min after reperfusion (that is just before DHP-PMX removal) for measuring serum IL-10 levels using a commercial sandwich ELISA (Predicta ELISA kit; Genzyme Corp., Cambridge, MA) according to the manufacturer's instructions. In each experiment, a standard curve was obtained with serial dilutions using linear regression analysis of specific samples *versus* expected concentrations. Interleukin measurements were done in duplicate.

Statistical analysis

All results are expressed as the mean ± SEM. The significance of the differences was determined using analysis of variance (ANOVA) or Mann-Whitney *U*-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

All 11 mongrel dogs were successfully observed for 6 h after reperfusion without any complications.

The changes in RPP

The changes in the RPP are shown in Figure 1A. The RPPs gradually decreased with time in the control group. Those in the PMX group also decreased gradually; however, it had improved at 6 h after reperfusion and was significantly different compared to the control group ($P < 0.05$).

The changes in SMA blood flow

The recovery rates of the SMA blood flow expressed as a percentage of the baseline control value obtained before I/R injury are shown in Figure 1B. The SMA blood flow remarkably decreased 1 h after reperfusion in both groups. Additionally, the SMA blood flow gradually decreased until 6 h after reperfusion in the control group. The changes in SMA blood flow in the PMX group, however, were consistently higher after reperfusion than those in the control group, especially with significant ($P < 0.05$) differences at 1 and 6 h after reperfusion (Figure 1B).

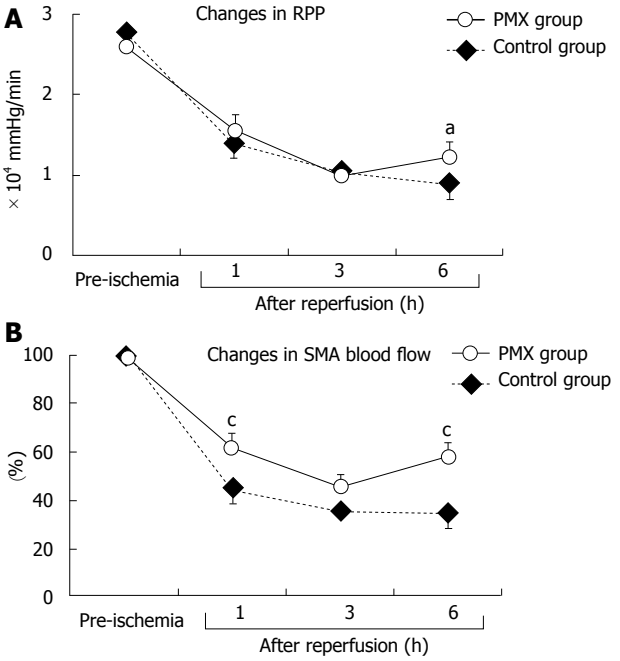


Figure 1 **A**: The changes in the RPP (HR × systolic pressure), $^aP < 0.05$; **B**: The changes in the recovery rates of SMA blood flow, $^cP < 0.05$.

The changes in mucosal tissue blood flow

The recovery rates of the mucosal tissue blood flow expressed as a percentage of the baseline control value obtained before I/R injury are shown in Figure 2A. In both groups, the mucosal tissue blood flow decreased gradually with time except at 6 h after reperfusion in the PMX group. The decreases in the mucosal tissue blood flow after reperfusion were smaller in the PMX group than in the control group. At 6 h after reperfusion, the mucosal tissue blood flow rate was significantly higher in the PMX group than in the control group after reperfusion.

The changes in pHi

As shown in Figure 2B, the changes in pHi also decreased remarkably in both groups. No significant differences in pHi levels were observed at 1 and 3 h after reperfusion in both groups; however, the pHi level in the PMX group was significantly higher ($P < 0.05$) than in the control group at 6 h after reperfusion.

Serum IL-10 levels

The serum IL-10 levels 170 min after reperfusion (that is just before DHP-PMX removal) in both groups are shown in Figure 3. As shown in Figure 3, the serum IL-10 level was significantly ($P < 0.05$) higher in the PMX group than in the control group.

DISCUSSION

Polymyxin B binds to endotoxin, an outer membrane component of gram-negative bacteria that is thought to be an important pathogenic trigger for the production of inflammatory mediators. Several preclinical studies have demonstrated that hemoperfusion or plasmapheresis

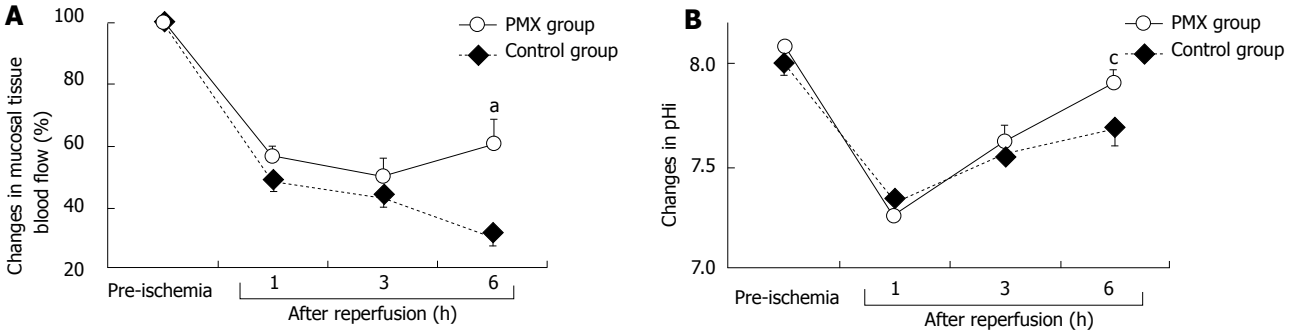


Figure 2 **A**: The changes in the recovery rates of mucosal tissue blood flow, $^{a}P < 0.05$; **B**: The changes in pHi, $^{c}P < 0.05$.

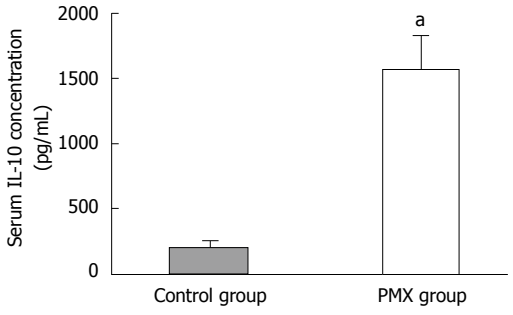


Figure 3 Serum IL-10 levels measured just before DHP-PMX removal, $^{a}P < 0.05$.

over immobilized polymyxin B can remove endotoxin from the blood[12-14]. Recently, some studies reported improved hemodynamic status[15] and improved survival[16] in patients with sepsis treated with PMX, and DHP-PMX therapy is effective for patients with septic shock who are infected not only by gram-negative bacteria but also by gram-positive bacteria without endotoxin release[17]. Therefore, DHP-PMX therapy has been conventionally used in patients with severe sepsis or septic shock, and its clinical effect has been confirmed. In addition, some authors have discussed the mechanism of DHP-PMX action. Kushi *et al* reported that the adsorption of pathogenic bacteria prevented the release of inflammatory cytokines and lessened the stimulation of vascular endothelial cells to lower PAI-1 level rather than directly inhibiting PAI-1 production by DHP-PMX therapy[18]. Tani *et al* speculated that a reduction in plasma endotoxins by endotoxin adsorption contributed to the cessation of cytokine gene expression and the excretion of cytokines[7]. Additionally, improvement in the PaO_2/FiO_2 ratio in patients with acute lung injury or acute respiratory distress syndrome caused by sepsis has been related to DHP-PMX therapy and decreases in blood neutrophil elastase (NE) and IL-8 levels[9]. We hypothesized that DHP-PMX therapy might be valuable in various inflammatory situations. Consequently, we evaluated the usefulness of DHP-PMX therapy on normothermic cardiopulmonary bypass in a pig model[19] and on pulmonary warm I/R injury in a canine model[10], and obtained satisfactory results. In the present study, we investigated the effectiveness of DHP-PMX therapy on small intestinal warm I/R injury in a canine model because intestinal I/R injury produces injury in both the intestines and distant organs including the lungs, kidneys, and liver[3] and is therefore associated with high rates of morbidity and mortality in both surgical and trauma patients[4].

As a result, the RPP was significantly ($P < 0.05$) better in the PMX group than in the control group at 6 h after reperfusion. The SMA and the mucosal tissue blood flow in the control group gradually decreased after reperfusion. Those parameters in the PMX group, however, had improved at 6 h after reperfusion and were significantly ($P < 0.05$) different compared to those parameters in the control group. The pHi level had also improved at 6 h after reperfusion and was significantly ($P < 0.05$) different from that in the control group. Our results showed the effectiveness of DHP-PMX therapy on small intestinal warm I/R injury.

IL-10 is a 35-kDa cytokine that regulates immune and inflammatory responses[20]. Systemic inflammatory response syndrome (SIRS) following major abdominal surgery is characterized by complex alterations in cytokine concentrations, and the balance between tumor necrosis factor (TNF)-α and IL-10 may be related to the occurrence of postoperative complications[21]. Wu *et al* reported that small intestinal ischemia reperfusion increased mucosal inflammatory modulator IL-6 concentration and inhibited anti-inflammatory cytokine IL-10 synthesis[22]. In addition, endogenous IL-10 exerts an anti-inflammatory role during reperfusion injury, possibly by regulating early stress-related genetic response, adhesion molecule expression, neutrophil recruitment, and subsequent cytokine and oxidant generation[23]. Malleo *et al* demonstrated that the absence of endogenous IL-10 enhanced organ dysfunction and mortality associated with multiple organ dysfunction syndrome in mice[24]. In this study, we focused on the serum IL-10 levels after reperfusion, and those in both groups were measured and compared. As a result, the serum IL-10 level measured 170 min (that is just before DHP-PMX removal) in the PMX group was significantly higher than in the control group. In addition, RPP, the recovery rates of SMA blood flow, the mucosal tissue blood flow, and the pHi levels after reperfusion were significantly better in the PMX group than in the control group. Therefore, we suggest that the IL-10 level is associated with the inhibition of small intestinal I/R injury using DHP-PMX therapy.

The authors of a recent study suggested that the absorption of anandamide by PMX might abolish

the diverse negative effects of anandamide such as hypotension, immunosuppression, and cytotoxicity[25]. Taking these results into consideration, the possibility exists that treatment with PMX not only removes endotoxin, but also reduces inflammatory reactions through the inhibition of various inflammatory cascades and has an effective role on I/R injury. Further studies that include the role of endotoxin and alterations of inflammatory factors such as cytokines and chemokines with DHP-PMX therapy are necessary.

In conclusion, DHP-PMX therapy may reduce warm I/R injury in the small intestine, and IL-10 could play an important role in this mechanism.

ACKNOWLEDGMENTS

We thank Toray Medical Co. for supplying the endotoxin adsorption cartridge (Toraymyxin).

COMMENTS

Background

Ischemia-reperfusion (I/R) injury of the small intestine is consequently a critical problem that is important in situations such as the interruption of blood flow to the intestines due to abdominal aortic aneurysm surgery, small intestinal transplantation, surgery involving cardiopulmonary bypass, strangulated hernias, and neonatal necrotizing enterocolitis. Intestinal I/R injury produces injury in both the intestines and distant organs including the lungs, kidneys, and liver, therefore, it is associated with high rates of morbidity and mortality in both surgical and trauma patients.

Research frontiers

A polymyxin B-immobilized fiber column (PMX cartridge, Toraymyxin), which was developed in Japan in 1994, is an extracorporeal hemoperfusion device that uses polymyxin-B fixed to α-chloroacetamide-methyl polystyrene-derived fibers packed in the cartridge. Direct hemoperfusion with PMX (DHP-PMX) therapy can remove circulating endotoxins and reduce various cytokines, even in patients with high levels of plasma cytokines.

Innovations and breakthroughs

DHP-PMX has been used for the treatment of endotoxemia and reported to lower inflammatory cytokine and plasminogen activator inhibitor-1 levels immediately. DHP-PMX therapy has also been attempted for severe sepsis secondary to intra-abdominal infection, acute lung injury, and acute respiratory distress syndrome caused by sepsis, and its effectiveness has been reported. Recently, we hypothesized that DHP-PMX therapy could reduce I/R injury and demonstrated the usefulness of this therapy on pulmonary warm I/R injury in a canine model.

Applications

DHP-PMX therapy may reduce warm I/R injury in the small intestine, and IL-10 could play an important role in this mechanism.

Terminology

The possibility exists that treatment with PMX not only removes endotoxin, but also reduces inflammatory reactions through the inhibition of various inflammatory cascades and has an effective role on I/R injury.

Peer review

The authors demonstrated that DHP with a polymyxin B-immobilized cartridge reduced reperfusion injury in the small intestine. This study was well designed and well investigated.

REFERENCES

1 **Tjärnström J**, Wikström T, Bagge U, Risberg B, Braide M. Effects of hyperbaric oxygen treatment on neutrophil activation and pulmonary sequestration in intestinal ischemia-reperfusion in rats. *Eur Surg Res* 1999; **31**: 147-154
2 **Collard CD**, Gelman S. Pathophysiology, clinical manifestations, and prevention of ischemia-reperfusion injury. *Anesthesiology* 2001; **94**: 1133-1138
3 **Köksoy C**, Kuzu MA, Kuzu I, Ergün H, Gürhan I, Ergun H, Gurhan I. Role of tumour necrosis factor in lung injury caused by intestinal ischaemia-reperfusion. *Br J Surg* 2001; **88**: 464-468
4 **Koike K**, Moore FA, Moore EE, Read RA, Carl VS, Banerjee A. Gut ischemia mediates lung injury by a xanthine oxidase-dependent neutrophil mechanism. *J Surg Res* 1993; **54**: 469-473
5 **Tsuzuki H**, Tani T, Ueyama H, Kodama M. Lipopolysaccharide: neutralization by polymyxin B shuts down the signaling pathway of nuclear factor kappaB in peripheral blood mononuclear cells, even during activation. *J Surg Res* 2001; **100**: 127-134
6 **Shoji H**. Extracorporeal endotoxin removal for the treatment of sepsis: endotoxin adsorption cartridge (Toraymyxin). *Ther Apher Dial* 2003; **7**: 108-114
7 **Tani T**, Hanasawa K, Kodama M, Imaizumi H, Yonekawa M, Saito M, Ikeda T, Yagi Y, Takayama K, Amano I, Shimaoka H, Ohta M, Okahisa T, Koga N, Fujita N, Yamasa H. Correlation between plasma endotoxin, plasma cytokines, and plasminogen activator inhibitor-1 activities in septic patients. *World J Surg* 2001; **25**: 660-668
8 **Vincent JL**, Laterre PF, Cohen J, Burchardi H, Bruining H, Lerma FA, Wittebole X, De Backer D, Brett S, Marzo D, Nakamura H, John S. A pilot-controlled study of a polymyxin B-immobilized hemoperfusion cartridge in patients with severe sepsis secondary to intra-abdominal infection. *Shock* 2005; **23**: 400-405
9 **Kushi H**, Miki T, Okamaoto K, Nakahara J, Saito T, Tanjoh K. Early hemoperfusion with an immobilized polymyxin B fiber column eliminates humoral mediators and improves pulmonary oxygenation. *Crit Care* 2005; **9**: R653-R661
10 **Oshima K**, Akao T, Kobayashi K, Muraoka M, Matsumoto K, Takeyoshi I. The effect of direct hemoperfusion with a polymyxin B-immobilized fiber column (DHP-PMX therapy) on pulmonary ischemia-reperfusion injury in a canine model. *J Invest Surg* 2008; **21**: 127-132
11 **Iwanami K**, Takeyoshi I, Ohwada S, Kobayashi J, Kawata K, Matsumoto K, Morishita Y. Intramucosal pH and intestinal mucosal damage in ischemia-reperfusion injury. *Transpl Int* 1998; **11**: 401-407
12 **King RC**, Binns OA, Rodriguez F, Kanithanon RC, Daniel TM, Spotnitz WD, Tribble CG, Kron IL. Reperfusion injury significantly impacts clinical outcome after pulmonary transplantation. *Ann Thorac Surg* 2000; **69**: 1681-1685
13 **Cohen J**, Aslam M, Pusey CD, Ryan CJ. Protection from endotoxemia: a rat model of plasmapheresis and specific adsorption with polymyxin B. *J Infect Dis* 1987; **155**: 690-695
14 **Aoki H**, Kodama M, Tani T, Hanasawa K. Treatment of sepsis by extracorporeal elimination of endotoxin using polymyxin B-immobilized fiber. *Am J Surg* 1994; **167**: 412-417
15 **Tetta C**, Gianotti L, Cavaillon JM, Wratten ML, Fini M, Braga M, Bisagni P, Giavaresi G, Bolzani R, Giardino R. Coupled plasma filtration-adsorption in a rabbit model of endotoxic shock. *Crit Care Med* 2000; **28**: 1526-1533
16 **Uriu K**, Osajima A, Hiroshige K, Watanabe H, Aibara K, Inada Y, Segawa K, Anai H, Takagi I, Ito A, Kamochi M, Kaizu K. Endotoxin removal by direct hemoperfusion with an adsorbent column using polymyxin B-immobilized fiber ameliorates systemic circulatory disturbance in patients with septic shock. *Am J Kidney Dis* 2002; **39**: 937-947
17 **Kawamata T**, Imaizumi H, Yoshida M, Kaneko M. Polymyxin B-immobilized fiber improves hyperdynamic state in MRSA septic patients. *Intensive Care Med* 1997; **23**: 130-131
18 **Kushi H**, Nakahara J, Miki T, Okamoto K, Saito T, Tanjo K. Hemoperfusion with an immobilized polymyxin B fiber column inhibits activation of vascular endothelial cells. *Ther Apher Dial* 2005; **9**: 303-307
19 **Ohki S**, Oshima K, Takeyoshi I, Matsumoto K, Morishita Y. Endotoxin removal with a polymyxin B-immobilized

hemoperfusion cartridge improves cardiopulmonary function after cardiopulmonary bypass. *J Surg Res* 2008; **145**: 74-79

20 **Fiorentino DF**, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. *J Immunol* 1991; **147**: 3815-3822

21 **Dimopoulou I**, Armaganidis A, Douka E, Mavrou I, Augustatou C, Kopterides P, Lyberopoulos P, Tzanela M, Orfanos SE, Pelekanou E, Kostopanagiotou G, Macheras A, Giamarellos-Bourboulis EJ. Tumour necrosis factor-alpha (TNFalpha) and interleukin-10 are crucial mediators in post-operative systemic inflammatory response and determine the occurrence of complications after major abdominal surgery. *Cytokine* 2007; **37**: 55-61

22 **Wu B**, Iwakiri R, Ootani A, Fujise T, Tsunada S, Fujimoto K. Platelet-activating factor promotes mucosal apoptosis via FasL-mediating caspase-9 active pathway in rat small intestine after ischemia-reperfusion. *FASEB J* 2003; **17**: 1156-1158

23 **Zingarelli B**, Yang Z, Hake PW, Denenberg A, Wong HR. Absence of endogenous interleukin 10 enhances early stress response during post-ischaemic injury in mice intestine. *Gut* 2001; **48**: 610-622

24 **Malleo G**, Mazzon E, Genovese T, Di Paola R, Caminiti R, Esposito E, Bramanti P, Cuzzocrea S. Absence of endogenous interleukin-10 enhanced organ dysfunction and mortality associated to zymosan-induced multiple organ dysfunction syndrome. *Cytokine* 2008; **41**: 136-143

25 **Wang Y**, Liu Y, Sarker KP, Nakashima M, Serizawa T, Kishida A, Akashi M, Nakata M, Kitajima I, Maruyama I. Polymyxin B binds to anandamide and inhibits its cytotoxic effect. *FEBS Lett* 2000; **470**: 151-155

S- Editor Li DL **L- Editor** Negro F **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5442

World J Gastroenterol 2008 September 21; 14(35): 5442-5447
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Metabolic syndrome is associated with erosive esophagitis

Jung Ho Park, Dong IL Park, Hong Joo Kim, Yong Kyun Cho, Chong IL Sohn, Woo Kyu Jeon, Byung Ik Kim

Jung Ho Park, Dong IL Park, Hong Joo Kim, Yong Kyun Cho, Chong IL Sohn, Woo Kyu Jeon, Byung Ik Kim, Department of Medicine, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Seoul, South Korea
Author contributions: Park JH, Park DI and Sohn CI designed the research; Park JH, Park DI, Kim HJ, Cho YK, Sohn CI, Jeon WK and Kim BI performed the research; Park JH, Park DI and Sohn CI analyzed data; Park JH wrote the paper.
Correspondence to: Jung Ho Park, MD, Department of Medicine, Sungkyunkwan University School of Medicine, Kangbuk Samsung Hospital, Seoul, South Korea. pjho3@hotmail.com
Telephone: +82-2-20012068 Fax: +82-2-20012485
Received: June 9, 2008 Revised: August 18, 2008
Accepted: August 25, 2008
Published online: September 21, 2008

Abstract

AIM: To clarify whether insulin resistance and metabolic syndrome are risk factors for erosive esophagitis.

METHODS: A case-control study was performed using the database of the Kangbuk Samsung Hospital Medical Screening Center.

RESULTS: A total of 1679 cases of erosive esophagitis and 3358 randomly selected controls were included. Metabolic syndrome was diagnosed in 21% of the cases and 12% of the controls ($P < 0.001$). Multiple logistic regressions confirmed the association between erosive esophagitis and metabolic syndrome (Odds ratio, 1.25; 95% CI, 1.04-1.49). Among the components of metabolic syndrome, increased waist circumference, elevated serum triglyceride levels and hypertension were significant risk factors for erosive esophagitis (all $P < 0.01$). Furthermore, increased insulin resistance (Odds ratio, 0.91; 95% CI, 0.85-0.98) and fatty liver, as diagnosed by ultrasonography (Odds ratio, 1.39; 95% CI, 1.20-1.60), were also related to erosive esophagitis even after adjustment for a series of confounding factors.

CONCLUSION: Metabolic syndrome and increased insulin resistance are associated with an increased risk of developing erosive esophagitis.

Key words: Metabolic syndrome; Erosive esophagitis; Insulin resistance; Fatty liver

Peer reviewer: Jeroen Maljaars, Department of Internal Medicine, Divison of Gastroenterology, University Hospital Maastricht (azM), PO Box 5800, Maastricht 6202 AZ, The Netherlands

Park JH, Park DI, Kim HJ, Cho YK, Sohn CI, Jeon WK, Kim BI. Metabolic syndrome is associated with erosive esophagitis. *World J Gastroenterol* 2008; 14(35): 5442-5447 Available from: URL: http://www.wjgnet.com/1007-9327/14/5442.asp DOI: http://dx.doi.org/10.3748/wjg.14.5442

INTRODUCTION

Metabolic syndrome is a cluster of metabolic abnormalities defined as the presence of an increased waist circumference and two of the following components: high blood pressure, hypertriglyceridemia, low levels of high density lipoprotein (HDL)-cholesterol, or diabetes/hyperglycemia. This syndrome helps to identify individuals at high risk for both cardiovascular disease and diabetes mellitus (DM); therefore, metabolic syndrome has become one of the major health problems worldwide.

Gastroesophageal reflux disease (GERD) and obesity are two of the most common diseases in Korea and the incidences of both have been increasing rapidly. Recently, GERD was shown to affect approximately 3.4%-3.8% of the Korean population[1,2] and in 1995 the prevalence of being overweight [body mass index (BMI) = 25-30 kg/m^2] or obese (BMI > 30 kg/m^2) was, respectively, reported as 11.7% and 2.1% in males, and 18.0% and 2.5% in females; in 2000 the prevalence of being overweight or obese was 33.1% and 3.2% in males, and 32.2% and 4.5% in females, respectively[3].

Several studies have shown the relationship between obesity, erosive esophagitis, and GERD symptoms[4-8]. Also, a recent study demonstrated that metabolic syndrome was associated with reflux esophagitis[9]. However, literature on whether metabolic syndrome and insulin resistance are risk factors for GERD is scant.

In this study, we therefore intended to determine whether metabolic syndrome and insulin resistance are associated with erosive esophagitis in a Korean population.

MATERIALS AND METHODS

Study population and selection of study participants

We conducted a cross-sectional case-control study. The study population consisted of subjects who visited the Medical Screening Center at Kangbuk Samsung Hospital from January to December 2006. Exclusion criteria

consisted of a history of prior gastric surgery, benign gastric or duodenal ulcer, gastric cancer or current proton pump inhibitor medication therapy.

Of the 83032 patients visited the Medical Screening Center, 44718 patients underwent upper gastrointestinal (UGI) endoscopic examination. However, only 28949 patients completed a questionnaire pertaining to their symptoms (heart burn and regurgitation) and provided weights, heights, and waist circumferences. The mean age of the participants was 45 ± 9.6 years and 11375 (39%) were females. After application of the exclusion criteria, 1679 (5.8%) subjects were included as patients with erosive esophagitis. Two controls for each case patient (3358 subjects) were randomly selected from the subjects with normal UGI endoscopic findings and no reflux symptoms.

Questionnaire

All participants completed a self-administered validated questionnaire that identified reflux symptoms, such as irritating heartburn and/or acid regurgitation experienced during the preceding year[6]. In addition, the questionnaire included questions about current smoking and other medical or surgical histories.

Measurement

BMI and metabolic syndrome components, such as waist circumference, lipid profile, blood pressure and fasting glucose level, were measured in all study participants. BMI was calculated as the ratio of weight (kg) to the square of height (kg/m^2) and abdominal obesity was defined as a waist circumference ≥ 80 cm in females and ≥ 90 cm in males[10]. Measurements were made at the World Health Organization (WHO) recommended site (the midpoint between the lower border of the rib cage and the iliac crest) by trained personnel[11]. The mean blood pressure was checked more than twice in the supine position with a sphygmomanometer after 10 min of rest. All blood testing was done after more than 12 h of fasting. Total cholesterol, triglycerides (TG), HDL-C and low-density lipoprotein (LDL)-C were measured with an automatic analyzer (Advia 1650, German). Total C and TG were analyzed by an enzymatic calorimetric test. A selective inhibition method was used in HDL-C measurement and a homogenous enzymatic calorimetric test was used in LDL-C measurement. Fasting glucose was measured by the hexokinase method with an automatic analyzer (Advia 1650) and fasting insulin was assayed *via* an immunoradiometric assay (Biosource, Belgium). The intraassay variation coefficients were 2.1%-4.5% and the interassay variation coefficients for the quality controls were 4.7%-12.2%. The degree of insulin resistance was evaluated by homeostasis model assessment (HOMA-IR) according to the following formula: (fasting insulin in μU/mL × fasting glucose in mmol/L)/22.5[12]. An experienced radiologist who was blind to the laboratory data performed ultrasonographic liver examinations. Fatty liver was defined as a bright liver on ultrasonography (USG). The diagnosis of bright liver was based on abnormally intense and high level echoes arising from the hepatic parenchyma with amplitudes similar to those of echoes arising from the diaphragm.

Participants were diagnosed with metabolic syndrome if they had an increased waist circumference and two of the following components: (1) high blood pressure (≥ 130 mmHg systolic or ≥ 85 mmHg diastolic), (2) hypertriglyceridemia (≥ 150 mg/dL), (3) low levels of HDL-C (≤ 40 mg/dL in males or ≤ 50 mg/dL in females), or (4) DM/hyperglycemia[10].

Upper gastrointestinal endoscopy

Standard endoscopic examination of the esophagus, stomach, and duodenum was performed in all subjects. The severity of erosive esophagitis was graded from A-D according to the LA classification[13]. We considered LA-A to be the cutoff for erosive esophagitis. We also considered a hiatal hernia to be present if diaphragmatic indentation was seen > 2 cm distal to the Z-line and the proximal margins of the gastric mucosal folds, which were observed with considerable air insufflation during inspiration. Distance was measured using the centimeter markings on the endoscope[14].

Statistical analyses

Statistical analysis was done using the χ^2 test for comparison of discrete variables and the *t*-test for comparison of continuous variables. The continuous variables measured in this study were expressed as the mean ± SD. Multivariate analysis was conducted using logistic regression. To examine the risks of potential confounders, including metabolic syndrome for erosive esophagitis, multivariate models included adjustments for age, gender, smoking, alcohol, and metabolic syndrome as categorical factors. For each variable, the odds ratio (OR) and 95% confidence interval (CI) were given. A two-tailed *P* value of < 0.05 was considered statistically significant.

RESULTS

Of the 28949 subjects, 1679 (5.8%) were confirmed to have erosive esophagitis; 1326 (78.9%) cases were classified as LA-A, 328 (19.5%) as LA-B, and 25 (1.6%) as LA-C or LA-D. The mean age was 45.19 ± 9.3 years and 86% of the subjects were men. The study characteristics are mentioned in Table 1. We found a significant increase in the mean BMI, waist circumference, systolic and diastolic blood pressure, fasting blood glucose, HbAlc, TG and HOMA in patients with erosive esophagitis as compared to the controls. Also, patients with erosive esophagitis were more likely to be male, obese, current smokers, regular consumers of alcohol, and more likely to have metabolic syndrome and fatty liver (as diagnosed by abdominal ultrasonography) and less than a college education.

Table 2 shows the results from the multivariate analysis examination of the association between erosive esophagitis and various risk factors. Male gender, current smoking, metabolic syndrome, reflux symptoms, regular

Table 1 Comparisons between participants with and without erosive esophagitis (n = 5037)

	With erosive esophagitis (n = 1679)	Without erosive esophagitis (n = 3358)	P
Age (yr, mean ± SD)	45.2 ± 9.3	45.2 ± 9.7	0.873
Gender (M/F, %)	86/14	59/41	< 0.001
BMI (kg/m^2)	24.8 ± 2.9	23.5 ± 3.0	< 0.001
Waist circumference (cm)	86.8 ± 8.7	81.5 ± 9.8	< 0.001
Current smoking	724 (43%)	786 (23%)	< 0.001
Alcohol use (≥ 3-4/wk)	360 (21%)	387 (12%)	< 0.001
Metabolic syndrome	352 (21%)	433 (13%)	< 0.001
Hiatal hernia	38 (2.2%)	24 (0.7%)	< 0.001
Reflux symptoms[1]	194 (12%)	265 (8.0%)	< 0.001
Systolic BP (mmHg)	118.3 ± 12.7	114.8 ± 13.5	< 0.001
Diastolic BP (mmHg)	77.7 ± 8.8	74.8 ± 9.5	< 0.001
Fatty liver on abdominal USG	809 (48%)	1014 30%)	< 0.001
Fasting plasma glucose	98.4 ± 20.5	95.2 ± 17.2	< 0.001
HbAlc	5.6 ± 0.7	5.5 ± 0.6	0.001
Triglycerides (mg/dL, mean ± SD)	158.9 ± 110.4	123.5 ± 78.6	< 0.001
HDL-C (mg/dL) (mean ± SD)	54.1 ± 12.0	56.4 ± 12.8	< 0.001
HOMA[2]	2.41 ± 1.10	2.18 ± 0.89	< 0.001
H pylori positive	211/555 (38%)	498/922 (54%)	< 0.001
Education (college and higher)	894/1184 (75%)	1639/2391 (69%)	< 0.001

[1]Reflux symptoms: Weekly heartburn and/or acid regurgitation; [2]HOMA: Homeostasis model assessment estimates steady state beta cell function and insulin sensitivity.

Table 2 Multivariate analyses of the risk for erosive esophagitis by gender, smoking, hiatal hernia, reflux symptoms, metabolic syndrome, fatty liver on abdominal USG and HOMA

	Adjusted odds ratio	95% CI	P
Gender	0.29	0.25-0.35	< 0.001
Current smoking	1.60	1.39-1.83	< 0.001
Alcohol use (≥ 3-4/wk)	1.80	1.53-2.14	< 0.001
Hiatal hernia	3.27	1.87-5.70	< 0.001
Reflux symptoms[1]	1.57	1.28-1.94	< 0.001
Metabolic syndrome	1.25	1.04-1.49	0.017
Fatty liver on abdominal USG	1.39	1.20-1.60	< 0.001
HOMA[2]	0.91	0.85-0.98	0.011

[1]Reflux symptoms: Weekly heartburn and/or acid regurgitation; [2]HOMA: Homeostasis model assessment estimates steady state beta cell function and insulin sensitivity.

Table 3 Risk of individual components of metabolic syndrome for erosive esophagitis

	OR (95% CI)[1]	P	OR (95% CI)[2]	P
Increased waist circumference	1.46 (1.28-1.67)	< 0.001	1.33 (1.15-1.54)	< 0.001
Hypertension	1.22 (1.06-1.40)	0.006	1.16 (1.00-1.35)	0.047
DM or elevated FBS	1.09 (0.77-1.54)	0.627	0.95 (0.66-1.38)	0.798
Increased TG	1.98 (1.74-2.26)	< 0.001	1.47 (1.14-1.90)	0.003
Low HDL-C	0.67 (0.56-0.80)	< 0.001	0.90 (0.74-1.09)	0.267

FBS: Fasting blood sugar; TG: Triglycerides; HDL-C: High-density lipoprotein-cholesterol. [1]Unadjusted; [2]Adjusted for gender, current smoking, hiatal hernia, reflux symptoms, alcohol use, fatty liver on abdominal USG, and HOMA.

alcohol use, HOMA and fatty liver (as diagnosed by abdominal ultrasonography) were significant independent risk factors for erosive esophagitis. Among the individual components of metabolic syndrome, increased waist circumference, hypertension, increased levels of TG, and low levels of HDL-C were significantly associated with erosive esophagitis. However, after adjusting for gender, smoking, hiatal hernia, reflux symptoms, regular alcohol use, HOMA and fatty liver (as diagnosed by abdominal ultrasonography), increased waist circumference, increased levels of TG, and hypertension were strongly associated with the development of erosive esophagitis (Table 3).

We also attempted to determine the relationship between the severity of erosive esophagitis, according to the LA classification, and various risk factors. Male gender, current smoking, regular alcohol use, hiatal hernia, metabolic syndrome, reflux symptoms, HOMA and fatty liver (as diagnosed by abdominal ultrasonography) were significantly associated with the severity of erosive esophagitis (Table 4). Among the individual components of metabolic syndrome, increased waist circumference and increased levels of TG were predictive factors for the severity of erosive esophagitis (Table 5).

DISCUSSION

This cross-sectional study in a Korean population showed that metabolic syndrome was strongly associated with the development and severity of erosive esophagitis. Also, insulin resistance, independent of metabolic syndrome, was another significant risk factor for erosive esophagitis.

Recently, the prevalence of metabolic syndrome has rapidly increased in Korea. According to the International Diabetes Federation (IDF) criteria, the age-adjusted prevalence of metabolic syndrome in males was 10.9% in 1997 and 23.3% in 2003. In females, the age-adjusted prevalence of metabolic syndrome was 42.2% in 1997 and 43.4% in 2003[15]. In the current study,

Table 4 Associations of grade of erosive esophagitis, according to LA classification, with risk factors for erosive esophagitis *n* (%)

	Control (*n* = 3358)	A (*n* = 1326)	B (*n* = 328)	C or D (*n* = 25)	*P* for linear trend
Age (yr, mean ± SD)	45.2 ± 9.7	44.8 ± 9.7	46.4 ± 9.3	49.9 ± 10.7	0.094
Males	1991 (59)	1122 (85)	300 (92)	22 (88)	< 0.001
Current smoking	786 (23)	554 (42)	162 (49)	8 (32)	< 0.001
Alcohol use (≥ 3-4/wk)	387 (12)	251 (19)	101 (31)	8 (32)	< 0.001
Hiatal hernia	24 (0.2)	32 (2)	3 (1)	3 (12)	< 0.001
Reflux symptoms[1]	265 (8.0)	141 (11)	50 (15)	3 (12)	< 0.001
Metabolic syndrome	433 (13)	255 (19)	87 (27)	10 (40)	0.001
Fatty liver on Abdominal USG	1014 (30)	625 (47)	166 (51)	18 (72)	< 0.001
HOMA[2] (mean ± SD)	2.18 ± 0.89	2.39 ± 1.10	2.50 ± 1.10	2.70 ± 1.12	0.007

[1]Reflux symptoms: Weekly heartburn and/or acid regurgitation; [2]HOMA: Homeostasis model assessment estimates steady state beta cell function and insulin sensitivity.

Table 5 Associations of grade of erosive esophagitis, according to LA classification, with individual components of the metabolic syndrome *n* (%)

	Control (*n* = 3358)	A (*n* = 1326)	B (*n* = 328)	C or D (*n* = 25)	*P* for linear trend[1]
Increased waist circumference	942 (28)	504 (38)	159 (48)	19 (76)	< 0.001
Hypertension	722 (22)	378 (29)	103 (31)	8 (32)	0.244
DM or elevated FBS	84 (2.5)	49 (4)	11 (3)	0	0.346
Increased TG	850 (25)	561 (42)	138 (42)	11 (44)	0.004
Low HDL-C	497 (15)	164 (12)	37 (11)	5 (20)	0.582

[1]Adjusted for gender, current smoking, hiatal hernia, reflux symptoms, alcohol use, fatty liver on abdominal USG, and HOMA.

the prevalence of metabolic syndrome was lower than previously reported, especially for females. A possible explanation for this difference is that the participants in this study were much younger than those in previous studies. Because the prevalence of metabolic syndrome increases with age[16,17], younger subjects are more likely to have a lower prevalence of metabolic syndrome than older subjects. Additionally, the higher educational level and economic status of the subjects could be another reason for the lower than expected prevalence of metabolic syndrome. Higher income was protective against metabolic syndrome[18] and females in the lower economic group were more likely to be at risk for metabolic syndrome when compared with females in the higher economic group[19,20].

With the increased prevalence of metabolic syndrome, GERD has also become more prevalent in Korea. The overall prevalence of erosive esophagitis was 3.4% in 2001[21] and 6.6% in 2006[6]. In terms of reflux symptoms, 2.5% of adults experienced heartburn and reflux symptoms in 2000[22]. In contrast, 7.1% reported that GERD symptoms were present at least once a week in 2007[1]. This increase may be due to extended life expectancy, greater intake of Westernized food[2], and/or increasing rates of obesity[6]. Moreover, an increase in alcohol consumption[23,24] and a decrease in *Helicobacter pylori (H pylori)* infections[25] could be possible reasons for the increase in erosive esophagitis in Korea.

This study demonstrated that metabolic syndrome and insulin resistance were also risk factors for erosive esophagitis. A recent study verified that elevated triglyceride levels, a component of metabolic syndrome, is an independent predictor for reflux esophagitis and suggested that humoral compounds might alter the lower esophageal sphincter pressure or affect esophageal clearance of refluxate[9]. In fact, human adipose tissue is a major site of IL-6 secretion[26]. IL-6 stimulates hepatic triglyceride secretion in rats[27] and plays an important role in insulin resistance in humans[28]. Moreover, IL-6 reduces esophageal circular muscle contraction[29]. Therefore, cytokines may play important roles in the pathogenesis of reflux esophagitis. However, given that very complex relationships exist among the risk factors for erosive esophagitis[23,30,31], we should be careful when interpreting the clinical significance of these relationships.

An interesting finding of this study was that among the individual components of metabolic syndrome, an elevated level of serum TG was a significant predictive factor not only for the presence of erosive esophagitis, but also for the severity of erosive esophagitis. This result is consistent with a recent study about metabolic syndrome and erosive esophagitis[9] as well as previous studies[32,33]; however, other reports did not find such a relationship between elevated serum TG and erosive esophagitis[34,35]. There are several possible explanations for this association. First, in view of the results of this study, insulin resistance and fatty liver may be responsible for increased serum TG levels because liver fat is a significant correlate of fasting glucose and triglyceride levels[36]. Also, hypertriglyceridemia is associated with increased insulin resistance[37]. Second, considering that *H pylori* infection has been suggested to be a protective factor for erosive esophagitis[38,39] and chronic *H pylori* infections can modify the serum lipid profile, including the increment of total C and TG[40,41], elevated serum TG levels could be just an epiphenomenon accompanying *H pylori* infection. Further studies are needed in order to

verify that point.

There were several limitations to our study. First, *H pylori* infections were not included in the multivariate analysis. *H pylori* infections in this study were diagnosed by histologic analysis of biopsy specimens. However, most of the subjects did not undergo the historical examination for *H pylori* because tissue biopsies were performed only when suspicious lesions were found by UGI endoscopic examination. Therefore, the prevalence of *H pylori* infection was lower in our subjects in comparison with the normal population. Nevertheless, patients undergoing a biopsy were randomly allocated to each comparison group; thus the rate of positive *H pylori* infection in patients with erosive esophagitis was significantly lower than in patients without erosive esophagitis, which is consistent with the result of a previous study[38]. Moreover, the result that metabolic syndrome was a significant risk factor for erosive esophagitis was still persistent after multivariate analysis, including *H pylori* infection ($P < 0.05$). Second, there is a possibility of selection bias because only one-half of the subjects who visited the health care center underwent UGI endoscopy and only a portion of them responded to the questionnaire regarding their symptoms. However, although subjects with reflux symptoms were more likely to participate in this study, the prevalence of patients with reflux symptoms was consistent with that of a recent study[1] and when the limited effects of reflux symptoms on erosive esophagitis are considered, this bias did not seem to affect the primary results of this study.

In conclusion, our study demonstrates that metabolic syndrome is an independent risk factor for erosive esophagitis. In addition, metabolic syndrome is significantly associated with the severity of erosive esophagitis. Therefore, we should take into account not only acid suppression, but also metabolic factors when consulting with patients who have erosive esophagitis.

COMMENTS

Background

Gastroesophageal reflux disease (GERD) and obesity are two of the most common diseases in Korea and the incidences of both have been increasing rapidly.

Research frontiers

Several studies have reported the relationship between obesity, erosive esophagitis, and GERD symptoms. A recent study demonstrated that metabolic syndrome was associated with reflux esophagitis. However, literature on whether metabolic syndrome and insulin resistance, suggested causes of metabolic syndrome, are risk factors for GERD remains scant.

Innovations and breakthroughs

One of the major findings of this study was that metabolic syndrome was strongly associated with the development and severity of erosive esophagitis. Moreover, insulin resistance, independent of metabolic syndrome, was a significant risk factor for erosive esophagitis.

Applications

This study should help to identify patients with particular risk for erosive esophagitis. An early identification of patients at risk for erosive esophagitis would allow more timely treatment and symptom relief.

Peer review

The present study showed a significant correlation between metabolic syndrome and erosive esophagitis and may be helpful for identifying the cause of erosive esophagitis. This study is interesting and valuable.

REFERENCES

1 **Yang SY**, Lee OY, Bak YT, Jun DW, Lee SP, Lee SH, Park GT, Yoon BC, Choi HS, Hahm JS, Lee MH, Lee DH. Prevalence of gastroesophageal reflux disease symptoms and uninvestigated dyspepsia in Korea: a population-based study. *Dig Dis Sci* 2008; **53**: 188-193

2 **Cho YS**, Choi MG, Jeong JJ, Chung WC, Lee IS, Kim SW, Han SW, Choi KY, Chung IS. Prevalence and clinical spectrum of gastroesophageal reflux: a population-based study in Asan-si, Korea. *Am J Gastroenterol* 2005; **100**: 747-753

3 **Youn YH**, Kang YW, Ahn SH, Park SK. Prevalence alteration of reflux esophagitis in recent years. *Korean J Gastrointest Endosc* 2001; **23**: 144-148

4 **Hampel H**, Abraham NS, El-Serag HB. Meta-analysis: obesity and the risk for gastroesophageal reflux disease and its complications. *Ann Intern Med* 2005; **143**: 199-211

5 **El-Serag HB**, Graham DY, Satia JA, Rabeneck L. Obesity is an independent risk factor for GERD symptoms and erosive esophagitis. *Am J Gastroenterol* 2005; **100**: 1243-1250

6 **Kang MS**, Park DI, Oh SY, Yoo TW, Ryu SH, Park JH, Kim HJ, Cho YK, Sohn CI, Jeon WK, Kim BI. Abdominal obesity is an independent risk factor for erosive esophagitis in a Korean population. *J Gastroenterol Hepatol* 2007; **22**: 1656-1661

7 **Ruhl CE**, Everhart JE. Overweight, but not high dietary fat intake, increases risk of gastroesophageal reflux disease hospitalization: the NHANES I Epidemiologic Followup Study. First National Health and Nutrition Examination Survey. *Ann Epidemiol* 1999; **9**: 424-435

8 **Locke GR 3rd**, Talley NJ, Fett SL, Zinsmeister AR, Melton LJ 3rd. Risk factors associated with symptoms of gastroesophageal reflux. *Am J Med* 1999; **106**: 642-649

9 **Chung SJ**, Kim D, Park MJ, Kim YS, Kim JS, Jung HC, Song IS. Metabolic syndrome and visceral obesity as risk factors for reflux oesophagitis: a cross-sectional case-control study of 7078 health check-up Koreans. *Gut* 2008 Apr 25 [Epub ahead of print]

10 **Alberti KG**, Zimmet P, Shaw J. The metabolic syndrome--a new worldwide definition. *Lancet* 2005; **366**: 1059-1062

11 **WHO**. Report of a WHO Consultation on Obesity: Prevention and Managing, the Global Epidemic. Geneva: World Health Organization, 1999

12 **Matthews DR**, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985; **28**: 412-419

13 **Armstrong D**, Bennett JR, Blum AL, Dent J, De Dombal FT, Galmiche JP, Lundell L, Margulies M, Richter JE, Spechler SJ, Tytgat GN, Wallin L. The endoscopic assessment of esophagitis: a progress report on observer agreement. *Gastroenterology* 1996; **111**: 85-92

14 **Johnson DA**, Younes Z, Hogan WJ. Endoscopic assessment of hiatal hernia repair. *Gastrointest Endosc* 2000; **52**: 650-659

15 **Won JC**, Park JY, Song KH, Lee WJ, Koh EH, Nam-Goong IS, Han SM, Lee MS, Kim MS, Lee KU. Changes in the prevalence of metabolic syndrome in a rural area of Korea defined by two criteria, Revised National Cholesterol Education Program and International Diabetes Federation. *J Korean Diabetes Assoc* 2007; **31**: 284-292

16 **Hildrum B**, Mykletun A, Hole T, Midthjell K, Dahl AA. Age-specific prevalence of the metabolic syndrome defined by the International Diabetes Federation and the National Cholesterol Education Program: the Norwegian HUNT 2 study. *BMC Public Health* 2007; **7**: 220

17 **Kuzuya M**, Ando F, Iguchi A, Shimokata H. Age-specific change of prevalence of metabolic syndrome: longitudinal observation of large Japanese cohort. *Atherosclerosis* 2007;

191: 305-312
18 **Lucove JC**, Kaufman JS, James SA. Association between adult and childhood socioeconomic status and prevalence of the metabolic syndrome in African Americans: the Pitt County Study. *Am J Public Health* 2007; **97**: 234-236
19 **Salsberry PJ**, Corwin E, Reagan PB. A complex web of risks for metabolic syndrome: race/ethnicity, economics, and gender. *Am J Prev Med* 2007; **33**: 114-120
20 **Dallongeville J**, Cottel D, Ferrieres J, Arveiler D, Bingham A, Ruidavets JB, Haas B, Ducimetiere P, Amouyel P. Household income is associated with the risk of metabolic syndrome in a sex-specific manner. *Diabetes Care* 2005; **28**: 409-415
21 **Lee SJ**, Song CW, Jeen YT, Chun HJ, Lee HS, Um SH, Lee SW, Choi JH, Kim CD, Ryu HS, Hyun JH. Prevalence of endoscopic reflux esophagitis among Koreans. *J Gastroenterol Hepatol* 2001; **16**: 373-376
22 **Choo KY**, Choi MG, Choi H, Lee DS, Kim JI, Kim SS, Bhang CS, Park SH, Kim JK, Han SW, Choi KY, Chung IS, Chung KW, Sun HS. The prevalence of gastrointestinal symptoms in a rural community in Korea. *Kor J Neurogastroenterol Motil* 2000; **6**: 31-43
23 **Yoon YS**, Oh SW, Baik HW, Park HS, Kim WY. Alcohol consumption and the metabolic syndrome in Korean adults: the 1998 Korean National Health and Nutrition Examination Survey. *Am J Clin Nutr* 2004; **80**: 217-224
24 **Chung WJ**, Chun HJ, Lee SM. [Socioeconomic costs of alcohol drinking in Korea] *J Prev Med Public Health* 2006; **39**: 21-29
25 **Yim JY**, Kim N, Choi SH, Kim YS, Cho KR, Kim SS, Seo GS, Kim HU, Baik GH, Sin CS, Cho SH, Oh BH. Seroprevalence of Helicobacter pylori in South Korea. *Helicobacter* 2007; **12**: 333-340
26 **Mohamed-Ali V**, Goodrick S, Rawesh A, Katz DR, Miles JM, Yudkin JS, Klein S, Coppack SW. Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. *J Clin Endocrinol Metab* 1997; **82**: 4196-4200
27 **Nonogaki K**, Fuller GM, Fuentes NL, Moser AH, Staprans I, Grunfeld C, Feingold KR. Interleukin-6 stimulates hepatic triglyceride secretion in rats. *Endocrinology* 1995; **136**: 2143-2149
28 **Rotter V**, Nagaev I, Smith U. Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor-alpha, overexpressed in human fat cells from insulin-resistant subjects. *J Biol Chem* 2003; **278**: 45777-45784
29 **Cao W**, Cheng L, Behar J, Fiocchi C, Biancani P, Harnett KM. Proinflammatory cytokines alter/reduce esophageal circular muscle contraction in experimental cat esophagitis. *Am J Physiol Gastrointest Liver Physiol* 2004; **287**: G1131-G1139
30 **Oh SW**, Yoon YS, Lee ES, Kim WK, Park C, Lee S, Jeong EK, Yoo T. Association between cigarette smoking and metabolic syndrome: the Korea National Health and Nutrition Examination Survey. *Diabetes Care* 2005; **28**: 2064-2066
31 **Nabipour I**, Vahdat K, Jafari SM, Pazoki R, Sanjdideh Z. The association of metabolic syndrome and Chlamydia pneumoniae, Helicobacter pylori, cytomegalovirus, and herpes simplex virus type 1: the Persian Gulf Healthy Heart Study. *Cardiovasc Diabetol* 2006; **5**: 25
32 **Furuta K**, Adachi K, Arima N, Yagi J, Tanaka S, Miyaoka Y, Miki M, Azumi T, Koshino K, Ishihara S, Amano Y, Kinoshita Y. Study of arteriosclerosis in patients with hiatal hernia and reflux esophagitis. *J Gastroenterol Hepatol* 2007; **22**: 1732-1736
33 **Yoon S**, Shim KN, Kim SE, Jung SA, Song HJ, Oh HJ, Ryu KH, Ha CY, Yeom HJ, Song JH, Jung HK, Kim TH, Yi SY, Yoo K, Moon I-H. The prevalence and clinical characteristics of patients with erosive esophagitis in a routine endoscopic check-up: Retrospective data in a Korean medical institute. *Gastrointest endosc* 2007; **65**: AB153
34 **Moki F**, Kusano M, Mizuide M, Shimoyama Y, Kawamura O, Takagi H, Imai T, Mori M. Association between reflux oesophagitis and features of the metabolic syndrome in Japan. *Aliment Pharmacol Ther* 2007; **26**: 1069-1075
35 **Kawanishi M**. Will symptomatic gastroesophageal reflux disease develop into reflux esophagitis? *J Gastroenterol* 2006; **41**: 440-443
36 **Nguyen-Duy TB**, Nichaman MZ, Church TS, Blair SN, Ross R. Visceral fat and liver fat are independent predictors of metabolic risk factors in men. *Am J Physiol Endocrinol Metab* 2003; **284**: E1065-E1071
37 **Moro E**, Gallina P, Pais M, Cazzolato G, Alessandrini P, Bittolo-Bon G. Hypertriglyceridemia is associated with increased insulin resistance in subjects with normal glucose tolerance: evaluation in a large cohort of subjects assessed with the 1999 World Health Organization criteria for the classification of diabetes. *Metabolism* 2003; **52**: 616-619
38 **Labenz J**, Blum AL, Bayerdorffer E, Meining A, Stolte M, Borsch G. Curing Helicobacter pylori infection in patients with duodenal ulcer may provoke reflux esophagitis. *Gastroenterology* 1997; **112**: 1442-1447
39 **Tsukada K**, Katoh H, Miyazaki T, Fukuchi M, Kuwano H, Kimura H, Fukai Y, Inose T, Motojima T, Toda N, Yamada S. Factors associated with the development of reflux esophagitis after Helicobacter pylori eradication. *Dig Dis Sci* 2006; **51**: 539-542
40 **Laurila A**, Bloigu A, Nayha S, Hassi J, Leinonen M, Saikku P. Association of Helicobacter pylori infection with elevated serum lipids. *Atherosclerosis* 1999; **142**: 207-210
41 **Niemela S**, Karttunen T, Korhonen T, Laara E, Karttunen R, Ikaheimo M, Kesaniemi YA. Could Helicobacter pylori infection increase the risk of coronary heart disease by modifying serum lipid concentrations? *Heart* 1996; **75**: 573-575

S- Editor Zhong XY **L- Editor** Kumar M **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5448

World J Gastroenterol 2008 September 21; 14(35): 5448-5453
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Clinical, virologic and phylogenetic features of hepatitis B infection in Iranian patients

Golnaz Bahramali, Majid Sadeghizadeh, Samad Amini-Bavil-Olyaee, Seyed-Moayed Alavian, Abbas Behzad-Behbahani, Ahmad Adeli, Mohammad-Reza Aghasadeghi, Safieh Amini, Fereidoun Mahboudi

Golnaz Bahramali, Majid Sadeghizadeh, Department of Genetics, Faculty of Basic Sciences, Tarbiat Modares University, Tehran 14115-175, Iran
Samad Amini-Bavil-Olyaee, Fereidoun Mahboudi, Ahmad Adeli, Biotechnology Department, Pasteur Institute of Iran, Tehran 13164, Iran
Mohammad-Reza Aghasadeghi, Safieh Amini, Hepatitis and HIV Department, Pasteur Institute of Iran, Tehran 13164, Iran
Seyed-Moayed Alavian, Baqiyatallah Research Center for Gastroenterology and Liver Diseases, Tehran Hepatitis Center, Tehran 14155-3651, Iran
Abbas Behzad-Behbahani, Medical Technology Research Center, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz 7143914693, Iran
Author contributions: Bahramali G performed the research; Amini-Bavil-Olyaee S and Sadeghizadeh M designed the research; Alavian SM and Behzad-Behbahani A did the sample collection; Mahboudi F, Adeli A, Aghasedeghi MR, Amini S contributed to the project; Amini-Bavil-Olyaee S and Bahramali G analyzed the data and wrote the manuscript; Sadeghizadeh M provided financial support; Bahramali G and Amini-Bavil-Olyaee S contributed equally to this work.
Supported by A grant from the Nanotechnology committee of the Ministry of Science, Research and Technology, Iran, No. 31.1895 on 05.03.2004 to Majid Sadeghizadeh
Corresponding to: Dr. Majid Sadeghizadeh, PhD, Department of Genetics, Faculty of Basic Sciences, Tarbiat Modares University, Tehran 14115-175, Iran. sadeghma@modares.ac.ir
Telephone: +98-218-2884409 Fax: +98-218-2883463
Received: June 15, 2008 Revised: July 30, 2008
Accepted: August 6, 2008
Published online: September 21, 2008

Abstract

AIM: To characterize the clinical, serologic and virologic features of hepatitis B virus (HBV) infection in Iranian patients with different stages of liver disease.

METHODS: Sixty two patients comprising of 12 inactive carriers, 30 chronic hepatitis patients, 13 patients with liver cirrhosis and 7 patients with hepatocellular carcinoma (HCC) were enrolled in the study. The HBV S, C and basal core promoter (BCP) regions were amplified and sequenced, and the clinical, serologic, phylogenetic and virologic characteristics were investigated.

RESULTS: The study group consisted of 16 HBeAg-positive and 46 HBeAg-negative patients. Anti-HBe-positive patients were older and had higher levels of ALT, ASL and bilirubin compared to HBeAg-positive patients. Phylogenetic analysis revealed that all patients were infected with genotype D (mostly *ayw2*). The G1896A precore (PC) mutant was detected in 58.1% patients. HBeAg-negative patients showed a higher rate of PC mutant compared to HBeAg-positive patients (χ^2 = 9.682, P = 0.003). The majority of patients with HCC were HBeAg-negative and were infected with PC mutant variants. There was no significant difference in the occurrence of BCP mutation between the two groups, while the rate of BCP plus PC mutants was higher in HBeAg-negative patients (χ^2 = 4.308, P = 0.04). In the HBV S region, the genetic variability was low, and the marked substitution was P120T/S, with a rate of 9.7% (n = 6).

CONCLUSION: In conclusion, HBV/D is the predominant genotype in Iran, and the nucleotide variability in the BCP and PC regions may play a role in HBV disease outcome in HBeAg-negative patients.

Key words: Hepatitis B virus; Clinical and virologic features; Genetic variability; Phylogenetic analysis

Peer reviewer: George V Papatheodoridis, PhD, 2nd Academic Department of Internal Medicine, Hippokration General Hospital, 114 Vas. Sofias ave., Athens 1527, Greece

Bahramali G, Sadeghizadeh M, Amini-Bavil-Olyaee S, Alavian SM, Behzad-Behbahani A, Adeli A, Aghasadeghi MR, Amini S, Mahboudi F. Clinical, virologic and phylogenetic features of hepatitis B infection in Iranian patients. *World J Gastroenterol* 2008; 14(35): 5448-5453 Available from: URL: http://www.wjgnet.com/1007-9327/14/5448.asp DOI: http://dx.doi.org/10.3748/wjg.14.5448

INTRODUCTION

Hepatitis B virus (HBV) infection is one of the most important infectious diseases worldwide and is a major global health problem. Approximately one million people die annually because of acute and chronic HBV infection despite the availability of effective vaccines and effective antiviral medications[1]. HBV replicates *via* the reverse transcriptase enzyme system which lacks proofreading ability; therefore, new virions

possess diverse genetic variability[2]. Different selection pressures such as host immunity (endogenous pressure), and vaccine or antiviral agents (exogenous pressure) influence the production of HBV quasispecies in infected individuals. It has been demonstrated that mutations in the HBV genome not only impact the replication fitness of the virus (phonotypical effect) but can also influence the disease outcome, as well as the response to treatment (clinical effect)[3]. Mutations in the HBV surface (*S*), precore (*PC*) and basal core promoter (*BCP*) genes are observed frequently in HBV infected patients, and studies show that these mutations are associated with the clinical outcomes of HBV disease[4-6]. The most clinically relevant mutations in the S region arise in the immunologic "a determinant" domain, and neutralizing antibodies (anti-HBs) are targeted against this epitope[7]. The most frequent and clinically important mutations in the PC and BCP regions are G1896A and A1762T/G1764A, respectively; which are often detected in HBeAg-negative chronic HBV infected patients[5]. Moreover, it has recently been documented that HBV genotypes may also contribute to the clinical features, disease outcome, and response to antiviral therapy[8].

Iran is located in the Middle East, and has an intermediate-to-low prevalence of the HBV infection[9]. The prevalence of HBV infection in Iran is around 2% and it appears that after the implementation of the HBV National Vaccination Programme (started in 1993), the HBV infection rate in young children has diminished significantly[9-11]. There are very few reports on the molecular epidemiology of HBV in Iran[12-14]. Recently, a study on the clinical and serological findings of HBV infection in Iran was published[15], however, there are no reports on the association of the clinical, serologic, virologic (HBV genetic variability) and phylogenetic features of HBV infection. In the present study, we have attempted to determine the HBV genetic variability, and its association with clinical outcome in HBV infected patients at different stages of liver disease.

MATERIALS AND METHODS

Patients

Sixty two HBsAg-positive patients who were referred to the Tehran Hepatitis Centre (2004-2006), were enrolled in a cross-sectional study. The study population consisted of 79% males (n = 49) and 21% (n = 13) females. The mean ± SD age was 37.3 ± 12.3 years (range: 15-64 years, median 36 years). All patients were interviewed and examined by gastroenterologists to evaluate the clinical findings and the results of the investigative workup (liver histology, ultrasonography, and laboratory tests such as serologic, biochemical and virological tests) in order to determine the clinical status of the patient. We followed the American Association for the Study of Liver Disease (AASLD) practice guidelines with regard to the diagnostic criteria. Briefly, inactive carriers had persistent HBV infection without significant necro-inflammatory disease. Chronic hepatitis was defined as HBsAg positivity with or without the presence of HBeAg and a high HBV DNA (> 100 000 copies/mL) level determined by the Amplicor HBV monitor, persistent or intermittent elevation in the serum ALT levels, and compatible liver biopsy. Liver cirrhosis and hepatocellular carcinoma (HCC) were confirmed by liver biopsy. Informed consent was obtained from the patients before collecting blood samples. Sera from the patients was frozen at -20℃ in aliquots, until used for virological examination.

Serologic, virologic and biochemical parameters

All patients were tested for HBV serological markers (HBsAg, anti-HBs, total anti-HBc, HBeAg, and anti-HBe), hepatitis D virus (HDV), hepatitis C virus (anti-HCV) and human immunodeficiency virus (anti-HIV) using commercial kits (DIA PRO Diagnostic Bioprobes, Srl., Italy). Coinfected patients with HIV, HDV and HCV were excluded from the study. Liver function tests such as serum albumin, total bilirubin, ALT, AST and ALP were measured by an auto-analyzer[16]. HBV DNA viral load was determined using the Cobas Amplicor HBV Monitor test (Roche Applied Science, Mannheim, Germany).

Detection of S and BCP/C mutations

HBV DNA was extracted using a nano-particle magnetic beads kit (BILATEC AG, Viernheim, Germany) according to the manufacturer's instructions. The HBV S/pol and BCP/C regions were amplified as previously described[17]. Negative serum samples from subjects with no HBV markers served periodically as a negative control. The PCR amplicons were purified using the AccuPrep™ Gel Purification Kit (Bioneer Inc, Alameda, CA), sequenced bi-directionally with inner primers using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and the data were collected by an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems).

Phylogenetic and sequence analysis

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1[18], as well as BioEdit, version 7.0.4.1 as described previously[14]. Briefly, sequences of BCP/C plus S regions (approximately 1000-bp) were block aligned by the CLUSTAL X program and corrected visually; the Kimura two-parameter algorithm was used for genetic distance calculation. A phylogenetic tree was generated by the neighbour-joining method, and bootstrap re-sampling and reconstruction was carried out 1000 × to confirm the reliability of the phylogenetic tree.

Statistical analysis

The data were statistically analyzed using the SPSS software, version 11.0 (SPSS, Inc., Chicago, IL). $P < 0.05$ was considered significant.

RESULTS

Clinical and demographic data

Based on the clinical and laboratory findings the

Table 1 Clinical, serological, virological and biochemical features of patients infected with HBV (mean ± SD)

Clinica-status	Number (%)	Sex		Age (yr)	HBs Ag+	Anti HBs+	Anti HBc+	HBe Ag+	Anti HBe+	ALT (IU/L)	AST (IU/L)	Alk (U/L)	T-Bil (mg/dL)	Albumin (g/dL)	HBV DNA (log copies /mL)
		M	F												
Inactive HBsAg carriers	12 (19.4)	9	3	30.6 ± 9.5	12	0	12	3	9	38.3 ± 25.8	31.4 ± 12.1	282.2 ± 199.9	0.7 ± 0.241	4.77 ± 1.25	4.3 ± 1.57
Chronic hepatitis	30 (48.4)	25	5	34.5 ± 11.6	30	0	30	8[1]	22[1]	250.9 ± 120.7	104.4 ± 74.7	243.6 ± 125.4	1.17 ± 0.49	4.2 ± 0.7	5.31 ± 1.48
Liver cirrhosis	13 (21.0)	9	4	44.5 ± 9.4	13	0	13	5	8	50.3 ± 18.8	64.6 ± 32.2	274.2 ± 229.3	1.41 ± 0.63	3.6 ± 0.67	5.8 ± 2.03
HCC	7 (11.3)	6	1	47.7 ± 13.7	7	0	7	0	7	123.5 ± 64.1	285.1 ± 196.8	402 ± 114.3	2.92 ± 1.5	3.1 ± 0.48	7.22 ± 2.01
Total	62 (100)	49 (79.00%)	13 (21.00%)	37.3 ± 12.3	62	0	62	16	46	178.7 ± 93.7	120.6 ± 91.1	274.2 ± 165.6	1.36 ± 0.89	3.83 ± 0.91	5.2 ± 1.57

[1]One patient had both positive HBeAg and anti-HBe status.

Table 2 Comparison of demographic and para-clinical features between HBeAg-positive and anti-HBe-positive individuals (mean ± SD)

	HBeAg-positive (n = 16)	Anti-HBe-positive (n = 46)	P
Age (yr)	35.06 ± 12.8	38 ± 12.2	NS
Sex (M/F)	8/8	41/5	0.002
Genotype	D	D	NS
ALT (IU/L)	70.7 ± 63.5	101.7 ± 204.3	NS
AST (IU/L)	69.3 ± 67.6	98.6 ± 134.07	NS
T-Bil (mg/dL)	1.18 ± 0.58	1.43 ± 0.97	NS
HBV DNA (log copies/mL)	6.21 ± 1.7	5.2 ± 1.57	NS

NS: Not significant.

Table 3 The rate and percentage of BCP/C region mutations in HBV isolated among different clinical groups n (%)

Mutation	Inactive HBsAg carriers (n = 12)	Chronic hepatitis (n = 30)	Liver cirrhosis (n = 13)	HCC (n = 7)	Total rate (%)
A1757	12 (100)	25 (83.3)	11 (84.61)	5 (71.42)	85.4
C1753	1 (8.3)	5 (16.6)	5 (38.46)	4 (57.14)	24.2
T1762/A1764	5 (41.6)	11 (36.6)	4 (30.76)	3 (42.85)	37.1
A1899	3 (25)	9 (30)	8 (61.5)	3 (42.85)	37.1
A1896	5 (41.6)	19 (63.3)	6 (46.15)	6 (85.71)	58.1
T1766/A1768	1 (8.3)	4 (13.3)	2 (15.38)	1 (14.28)	12.9
T1764/G1766	1 (8.3)	5 (16.6)	1 (7.69)	2 (28.5)	14.5

patients were divided into four categories: 19.4% patients (n = 12) were inactive HBsAg carriers, 48.4% (n = 30) had chronic hepatitis B infection, 21.0% (n = 13) were diagnosed with cirrhosis, and 11.3% (n = 7) had HCC. The clinical and laboratory findings (serologic, biochemical and virologic) are summarized in Table 1. The clinical features of HBeAg-positive patients and anti-HBe-positive patients are shown in Table 2. Based on the HBeAg serology status, 16 patients were HBeAg-positive and 46 patients were anti-HBe-positive. There was no significant difference in age, ALT, AST, and bilirubin levels (biochemical parameters), and HBV viral load between HBeAg-positive and HBeAg-negative groups; however, a significant difference in the gender distribution was observed (χ^2 = 10.96, P = 0.003) (Table 2). All chronic hepatitis B patients with HCC were HBeAg-negative and were older than the other groups.

HBV genotype and subtype

The phylogenetic tree was constructed using the block alignment of HBV S plus BCP/C gene sequences (62 HBV isolates from this study) along with different HBV genotype (A to H) sequences retrieved from the GenBank[19] as reference genes. The phylogenetic tree revealed that all Iranian isolates were branched with other genotype D of HBV reference isolates with a high bootstrap value, 99%, 1000 × replicates (Figure 1). Thus, all Iranian patients were infected with only genotype D. To assess the HBV subtype, the amino acid mapping on the HBV *S* gene protein was performed. Based on the presence of Arg[122], Thr[125], Pro[127], and Lys[160] residues, 98.4% (n = 61) and 1.6% (n = 1) of isolated HBV were subtyped as *ayw2* and *ayw3*, respectively.

Characteristics of nucleotide substitution in the S and BCP/C regions

Amino acid sequences of a portion of the S region of all 62 isolates of the study patients were compared with the amino acid sequences of the reference genes. Amino acid mapping revealed that the S region was relatively conserved; however, an important substitution of P120T/S was observed. P120T/S substitutions were detected in 9.7% of chronic hepatitis and cirrhotic patients (n = 6). No G145R substitution was identified in the isolates; whereas, some substitutions such as P127T, T131I, Y134H, D144N and I152T were observed in the immunologic domain of the "a determinant" region.

With regard to the mutations in the BCP and C regions (Table 3), a high rate of G1896A PC mutant variants (58.1%, 36/62) was detected in the isolates. The rate of precore mutant isolates was significantly higher (χ^2 = 9.682, P = 0.003) in HBeAg-negative patients (69.5%, n = 32) compared to HBeAg-positive patients (25%, n = 4). In the HBV precore region, mutation of G1899A (Gly-to-Asp, at codon 29) was found in 37.1% isolates (n = 23), and was mostly detected in patients with cirrhosis (61.5%), and HCC (42.8%). All isolates had T1585 which is specific for genotype D.

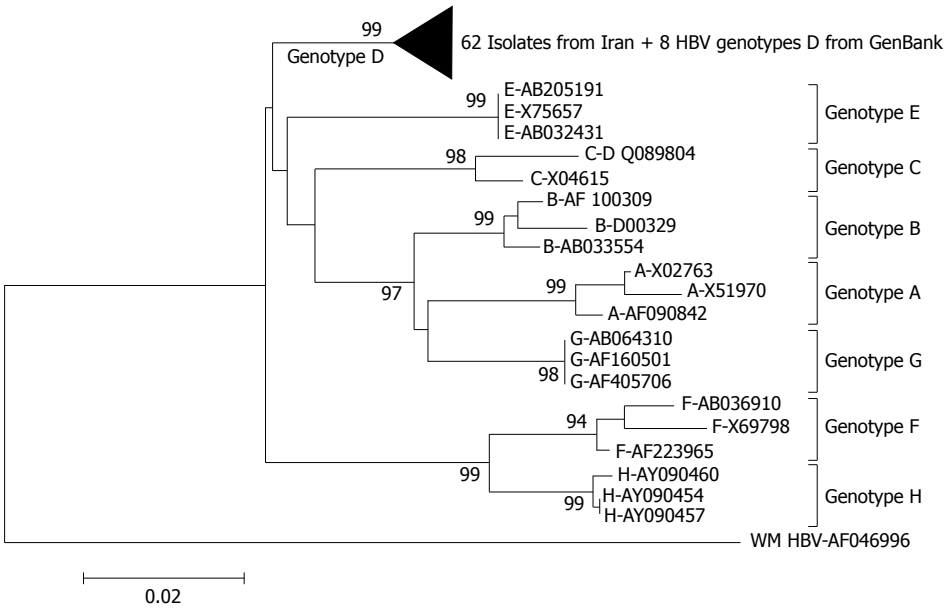


Figure 1 Neighbour joining phylogenetic analysis based on the block-alignment of the S/BCP/C gene regions (approximately 1000-bp) of 62 HBV isolates from Iran and other HBV genotypes form GenBank as reference genes. Bootstrap values indicate 1000-fold replicates. Due to clarity, the 62 isolates from Iran and 8 reference genes of HBV genotype D were collapsed. Woolly monkey HBV was used as an out-group.

BCP double mutation (T1762/A1764) was observed in 37.1% isolates (n = 23); whereas, T1762 and A1764 were also found alone. There was no significant difference in the rate of BCP double mutation between HBeAg-positive and HBeAg-negative patients. The occurrence of precore mutant plus BCP double mutation was detected in 28.2% of HBeAg-negative patients (n = 13); and in 12.5% (n = 2) HBeAg-positive individuals. The G1862T mutation was observed in 4.8% of chronic hepatitis patients (n = 3). This mutation, in which valine (Val) was replaced by phenylalanine (Phe) at position 17 in the PC region, was observed only in HBeAg-negative patients.

DISCUSSION

In the present study, we examined the clinical, serologic, virologic and phylogenetic features in patients with different clinical stages of HBV-related liver disease (inactive HBsAg carriers, chronic hepatitis B, chronic hepatitis B with cirrhosis and HCC). We believe this is the first such study from Iran. Our previous studies on the molecular analysis of HBV, revealed the presence of genotype D in Iran[14,20]. As expected, the present study also showed that genotype D with *ayw2* subtype was present in all 62 patients studied. Genotype D has been reported globally[21]; but has a high prevalence in the Mediterranean area and in the Middle East[22].

The relationship between HBV genotype(s), and the outcome of liver disease, and the response to treatment has been well documented, and has an important impact on public health[8,23]. For example, several studies have shown differences in disease progression between genotype B and C in Asian patients. HBV genotype C is associated with more severe cirrhosis and HCC, and poorer response to interferon therapy[24,25]. Moreover, it has been observed that genotypes can influence HBV replication. For example, HBeAg-negative patients harbouring genotype B had lower viral replication efficiency[25]. Since HBV genotype D was the only predominant genotype in the present study, a comparison between different genotypes was not possible. Non D HBV genotypes are not seen in Iran; whereas, different subtypes of genotype D have been reported[26].

In the present study, amino acid mapping of the *S* gene showed a high rate of homology between the sequences. It has been shown that amino acid substitutions within the "a determinant" domain in the HBV *S* region may lead to conformational changes in the S protein. Some of these changes may create important medical and public health problems including vaccine escape, failure of hepatitis B immune globulin (HBIG) to protect liver transplant patients and babies born to HBV carrier mothers, and failure to detect HBV carriers with certain diagnostic tests[7,27]. In this study, the P120T/S was the most important substitution. This substitution was located at the outside of the "a determinant" immunologic domain. The P120T/S was detected in six isolates. As previously reported, the P120T/S substitution may cause problems with diagnostic assays, and may also cause vaccine escape and poor response to HBIG therapy[27,28].

The G1896A PC mutation truncates the HBeAg protein product by creating a stop codon at position 28 within the precore mRNA. Therefore, patients with HBV variants carrying the A1896 mutant in the genome are usually HBeAg-negative. The G1896A PC mutant may be detected in 20%-95% of HBeAg-negative patients worldwide[3], and is highly predominant in the Mediterranean area where HBV genotype D has a high rate of infection[29]. In a previous study, the rate of HBV precore mutant variants in Iran was reported to be 54%[14]; in the present study, the rate was 58.1%. We observed that patients with a precore mutant variant were older and had a higher rate of AST and ALT elevation (but not statistically significant) compared to patients without this variant, suggesting that this variant occurs in patients with a longer history of HBV infection and worse liver disease. In this study, 85.7%

of patients with HCC (n = 6) carried A1896 variants; whereas, this rate was less in the other study groups. These results are in accordance with previous reports[6,30]. The precore mutant variants have also been reported in HBeAg-positive patients, ranging from 0%-80%[6,31]. In our study, two patients with cirrhosis were infected with the HBV A1896 variants despite HBeAg positivity.

The BCP T1762/A1764 double mutations located at the HBV X gene diminishes HBeAg production, and is associated with more active liver disease[2,32]. In the present study, BCP double mutation T1762/A1764 was detected in 37.1% of patients; but there was no association between these mutations and the status of liver disease (P = 0.7). Moreover, no significant difference (P = 0.9) was observed between the frequency of BCP double mutation in patients with HBeAg-positive and HBeAg-negative phenotypes. By contrast, other studies have shown a relationship between BCP double mutations and the clinical manifestations of HBV infection[6,30]. Moreover, the T1764/G1766 double mutation in the BCP region was detected in 14.5% of isolates (n = 9) (Table 2). However, this study utilized a relatively small study population, and the results suggest that mutations in the PC region were related to more severe liver disease. More studies in larger populations are required to better understand these associations.

In conclusion, the present study has shown that genotype D (predominantly subtype *ayw2*) is the only genotype in Iranian patients. Moreover, a high rate of the precore mutation and BCP double mutation was detected in our study.

ACKNOWLEDGMENTS

We are grateful to Abbas Padeganeh for his comments and critical reading of the manuscript.

COMMENTS

Background

Heterogeneity of hepatitis B virus (HBV) genome and its mutations may influence the outcome of liver disease as well as the response to antiviral treatment. Considering this fact, we studied the clinical, virologic and phylogenetic features of HBV infection in four groups of patients: inactive carriers, chronic hepatitis, cirrhosis and hepatocellular carcinoma.

Research frontiers

The present study revealed an association between certain HBV mutations and the outcome of liver disease, and the response to treatment.

Innovations and breakthroughs

This is the first report on the association between different clinical presentations of HBV infection and mutations in three regions of the HBV genome: basal core promoter (BCP)/Precore, P and S.

Peer review

The present study is relatively small, but it provides useful information on HBV characteristics in Iranian patients with chronic HBV infection.

REFERENCES

1 **Kao JH**, Chen DS. Global control of hepatitis B virus infection. *Lancet Infect Dis* 2002; **2**: 395-403
2 **Hannoun C**, Horal P, Lindh M. Long-term mutation rates in the hepatitis B virus genome. *J Gen Virol* 2000; **81**: 75-83
3 **Kidd-Ljunggren K**, Miyakawa Y, Kidd AH. Genetic variability in hepatitis B viruses. *J Gen Virol* 2002; **83**: 1267-1280
4 **Kidd-Ljunggren K**, Myhre E, Blackberg J. Clinical and serological variation between patients infected with different Hepatitis B virus genotypes. *J Clin Microbiol* 2004; **42**: 5837-5841
5 **Tacke F**, Manns MP, Trautwein C. Influence of mutations in the hepatitis B virus genome on virus replication and drug resistance--implications for novel antiviral strategies. *Curr Med Chem* 2004; **11**: 2667-2677
6 **Chen CH**, Lee CM, Hung CH, Hu TH, Wang JH, Wang JC, Lu SN, Changchien CS. Clinical significance and evolution of core promoter and precore mutations in HBeAg-positive patients with HBV genotype B and C: a longitudinal study. *Liver Int* 2007; **27**: 806-815
7 **Carman WF**. S gene variation of HBV. *Acta Gastroenterol Belg* 2000; **63**: 182-184
8 **Verschuere V**, Yap PS, Fevery J. Is HBV genotyping of clinical relevance? *Acta Gastroenterol Belg* 2005; **68**: 233-236
9 **Alavian SM**. Ministry of Health in Iran Is Serious about Controlling Hepatitis B. *Hep Mon* 2007; **7**: 3-5
10 **Alavian SM**, Fallahian F, Lankarani KB. The changing epidemiology of viral hepatitis B in Iran. *J Gastrointestin Liver Dis* 2007; **16**: 403-406
11 **Alizadeh AH**, Ranjbar M, Ansari S, MirArab A, Alavian SM, Mohammad K, Adibi P, Sadri GH, Keramat F, Ardalan A, Arabi M, Gharekhani S, Ataei A, Amraei GR, Hosseinzadeh M, Hatami S, Zali M. Seroprevalence of hepatitis B in Nahavand, Islamic Republic of Iran. *East Mediterr Health J* 2006; **12**: 528-537
12 **Amini-Bavil-Olyaee S**, Hosseini SY, Sabahi F, Alavian SM. Hepatitis B virus (HBV) genotype and YMDD motif mutation profile among patients infected with HBV and untreated with lamivudine. *Int J Infect Dis* 2008; **12**: 83-87
13 **Alavian SM**, Keyvani H, Rezai M, Ashayeri N, Sadeghi HM. Preliminary report of hepatitis B virus genotype prevalence in Iran. *World J Gastroenterol* 2006; **12**: 5211-5213
14 **Amini-Bavil-Olyaee S**, Sarrami-Forooshani R, Mahboudi F, Sabahi F, Adeli A, Noorinayer B, Azizi M, Reza Zali M. Genotype characterization and phylogenetic analysis of hepatitis B virus isolates from Iranian patients. *J Med Virol* 2005; **75**: 227-234
15 **Mojiri A**, Behzad-Behbahani A, Saberifirozi M, Ardabili M, Beheshti M, Rahsaz M, Banihashemi M, Azarpira N, Geramizadeh B, Khadang B, Moaddeb A, Ghaedi M, Heidari T, Torab A, Salah A, Amirzadeh S, Jowkar Z, Mehrabani D, Amini-Bavil-Olyaee S, Dehyadegari MA. Hepatitis B virus genotypes in southwest Iran: molecular, serological and clinical outcomes. *World J Gastroenterol* 2008; **14**: 1510-1513
16 **Mohamadnejad M**, Pourshams A, Malekzadeh R, Mohamadkhani A, Rajabiani A, Asgari AA, Alimohamadi SM, Razjooyan H, Mamar-Abadi M. Healthy ranges of serum alanine aminotransferase levels in Iranian blood donors. *World J Gastroenterol* 2003; **9**: 2322-2324
17 **Amini-Bavil-Olyaee S**, Alavian SM, Adeli A, Sarrami-Forooshani R, Sabahi F, Sabouri E, Tavangar HR, Azizi M, Mahboudi F. Hepatitis B virus genotyping, core promoter, and precore/core mutations among Afghan patients infected with hepatitis B: a preliminary report. *J Med Virol* 2006; **78**: 358-364
18 **Kumar S**, Tamura K, Nei M. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* 2004; **5**: 150-163
19 **Rozanov M**, Plikat U, Chappey C, Kochergin A, Tatusova T. A web-based genotyping resource for viral sequences. *Nucleic Acids Res* 2004; **32**: W654-W659
20 **Amini-Bavil-Olyaee S**, Sarrami-Forooshani R, Adeli A, Sabahi F, Abachi M, Azizi M, Mahboudi F. Complete genomic sequence and phylogenetic relatedness of hepatitis B virus isolates from Iran. *J Med Virol* 2005; **76**: 318-326
21 **McMahon BJ**. Epidemiology and natural history of hepatitis

B. *Semin Liver Dis* 2005; **25** Suppl 1: 3-8
22 **Qirbi N**, Hall AJ. Epidemiology of hepatitis B virus infection in the Middle East. *East Mediterr Health J* 2001; **7**: 1034-1045
23 **Kao JH**. Hepatitis B viral genotypes: clinical relevance and molecular characteristics. *J Gastroenterol Hepatol* 2002; **17**: 643-650
24 **Kao JH**, Chen PJ, Lai MY, Chen DS. Genotypes and clinical phenotypes of hepatitis B virus in patients with chronic hepatitis B virus infection. *J Clin Microbiol* 2002; **40**: 1207-1209
25 **Tseng TC**, Kao JH. HBV genotype and clinical outcome of chronic hepatitis B: facts and puzzles. *Gastroenterology* 2008; **134**: 1272-1273; author reply 1273
26 **Mohebbi SR**, Amini-Bavil-Olyaee S, Noorinayer B, Derakhshan F, Chiani M, Rostami Nejad M, Antikchi MH, Sabahi F, Zali MR. Hepatitis B virus molecular epidemiology in Iran. *Clin Microbiol Infect* 2008; **14**: 858-866
27 **Carman WF**. The clinical significance of surface antigen variants of hepatitis B virus. *J Viral Hepat* 1997; **4** Suppl 1: 11-20
28 **Tian Y**, Xu Y, Zhang Z, Meng Z, Qin L, Lu M, Yang D. The amino Acid residues at positions 120 to 123 are crucial for the antigenicity of hepatitis B surface antigen. *J Clin Microbiol* 2007; **45**: 2971-2978
29 **Funk ML**, Rosenberg DM, Lok AS. World-wide epidemiology of HBeAg-negative chronic hepatitis B and associated precore and core promoter variants. *J Viral Hepat* 2002; **9**: 52-61
30 **Chen CH**, Lee CM, Lu SN, Changchien CS, Eng HL, Huang CM, Wang JH, Hung CH, Hu TH. Clinical significance of hepatitis B virus (HBV) genotypes and precore and core promoter mutations affecting HBV e antigen expression in Taiwan. *J Clin Microbiol* 2005; **43**: 6000-6006
31 **Brunetto MR**, Oliveri F, Coco B, Leandro G, Colombatto P, Gorin JM, Bonino F. Outcome of anti-HBe positive chronic hepatitis B in alpha-interferon treated and untreated patients: a long term cohort study. *J Hepatol* 2002; **36**: 263-270
32 **Lindh M**, Hannoun C, Dhillon AP, Norkrans G, Horal P. Core promoter mutations and genotypes in relation to viral replication and liver damage in East Asian hepatitis B virus carriers. *J Infect Dis* 1999; **179**: 775-782

S- Editor Li DL **L- Editor** Anand BS **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5454

World J Gastroenterol 2008 September 21; 14(35): 5454-5460
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Polymorphisms of microsomal triglyceride transfer protein in different hepatitis B virus-infected patients

Zhi-Tao Yang, Xin-Xin Zhang, Xiao-Fei Kong, Dong-Hua Zhang, Shen-Ying Zhang, Jie-Hong Jiang, Qi-Ming Gong, Gen-Di Jin, Zhi-Meng Lu

Zhi-Tao Yang, Xin-Xin Zhang, Xiao-Fei Kong, Dong-Hua Zhang, Shen-Ying Zhang, Zhi-Meng Lu, Pole Sino-Français de Recherches en Science du Vivant et Genomique, Ruijin Hospital, 197 Ruijin Er Road, Shanghai 200025, China
Xin-Xin Zhang, Xiao-Fei Kong, Dong-Hua Zhang, Shen-Ying Zhang, Jie-Hong Jiang, Qi-Ming Gong, Gen-Di Jin, Zhi-Meng Lu, Department of Infectious Diseases, Ruijin Hospital, 197 Ruijin Er Road, Shanghai 200025, China
Author contributions: Yang ZT, Kong XF, Zhang SY and Zhang XX designed the research; Yang ZT and Kong XF performed the research; Jiang JH, Jin GD and Zhang DH carried out the HBV serological test and detected the PCR HBV-DNA; Zhang XX, Gong QM, Lu ZM, Jin GD, Yang ZT and Kong XF collected the clinical data; Yang ZT, Kong XF and Zhang XX analyzed the data; Yang ZT and Kong XF wrote the paper; Zhang XX corrected the paper.
Supported by F.Hoffmann-La Roche Ltd Switzerland and the National High Technology Research and Development Program of China (863 Program), No. 2006AA02A411
Correspondence to: Xin-Xin Zhang, Department of Infectious Diseases, Ruijin Hospital, 197 Ruijin Er Road, Shanghai 200025, China. xin-xin-zhang@163.com
Telephone: +86-21-64370045-360409 Fax: +86-21-64668720
Received: May 19, 2008 Revised: July 7, 2008
Accepted: July 14, 2008
Published online: September 21, 2008

Abstract

AIM: To identify the two polymorphisms of microsomal triglyceride transfer protein (*MTP*) gene in the Chinese population and to explore their correlation with both hepatitis B virus (HBV) self-limited infection and persistent infection.

METHODS: A total of 316 subjects with self-limited HBV infection and 316 patients with persistent HBV infection (195 subjects without familial history), matched with age and sex, from the Chinese Han population were enrolled in this study. Polymorphisms of MTP at the promoter region -493 and at H297Q were determined by the allele specific polymerase chain reaction (PCR).

RESULTS: The ratio of males to females was 2.13:1 for each group and the average age in the self-limited and chronic infection groups was 38.36 and 38.28 years, respectively. None of the allelic distributions deviated significantly from that predicted by the Hardy-Weinberg equilibrium. There was a linkage disequilibrium between H297Q and -493G/T (D' = 0.77). As the χ^2 test was used, the genotype distribution of *MTP*-493G/T demonstrated a significant difference between the self-limited infection group and the entire chronic group or the chronic patients with no family history (χ^2 = 8.543, P = 0.015 and χ^2 = 7.199, P = 0.019). The allele distribution at the *MTP*-493 position also demonstrated a significant difference between the study groups without family history (χ^2 = 6.212, P = 0.013). The T allele emerged as a possible protective factor which may influence the outcomes of HBV infection (OR: 0.59; 95% CI: 0.389-0.897).

CONCLUSION: The polymorphism of the *MTP* gene, T allele at -493, may be involved in determining the HBV infection outcomes, of which the mechanism needs to be further investigated.

Key words: Hepatitis B virus; Microsomal triglyceride transfer protein; Single nucleotide polymorphism; Self-limited HBV infection; Chronic hepatitis B; Clinical outcomes

Peer reviewer: Eva Herrmann, Professor, Saarland University, Kirrberger Str., Homburg/Saar 66421, Germany

Yang ZT, Zhang XX, Kong XF, Zhang DH, Zhang SY, Jiang JH, Gong QM, Jin GD, Lu ZM. Polymorphisms of microsomal triglyceride transfer protein in different hepatitis B virus-infected patients. *World J Gastroenterol* 2008; 14(35): 5454-5460 Available from: URL: http://www.wjgnet.com/1007-9327/14/5454.asp DOI: http://dx.doi.org/10.3748/wjg.14.5454

INTRODUCTION

Hepatitis B virus (HBV) is the most common cause of acute and chronic liver disease worldwide, especially in several areas of Asia and Africa. Most infected individuals can clear the virus, while only 5%-10% develop chronic hepatitis and remain in a persistent viral state[1,2]. The reasons for viral persistence are poorly understood, but host genetic factors are likely to influence the disease outcome[3].

Molecular genetics methods have increased our

ability to discover variations in the human genome and to correlate them with disease. Single nucleotide polymorphisms (SNPs) are used to characterize gene variations. Genetic associations can provide clues to fundamental questions about the pathogenesis of diseases and lead to new therapeutic avenues[4]. For chronic hepatitis B, this approach may help determine the basis for viral persistence and the development of end-stage complications such as cirrhosis or hepatocellular carcinoma. Initial genetic studies of viral hepatitis focused on human leukocyte antigen (*HLA*) associations[5] and polymorphisms in the promoter or coding region of several genes, such as interleukin-10 (*IL*-10), interferon-γ (*IFN*-γ), vitamin-D receptor (*VDR*), *etc*[6-10], and demonstrate some relationships with the outcome of HBV infection.

The liver is the major organ for the production of plasma lipoproteins, their uptake from plasma and their catabolism[11]. The production of apolipoprotein B (apoB)-containing lipoproteins by the liver is required for the assembly and secretion of very low-density lipoproteins (VLDLs) and low-density lipoproteins (LDLs)[12-16]. The microsomal triglyceride transfer protein (MTP) also plays a key role in apoB secretion by catalyzing the transfer of lipids to the nascent apoB molecule as it is co-translationally translocated across the endoplasmic reticulum membrane[17,18]. Recent studies have shown that the polymorphism at *MTP*-493 is responsible for a change in the *MTP* gene at the transcription level, and that this is prone to influence the intrahepatic triglyceride content[19-21].

Hepatic steatosis frequently occurs during chronic hepatitis B and C. In a transgenic mouse model, hepatitis C virus (HCV) core protein has been shown to inhibit the MTP activity and to modify the hepatic VLDL assembly and secretion[22]. However, no data are available to demonstrate the functional polymorphism of *MTP*-493T/G in HBV-infected patients. The aim of this pilot study was to identify the two polymorphisms of the *MTP* gene in the Chinese population by SNP and to explore their correlation with both HBV self-limited infection and persistent infection.

PATIENTS AND METHODS

Human subjects

In China, 90% of Chinese people are Han and the other 10% derive from 55 minority populations. We enrolled 632 Han Chinese subjects from Ruijin Hospital of Shanghai Jiaotong University Medical School. Among them, 316 had persistent HBV infection (including 195 patients with no family history of chronic hepatitis B) and 316 had previously self-limited HBV infection with no family history. Age and sex were matched between these groups.

The diagnostic criteria for persistent HBV infection were based on the presence of hepatitis B surface antigen (HBsAg) and anti-core IgG-antibody (Anti-HBc), and the absence of anti-hepatitis B surface antibody (Anti-HBs) for more than 6 mo. The mean time from the presumed onset of HBV infection was defined as the first documented seropositivity for HBsAg with or without elevated serum liver enzyme.

Self-limited hepatitis B virus infection was defined as being positive for anti-HBs and anti-HBc, in the absence of previous HBV vaccination, and a negative family history of chronic hepatitis B. Serum HBV-DNA was analyzed to exclude patients with occult HBV infection.

Subjects negative for all HBV markers were not included in the study as these subjects were unlikely to have been exposed to HBV. If they had been exposed to the virus, it would be impossible to predict their outcome.

Patients with concurrent hepatitis A, C, D, E or human immunodeficiency virus (HIV) infection were excluded from the study. Patients with liver disease caused by other factors, such as excess alcohol consumption and autoimmune hepatitis, were also excluded from the study. Our study conforms to the ethical guideline of the 2004 Declaration of Helsinki.

Serological test

Five milliliters of whole blood samples was collected from each subject, the sera were stored at -20℃. Serology for HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HBc was conducted in accordance with the manufacturer's protocol (Axsym, Abbott).

Genomic DNA extraction

Genomic DNA was isolated using a genomic DNA purification kit (PUREGENE) according to its manufacturer's instructions. DNA samples were quantified with a biophotometer (Eppendorf) and subjected to allele specific real-time polymerase chain reaction (PCR).

Genotyping of gene polymorphisms

Polymorphisms of MTP, including *MTP*-493G/T and H297Q were analyzed. We used the Allele Specific PCR Primer Design Program provided by Roche to design primers (Table 1).

All amplifications were performed on ABI-7000 (real-time PCR) with a 50 μL reaction mixture containing 30 ng of genomic DNA, 0.2 μmol/L per primer, PCR buffer, 0.2 μmol/L of each dNTP (Promega), 4% DMSO (Fisherbrand), 2.4% glycerine, 5 units of Delta Z05 DNA polymerase (Roche), 1 × SYB green (Cambrex). Each genotyping contains 2 amplifications, with one common primer and two specific primers, respectively. To genotype the polymorphisms at the promoter region-493 and at the coding region of MTP at amino acid position 297, primers *MTP*-493-1, *MTP*-493-2, *MTP*-493-cp and MTP H297Q-1, MTP H297Q-2, MTP H297Q-cp were used to analyze the *MTP*-493 G/T and MTP H297Q polymorphisms, respectively (Table 1). Amplification was performed with activation and denaturation at 94℃ and at 95℃ and an annealing temperature of 60℃, respectively.

Genotypes were determined by the difference in cycling numbers (ΔCT) of 2 amplification curbs with the same genomic DNA and the melting curbs, according to the manufacturer's (Roche) instructions.

Statistical analysis

The frequencies of *MTP*-493G/T and *MTP* H297Q alleles were compared between the chronic infection and self-limited infection groups by the χ^2 test. Hardy-Weinberg equilibrium was tested by comparing the expected and observed genotype frequencies using the χ^2 test. To analyze the linkage disequilibrium (LD), pair wise LD was analyzed between two loci on MTP by evaluating the measurement of D'. The difference between the probabilities of observing the alleles independently in the population is: $f(D) = f(A_1B_1) - f(A_1)f(B_1)$, where A and B refer to two genetic markers and *f* is their frequency. D' is obtained from D/Dmax and a value of 0.0 suggests independent assortment, whereas 1.0 means that copies of an allele occur exclusively with one of the possible alleles of the other marker. Analysis of D' was performed using HAPLOVIEW 3.0. The odds ratio with a 95% confidence interval, *P* values and Mantel-Haenszel test were calculated using SAS 8.0 to explore the SNP which may independently influence the outcome of HBV infection.

Table 1 Positions of analyzed SNP and primers used in this study

Genes (Ref.)	SNP	Primers	Sequences (5'-3')
MTP H297Q rs#2306985[23]	C/G	-1	CAGGTCTTCCAGAGCCAC
		-2	CAGGTCTTCCAGAGCCAG
		-cp	ATTTGTCTGCACTTACAGAAGGA
MTP-493[20]	G/T	-1	ATTTAAACTGTTAATTCATACCACA
		-2	TTTAAACTGTTAATTCATACCACC
		-cp	CTTTAACATTATTTTGAAGTGATTGG

Table 2 General characteristics of 632 subjects

	Self-limited HBV infection (*n* = 316)	Chronic hepatitis B (*n* = 316)	
		With family history (*n* = 121)	No family history (*n* = 195)
Male	215	80 (25.3%)	135 (42.7%)
Female	105	41 (13.0%)	60 (19.0%)
Age (yr)	38.28 ± 11.44	36.93 ± 12.28	39.12 ± 10.84
Age range (yr)	9-75	9-69	16-75
ALT level (× ULN)	Normal	3.62 ± 3.48	3.12 ± 2.62
HBV-DNA level (Log)	Undetectable	5.45 ± 2.34	

RESULTS

Demographic characteristics of subjects

In the 2 groups matched for age and sex, the male to female ratio was 2.13:1 (215:101) in each group. The distribution of age in the chronic hepatitis B and self-limited groups, calculated by SAS respectively, was normal ($P = 0.07$ and 0.182). The mean age of subjects in the two groups was 38.28 years and 38.36 years, respectively, with no significant deviation (STDEV was 11.44 and 11.12). In the chronic hepatitis B subgroups, 121 patients (80 males and 41 females) had a family history while 195 (135 males and 60 females) had no family history of liver disease. Serum alanine aminotransferase (ALT) levels in the self-limited group were normal, and 3 times higher than the upper normal limit in the chronic hepatitis B group. Serum HBV-DNA was detectable in each study subject but undetectable (< 3 $\log_{10}$) in the self-limited group, whereas it was positive in the chronic hepatitis B group (5.45 ± 2.34 $\log_{10}$).

The general characteristics of our study subjects are summarized in Table 2.

Allele frequencies and linkage disequilibrium

The polymorphisms of *MTP* H297Q and *MTP*-493G/T were analyzed in 632 subjects of the Chinese Han population in Shanghai. The T minor allele frequency of promoter polymorphisms-493 in the *MTP* gene was 0.123, whereas the G frequency of missense polymorphism H297Q was 0.668. The 2 SNPs of the *MTP* gene showed a statistically significant linkage disequilibrium (D' = 0.77, $P < 0.05$). None of the allelic distributions deviated significantly from that predicted by the Hardy-Weinberg equilibrium (calculated by SAS, Chi-Square $P > 0.05$).

Association of SNP genotypes with outcomes of HBV infection

The genotype distribution of *MTP*, depending on the outcome of HBV infection, is shown in Table 3. The genotype frequencies of *MTP* H297Q, CC, CG and GG were 0.104, 0.468 and 0.428 in the self-limited group while 0.104, 0.443 and 0.453 in the chronic group, respectively. The frequencies of the TT, TG and GG genotypes of *MTP*-493G/T were 0.013, 0.253 and 0.734 in the self-limited group, and 0.025, 0.165 and 0.810 in the chronic group, respectively. The χ^2 test was used to analyze the association of genotype distribution with HBV infection outcomes. The distribution of *MTP*-493G/T was significantly different between the self-limited and chronic hepatitis B groups, both before and after adjustment for family history ($\chi^2 = 8.543$, $P = 0.015$; $\chi^2 = 7.199$, $P = 0.019$). The genotype distributions of *MTP* H297Q demonstrated no significant difference between the two groups and subgroups (i.e. with and without family history).

A significant difference was demonstrated in the allele distribution of *MTP*-493G/T between the self-limited and chronic hepatitis B groups without familial history ($\chi^2 = 6.212$, $P = 0.013$, Table 4). As calculated by Mantel-Haenszel, the T allele emerged as a potential protective factor positively influencing the HBV infection outcomes in the self-limiting group compared with the chronic hepatitis B group without family history ($P = 0.013$, OR = 0.59 < 1).

DISCUSSION

Several studies suggested that HBV-associated chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) are more common in men than in women[24-26], showing that the relative risk for chronic HBV infection is increased in men when compared to that in women. In China, most HBV infections occur during the neonatal or perinatal period, following materno-foetal transmission where the mothers are HBeAg-positive

Table 3 Genotype distributions of *MTP* H297Q and *MTP*-493G/T *n* (%)

SNP	Genotype	Self-limited HBV infection (*n* = 316)	Chronic hepatitis B total (*n* = 316)	Chronic hepatitis B without family history (*n* = 195)	χ^2 test			
					SLHBV *vs* CHB Total		SLHBV *vs* CHB no FH	
					Value	*P*	Value	*P*
MTP H297Q	CC	33 (10.4)	33 (10.4)	20 (10.3)	0.452	0.798	2.588	0.274
	CG	148 (46.8)	140 (44.3)	78 (40.0)				
	GG	135 (42.8)	143 (45.3)	97 (49.7)				
MTP-493G/T	GG	232 (73.4)	256 (81.0)	163 (83.6)	8.543	0.015	7.199	0.019[1]
	GT	80 (25.3)	52 (16.5)	30 (15.4)				
	TT	4 (1.3)	8 (2.5)	2 (1.0)				

[1]Fisher's exact test (2-Tail). SLHBV: Self-limited HBV infection group; CHB total: Chronic hepatitis B total group; CHB no FH: Chronic hepatitis B without chronic hepatitis B family history group.

Table 4 Allele Distributions of *MTP* H297Q and *MTP*-493G/T *n* (%)

SNP	Allele	Self-Limited HBV infection (*n* = 632)	Chronic hepatitis B total (*n* = 632)	Chronic hepatitis B without family history (*n* = 390)	χ^2 test and Mantel-Haenszel logit					
					SLHBV *vs* CHB total			SLHBV *vs* CHB no FH		
					P	OR	95% CI	*P*	OR	95% CI
MTP H297Q	C	214 (33.9)	206 (32.6)	118 (30.3)	0.52	0.903	0.659-1.236	0.232	1.18	0.899-1.549
	G	418 (66.1)	426 (67.4)	272 (69.7)						
MTP-493G/T	G	544 (86.1)	564 (89.2)	356 (91.3)	0.023	0.645	0.442-0.940	0.013	0.59	0.389-0.897
	T	88 (13.9)	68 (10.8)	34 (8.7)						

SLHBV: Self-limited HBV infection group; CHB total: Chronic hepatitis B total group; CHB no FH: Chronic hepatitis B without chronic hepatitis B family history group.

and the infants subsequently become chronic HBV carriers. The predominant mode of HBV spread is intra-familial from mothers to infants or siblings to siblings[25]. This should mean an equal exposure rate of males and females to HBV. The mechanism underlying such a male predominance is unknown. As age and sex were matched when the subjects were enrolled in our study, there was no significant difference in sex and age ($P > 0.05$). We also studied the family history of 632 subjects, in which all of the self-limited subjects and 195 chronic hepatitis B patients had no family history of the disease.

The majority of published studies on HBV persistence correlate to the role of the major histo-compati-bility complex (MHC) in determining the infection outcomes. The most convincing evidence refers to the association between HBV carriage and MHC class Ⅱ and Ⅰ molecules, such as human leukocyte antigen allele *DRB1*1302*[27-29] and allele A*0301[30], which are associated with viral clearance, whilst B*08 is associated with persistent infection[30]. Non-MHC genes have also proved interesting and successful candidates for association studies of hepatitis B viral infection. It was reported that SNPs in the *VDR*[31,32] and *TNF-α* genes, at position-857[33] as TT, are associated with the clearance of HBV in Gambians and the Chinese population, respectively. Two studies showed that the *TNF-α* SNP at position-238 may be associated with persistent infection[33,34]. Several population-based studies also revealed that *IFN-γ* with its +847 and CA repeat allele[35], cytotoxic T-lymphocyte antigen 4 (*CTLA4*) with its -318[36] and *IL-18* with its -607 and -137[37], interferon alpha receptor 1 with its -568C and -408T[38], CC chemokine receptor 5 (*CCR5*) with its 59029G and 59353, heterozygosity of *CCR5* delta 32[31,39], and mannose binding lectin (*MBL*)[40,41] are all associated with chronic HBV infection or HBV clearance.

MTP catalyzes the transport of triglyceride, cholesteryl ester, and phospholipids on phospholipid surfaces[42]. The large subunit of the human *MTP* gene is situated on chromosome 4q22-q24[43]. It has a key function in intracellular apolipoprotein (apo) B lipidation and secretion of VLDL[44]. Abundant MTP has been found on the luminal side of the endoplasmic reticulum and in the liver, intestine, and heart[45]. In the present study, we investigated the two polymorphisms of *MTP*; one is located at -493 of the promoter, the other at the 297th amino acid of the coding region. The polymorphism of *MTP*-493 G-to-T substitution affects the promoter activity of the *MTP* gene[19,20]. It was recently reported that the G allele, which decreases the *MTP* gene transcription, increases intrahepatic triglyceride content[21]. The T allele is associated with an increased expression of the MTP gene[19]. There is linkage disequilibrium between the 2 SNPs[46]. In our study, the genotype of *MTP*-493G/T distribution was significantly different between the two study groups, with different outcomes of HBV infection ($P = 0.015$). This significant difference was observed in allele distribution after adjustment for sex, age and family history ($P = 0.013$), indicating that the T allele may be one of the protective factors against HBV infection, especially against postnatal infection (OR: 0.59 < 1; 95% CI: 0.389-0.897).

A recent study showed that hepatitis C, as a metabolic disease, is associated with liver steatosis involving accumulation of intracytoplasmic lipid droplets[47].

The function of MTP is to lipidate the growing apoB polypeptide chain during translation, allowing apoB to fold correctly and assemble a lipoprotein with a neutral lipid core before secretion[48,49]. It appears to be obligatory for hepatic secretion of apoB[50,51]. It has been shown that the G allele in *MTP*-493 G/T influences the transcriptional activity and is associated with low plasma levels of LDL cholesterol in healthy middle-aged men, and the T allele is associated with an increased expression of the *MTP* gene *in vitro*, and may enhance hepatic secretion of larger VLDL[19]. It was reported that TT and TG in *MTP*-493 increase the *MTP* gene expression and hepatic secretion of VLDL. However, one French study demonstrated that the functional G/T *MTP* polymorphism does not play a role in the development of steatosis in chronic hepatitis C[52].

Brozovic S[53] recently showed that CD1d, a MHC class I-related molecule that functions in glycolipid antigen presentation to distinct subsets of T cells that express natural killer receptors and an invariant T-cell receptor-α chain (invariant NKT cells), is regulated by MTP in hepatocytes. MTP deletion affects the ability of hepatocytes CD1d to activate invariant NKT cells.

In self-limited infections, HBV-DNA falls by more than 90% within 2-3 wk following the viral replication peak and before the peak of antigen-specific CD8 response and liver damage[54]. The role of NK cells in the initial viral containment is confirmed by the observation that the NK cell peak in the circulation of patients infected with HBV precedes the decline of HBV replication[55]. This maximal elevation of the NK cell frequency is then followed by the peak of HBV-specific CD8 cells a few weeks later[55]. Experiments of T cell depletion with anti-CD4 or anti-CD8 antibodies injected into infected chimpanzees showed that NK and NKT cells can contribute substantially to early viral containment[56]. MTP may influence the outcomes of HBV infection by a mechanism mediated by CD1d regulation and NKT cells' activation during the early period of infection.

In summary, the 2 SNPs in MTP have linkage disequilibrium and the T at *MTP*-493G/T may be associated with the clearance of HBV leading to self-limited infection. This might be mediated by CD1d regulation and activation of NKT cells during the early period of infection. The mechanism needs to be further investigated. With the development of SNP detection technology, more SNPs in different genes are likely to be found to be associated with HBV infection. The combination of several SNPs can serve as a predictor for the HBV infection outcomes, leading to new therapeutic methods for HBV infection.

ACKNOWLEDGMENTS

The authors thank Dr. Alexendre Alcais and Dr. Emmanuelle Jouanguy, Faculte de Medecine Necker Paris France for their statistical analysis and helpful discussion.

COMMENTS

Background

Most infected individuals infected with hepatitis B virus (HBV) can clear the virus, while only 5%-10% develop chronic hepatitis and remain in a persistent viral state. The reasons for viral persistence are poorly understood, but host genetic factors are likely to influence the disease outcome.

Research frontiers

This study looked for the potential host genetic factors which may influence the outcomes of HBV infection.

Innovations and breakthroughs

The results show, for the first time, that single nucleotide polymorphisms (SNPs) of microsomal triglyceride transfer protein (*MTP*)-493G/T, which is responsible for MTP transcription level, might be involved in determining the outcomes of HBV infection.

Applications

Based on the results of our study, further investigation should be focused on the mechanism underlying the association between MTP-dependent lipid metabolism and HBV infection, which may lead to new therapeutic methods.

Peer review

The manuscript reports results of an interesting clinical trial. The authors analyzed the polymorphisms of *MTP*-493 at 297 positions in correlation with self-limited and persistent HBV infection in 632 Han Chinese patients. Such an analysis is of importance. The study is well designed and the paper is written in rather good English.

REFERENCES

1 **Lau GK**, Carman WF, Locarnini SA, Okuda K, Lu ZM, Williams R, Lam SK. Treatment of chronic hepatitis B virus infection: an Asia-Pacific perspective. *J Gastroenterol Hepatol* 1999; **14**: 3-12
2 **Seeger C**, Mason WS. Hepatitis B virus biology. *Microbiol Mol Biol Rev* 2000; **64**: 51-68
3 **Lin TM**, Chen CJ, Wu MM, Yang CS, Chen JS, Lin CC, Kwang TY, Hsu ST, Lin SY, Hsu LC. Hepatitis B virus markers in Chinese twins. *Anticancer Res* 1989; **9**: 737-741
4 **Thio CL**, Thomas DL, Carrington M. Chronic viral hepatitis and the human genome. *Hepatology* 2000; **31**: 819-827
5 **Thio CL**, Carrington M, Marti D, O'Brien SJ, Vlahov D, Nelson KE, Astemborski J, Thomas DL. Class II HLA alleles and hepatitis B virus persistence in African Americans. *J Infect Dis* 1999; **179**: 1004-1006
6 **Hohler T**, Kruger A, Gerken G, Schneider PM, Meyer zum Buschenefelde KH, Rittner C. A tumor necrosis factor-alpha (TNF-alpha) promoter polymorphism is associated with chronic hepatitis B infection. *Clin Exp Immunol* 1998; **111**: 579-582
7 **Ben-Ari Z**, Mor E, Papo O, Kfir B, Sulkes J, Tambur AR, Tur-Kaspa R, Klein T. Cytokine gene polymorphisms in patients infected with hepatitis B virus. *Am J Gastroenterol* 2003; **98**: 144-150
8 **Miyazoe S**, Hamasaki K, Nakata K, Kajiya Y, Kitajima K, Nakao K, Daikoku M, Yatsuhashi H, Koga M, Yano M, Eguchi K. Influence of interleukin-10 gene promoter polymorphisms on disease progression in patients chronically infected with hepatitis B virus. *Am J Gastroenterol* 2002; **97**: 2086-2092
9 **Thomas HC**, Foster GR, Sumiya M, McIntosh D, Jack DL, Turner MW, Summerfield JA. Mutation of gene of mannose-binding protein associated with chronic hepatitis B viral infection. *Lancet* 1996; **348**: 1417-1419
10 **Bellamy R**, Ruwende C, Corrah T, McAdam KP, Thursz M, Whittle HC, Hill AV. Tuberculosis and chronic hepatitis B virus infection in Africans and variation in the vitamin D receptor gene. *J Infect Dis* 1999; **179**: 721-724
11 **Davis RA**, Hui TY. 2000 George Lyman Duff Memorial Lecture: atherosclerosis is a liver disease of the heart. *Arterioscler Thromb Vasc Biol* 2001; **21**: 887-898
12 **Davis RA**. Cell and molecular biology of the assembly and

secretion of apolipoprotein B-containing lipoproteins by the liver. *Biochim Biophys Acta* 1999; **1440**: 1-31
13 **Olofsson SO**, Asp L, Boren J. The assembly and secretion of apolipoprotein B-containing lipoproteins. *Curr Opin Lipidol* 1999; **10**: 341-346
14 **Davidson NO**, Shelness GS. APOLIPOPROTEIN B: mRNA editing, lipoprotein assembly, and presecretory degradation. *Annu Rev Nutr* 2000; **20**: 169-193
15 **Fisher EA**, Ginsberg HN. Complexity in the secretory pathway: the assembly and secretion of apolipoprotein B-containing lipoproteins. *J Biol Chem* 2002; **277**: 17377-17380
16 **Yao Z**, McLeod RS. Synthesis and secretion of hepatic apolipoprotein B-containing lipoproteins. *Biochim Biophys Acta* 1994; **1212**: 152-166
17 **Jamil H**, Dickson JK Jr, Chu CH, Lago MW, Rinehart JK, Biller SA, Gregg RE, Wetterau JR. Microsomal triglyceride transfer protein. Specificity of lipid binding and transport. *J Biol Chem* 1995; **270**: 6549-6554
18 **Wetterau JR**, Gregg RE, Harrity TW, Arbeeny C, Cap M, Connolly F, Chu CH, George RJ, Gordon DA, Jamil H, Jolibois KG, Kunselman LK, Lan SJ, Maccagnan TJ, Ricci B, Yan M, Young D, Chen Y, Fryszman OM, Logan JV, Musial CL, Poss MA, Robl JA, Simpkins LM, Slusarchyk WA, Sulsky R, Taunk P, Magnin DR, Tino JA, Lawrence RM, Dickson JK Jr, Biller SA. An MTP inhibitor that normalizes atherogenic lipoprotein levels in WHHL rabbits. *Science* 1998; **282**: 751-754
19 **Karpe F**, Lundahl B, Ehrenborg E, Eriksson P, Hamsten A. A common functional polymorphism in the promoter region of the microsomal triglyceride transfer protein gene influences plasma LDL levels. *Arterioscler Thromb Vasc Biol* 1998; **18**: 756-761
20 **Garcia-Garcia AB**, Gonzalez C, Real JT, Martin de Llano JJ, Gonzalez-Albert V, Civera M, Chaves FJ, Ascaso JF, Carmena R. Influence of microsomal triglyceride transfer protein promoter polymorphism -493 GT on fasting plasma triglyceride values and interaction with treatment response to atorvastatin in subjects with heterozygous familial hypercholesterolaemia. *Pharmacogenet Genomics* 2005; **15**: 211-218
21 **Bernard S**, Touzet S, Personne I, Lapras V, Bondon PJ, Berthezene F, Moulin P. Association between microsomal triglyceride transfer protein gene polymorphism and the biological features of liver steatosis in patients with type II diabetes. *Diabetologia* 2000; **43**: 995-999
22 **Perlemuter G**, Sabile A, Letteron P, Vona G, Topilco A, Chretien Y, Koike K, Pessayre D, Chapman J, Barba G, Brechot C. Hepatitis C virus core protein inhibits microsomal triglyceride transfer protein activity and very low density lipoprotein secretion: a model of viral-related steatosis. *FASEB J* 2002; **16**: 185-194
23 **Reference SNP (refSNP) Cluster Report: rs2306985**. Available from URL: http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=2306985
24 **Chen DS**. Natural history of chronic hepatitis B virus infection: new light on an old story. *J Gastroenterol Hepatol* 1993; **8**: 470-475
25 **Tsai JF**, Jeng JE, Ho MS, Chang WY, Lin ZY, Tsai JH. Independent and additive effect modification of hepatitis C and B viruses infection on the development of chronic hepatitis. *J Hepatol* 1996; **24**: 271-276
26 **Tsai JF**, Chuang LY, Jeng JE, Ho MS, Lin ZY, Hsieh MY, Wang LY, Tsai JH. Sex differences in relation to serum hepatitis B e antigen and alanine aminotransferase levels among asymptomatic hepatitis B surface antigen carriers. *J Gastroenterol* 2000; **35**: 690-695
27 **Thursz MR**, Kwiatkowski D, Allsopp CE, Greenwood BM, Thomas HC, Hill AV. Association between an MHC class II allele and clearance of hepatitis B virus in the Gambia. *N Engl J Med* 1995; **332**: 1065-1069
28 **Hohler T**, Gerken G, Notghi A, Lubjuhn R, Taheri H, Protzer U, Lohr HF, Schneider PM, Meyer zum Buschenfelde KH, Rittner C. HLA-DRB1*1301 and *1302 protect against chronic hepatitis B. *J Hepatol* 1997; **26**: 503-507
29 **Almarri A**, Batchelor JR. HLA and hepatitis B infection. *Lancet* 1994; **344**: 1194-1195
30 **Thio CL**, Thomas DL, Karacki P, Gao X, Marti D, Kaslow RA, Goedert JJ, Hilgartner M, Strathdee SA, Duggal P, O'Brien SJ, Astemborski J, Carrington M. Comprehensive analysis of class I and class II HLA antigens and chronic hepatitis B virus infection. *J Virol* 2003; **77**: 12083-12087
31 **Suneetha PV**, Sarin SK, Goyal A, Kumar GT, Shukla DK, Hissar S. Association between vitamin D receptor, CCR5, TNF-alpha and TNF-beta gene polymorphisms and HBV infection and severity of liver disease. *J Hepatol* 2006; **44**: 856-863
32 **Bellamy R**, Ruwende C, Corrah T, McAdam KP, Thursz M, Whittle HC, Hill AV. Tuberculosis and chronic hepatitis B virus infection in Africans and variation in the vitamin D receptor gene. *J Infect Dis* 1999; **179**: 721-724
33 **Du T**, Guo XH, Zhu XL, Li JH, Lu LP, Gao JR, Gou CY, Li Z, Liu Y, Li H. Association of TNF-alpha promoter polymorphisms with the outcomes of hepatitis B virus infection in Chinese Han population. *J Viral Hepat* 2006; **13**: 618-624
34 **Hohler T**, Kruger A, Gerken G, Schneider PM, Meyer zum Buschenefelde KH, Rittner C. A tumor necrosis factor-alpha (TNF-alpha) promoter polymorphism is associated with chronic hepatitis B infection. *Clin Exp Immunol* 1998; **111**: 579-582
35 **Abbott W**, Gane E, Winship I, Munn S, Tukuitonga C. Polymorphism in intron 1 of the interferon-gamma gene influences both serum immunoglobulin E levels and the risk for chronic hepatitis B virus infection in Polynesians. *Immunogenetics* 2007; **59**: 187-195
36 **Mohammad Alizadeh AH**, Hajilooi M, Ranjbar M, Fallahian F, Mousavi SM. Cytotoxic T-lymphocyte antigen 4 gene polymorphisms and susceptibility to chronic hepatitis B. *World J Gastroenterol* 2006; **12**: 630-635
37 **Zhang PA**, Wu JM, Li Y, Yang XS. Association of polymorphisms of interleukin-18 gene promoter region with chronic hepatitis B in Chinese Han population. *World J Gastroenterol* 2005; **11**: 1594-1598
38 **Zhou J**, Lu L, Yuen MF, Lam TW, Chung CP, Lam CL, Zhang B, Wang S, Chen Y, Wu SH, Poon VK, Ng F, Chan CC, Jiang S, Yuen KY, Zheng BJ. Polymorphisms of type I interferon receptor 1 promoter and their effects on chronic hepatitis B virus infection. *J Hepatol* 2007; **46**: 198-205
39 **Ahn SH**, Kim do Y, Chang HY, Hong SP, Shin JS, Kim YS, Kim H, Kim JK, Paik YH, Lee KS, Chon CY, Moon YM, Han KH. Association of genetic variations in CCR5 and its ligand, RANTES with clearance of hepatitis B virus in Korea. *J Med Virol* 2006; **78**: 1564-1571
40 **Thio CL**, Mosbruger T, Astemborski J, Greer S, Kirk GD, O'Brien SJ, Thomas DL. Mannose binding lectin genotypes influence recovery from hepatitis B virus infection. *J Virol* 2005; **79**: 9192-9196
41 **Chong WP**, To YF, Ip WK, Yuen MF, Poon TP, Wong WH, Lai CL, Lau YL. Mannose-binding lectin in chronic hepatitis B virus infection. *Hepatology* 2005; **42**: 1037-1045
42 **Shoulders CC**, Shelness GS. Current biology of MTP: implications for selective inhibition. *Curr Top Med Chem* 2005; **5**: 283-300
43 **Narcisi TM**, Shoulders CC, Chester SA, Read J, Brett DJ, Harrison GB, Grantham TT, Fox MF, Povey S, de Bruin TW. Mutations of the microsomal triglyceride-transfer-protein gene in abetalipoproteinemia. *Am J Hum Genet* 1995; **57**: 1298-1310
44 **Bjorn Lundahl**, Leren TP, Ose L, Hamsten A, Karpe F. A functional polymorphism in the promoter region of the microsomal triglyceride transfer protein (MTP -493G/T) influences lipoprotein phenotype in familial hypercholesterolemia. *Arterioscler Thromb Vasc Biol* 2000; **20**: 1784-1788

45 **Ledmyr H**, McMahon AD, Ehrenborg E, Nielsen LB, Neville M, Lithell H, MacFarlane PW, Packard CJ, Karpe F. The microsomal triglyceride transfer protein gene-493T variant lowers cholesterol but increases the risk of coronary heart disease. *Circulation* 2004; **109**: 2279-2284

46 **Ledmyr H**, Karpe F, Lundahl B, McKinnon M, Skoglund-Andersson C, Ehrenborg E. Variants of the microsomal triglyceride transfer protein gene are associated with plasma cholesterol levels and body mass index. *J Lipid Res* 2002; **43**: 51-58

47 **Andre P**, Perlemuter G, Budkowska A, Brechot C, Lotteau V. Hepatitis C virus particles and lipoprotein metabolism. *Semin Liver Dis* 2005; **25**: 93-104

48 **Gordon DA**, Jamil H, Sharp D, Mullaney D, Yao Z, Gregg RE, Wetterau J. Secretion of apolipoprotein B-containing lipoproteins from HeLa cells is dependent on expression of the microsomal triglyceride transfer protein and is regulated by lipid availability. *Proc Natl Acad Sci USA* 1994; **91**: 7628-7632

49 **Leiper JM**, Bayliss JD, Pease RJ, Brett DJ, Scott J, Shoulders CC. Microsomal triglyceride transfer protein, the abetalipoproteinemia gene product, mediates the secretion of apolipoprotein B-containing lipoproteins from heterologous cells. *J Biol Chem* 1994; **269**: 21951-21954

50 **Sharp D**, Blinderman L, Combs KA, Kienzle B, Ricci B, Wager-Smith K, Gil CM, Turck CW, Bouma ME, Rader DJ. Cloning and gene defects in microsomal triglyceride transfer protein associated with abetalipoproteinaemia. *Nature* 1993; **365**: 65-69

51 **Jamil H**, Chu CH, Dickson JK Jr, Chen Y, Yan M, Biller SA, Gregg RE, Wetterau JR, Gordon DA. Evidence that microsomal triglyceride transfer protein is limiting in the production of apolipoprotein B-containing lipoproteins in hepatic cells. *J Lipid Res* 1998; **39**: 1448-1454

52 **Petit JM**, Masson D, Minello A, Duvillard L, Galland F, Verges B, Gambert P, Hillon P. Lack of association between microsomal triglyceride transfer protein gene polymorphism and liver steatosis in HCV-infected patients. *Mol Genet Metab* 2006; **88**: 196-198

53 **Brozovic S**, Nagaishi T, Yoshida M, Betz S, Salas A, Chen D, Kaser A, Glickman J, Kuo T, Little A, Morrison J, Corazza N, Kim JY, Colgan SP, Young SG, Exley M, Blumberg RS. CD1d function is regulated by microsomal triglyceride transfer protein. *Nat Med* 2004; **10**: 535-539

54 **Guidotti LG**, Rochford R, Chung J, Shapiro M, Purcell R, Chisari FV. Viral clearance without destruction of infected cells during acute HBV infection. *Science* 1999; **284**: 825-829

55 **Webster GJ**, Reignat S, Maini MK, Whalley SA, Ogg GS, King A, Brown D, Amlot PL, Williams R, Vergani D, Dusheiko GM, Bertoletti A. Incubation phase of acute hepatitis B in man: dynamic of cellular immune mechanisms. *Hepatology* 2000; **32**: 1117-1124

56 **Thimme R**, Wieland S, Steiger C, Ghrayeb J, Reimann KA, Purcell RH, Chisari FV. CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. *J Virol* 2003; **77**: 68-76

S- Editor Li DL **L- Editor** Wang XL **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5461

World J Gastroenterol 2008 September 21; 14(35): 5461-5466
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Dendroaspis natriuretic peptide relaxes gastric antral circular smooth muscle of guinea-pig through the cGMP/cGMP-dependent protein kinase pathway

Chun-Yu Cai, Zheng-Xu Cai, Xin-Yi Gu, Lu-Juan Shan, Yong-Xiao Wang, Xue-Zhe Yin, Qing-Hui Qi, Hui-Shu Guo

Chun-Yu Cai, Hui-Shu Guo, Lu-Juan Shan, Qing-Hui Qi, Centralab, the first Affiliated Hospital of Dalian Medical Universty, Dalian 116011, Liaoning Province, China
Chun-Yu Cai, Xue-Zhe Yin, Department of internal medicine, the first Affiliated Hospital of Yanbian University College of Medicine, Yanji 133000, Jilin Province, China
Zheng-Xu Cai, Department of Neurology, the first Affiliated Hospital of Dalian Medical University, Dalian 116011, Liaoning Province, China
Hui-Shu Guo, Xin-Yi Gu, Centralab, Zhongshan Affiliated Hospital of Dalian University, Dalian 116001, Liaoning Province, China
Yong-Xiao Wang, Center for Cardiovascular Sciences, Albany Medical College, Albany, NY 12208, United States
Author contributions: Guo HS and Qi QH designed research; Cai CY, Cai ZX, Gu XY and Shan LJ performed research; Yin XZ contributed new analytic tools; Cai CY and Cai ZX analyzed data; Wang YX corrected syntax; Cai CY, Cai ZX and Guo HS wrote the paper.
Supported by The National Natural Science Foundation of China, No. 30800382 and the Youth Science Foundation of Dalian to Professor Hui-Shu Guo, No. 2006B3NS218
Correspondence to: Hui-Shu Guo, Professor, Centralab, the first Affiliated Hospital of Dalian Medical Universty and Zhongshan Affiliated Hospital of Dalian University, Dalian 116011, Liaoning Province, China. guohuishu1@msn.com
Telephone: +86-411-83635963-2237 Fax: +86-411-83622844
Received: April 18, 2008 Revised: August 22, 2008
Accepted: August 29, 2008
Published online: September 21, 2008

Abstract

AIM: To systematically investigate if cGMP/cGMP-dependent protein kinase G (PKG) signaling pathway may participate in dendroaspis natriuretic peptide (DNP)-induced relaxation of gastric circular smooth muscle.

METHODS: The content of cGMP in guinea pig gastric antral smooth muscle tissue and perfusion solution were measured using radioimmunoassay; spontaneous contraction of gastric antral circular muscles recorded using a 4-channel physiograph; and Ca^{2+}-activated K^+ currents ($I_{K(Ca)}$) and spontaneous transient outward currents (STOCs) in isolated gastric antral myocytes were recorded using the whole-cell patch clamp technique.

RESULTS: DNP markedly enhanced cGMP levels in gastric antral smooth muscle tissue and in the perfusion medium. DNP induced relaxation in gastric antral circular smooth muscle, which was inhibited by KT5823, a cGMP-dependent PKG inhibitor. DNP increased $I_{K(Ca)}$. This effect was almost completely blocked by KT5823, and partially blocked by LY83583, an inhibitor of guanylate cyclase to change the production of cGMP. DNP also increased STOCs. The effect of DNP on STOCs was abolished in the presence of KT5823, but not affected by KT-5720, a PKA-specific inhibitor.

CONCLUSION: DNP activates $I_{K(Ca)}$ and relaxes guinea-pig gastric antral circular smooth muscle *via* the cGMP/PKG-dependent singling axis instead of cAMP/PKA pathway.

Key words: Dendroaspis natriuretic peptide; Cyclic guanosine monophosphate; Protein kinase G; Protein kinase A; Gastric motility

Peer reviewers: Leonard R Johnson, Professor, Department of Physiology, University Tennessee College of Medicine, 894 Union Ave, Memphis, TN 38163, United States; Per M Hellström, MD, Professor, Gastrocentre Medicine, Karolinska University Hospital Solna, Stockholm SE-17176, Sweden

Cai CY, Cai ZX, Gu XY, Shan LJ, Wang YX, Yin XZ, Qi QH, Guo HS. Dendroaspis natriuretic peptide relaxes gastric antral circular smooth muscle of guinea-pig through the cGMP/cGMP-dependent protein kinase pathway. *World J Gastroenterol* 2008; 14(35): 5461-5466 Available from: URL: http://www.wjgnet.com/1007-9327/14/5461.asp DOI: http://dx.doi.org/10.3748/wjg.14.5461

INTRODUCTION

Natriuretic peptides (NP) include atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), dendroaspis natriuretic peptide (DNP) and urodilatin[1]. DNP is a recently isolated peptide that contains 38 amino residues and shares structural and functional properties with the other members of the natriuretic peptide family[2]. Studies about its physiologic functions mainly focus on cardiovascular[3-5], genital[6], and urinary systems[7]. Evolutionary studies[8] have suggested the presence of DNP-like immunoreactivity

in rat colon, and the DNP-like molecule may control colonic motility as a local regulator. Interestingly, we found that DNP inhibits spontaneous contraction in gastric circular smooth muscle[9]. NPs are similar to nitric oxide (NO), which play important physiological functions by affecting the activity of cGMP and cAMP. Sabbatini *et al* reported[10] that CNP enhances amylase release by reducing cAMP in the exocrine pancreas. Borán *et al*[11] showed that ANP could play a beneficial role in the resolution of neuroinflammation by removing dead cells and decreasing levels of proinflammatory mediators in microglia *via* the cGMP-dependent protein kinase G (PKG) signaling pathway. ANP stimulates lipolysis in human adipocyte through a cGMP signaling pathway[12]. However, Wen *et al*[13] found that CNP activates the pGC-cGMP- phosphodiesterases 3 (PDE3)-cAMP signaling to play a role in hyperthyroid beating of rabbit atria. It is indicated that NP exerts its physiological function by a different pathway. In our previous study, we simply observed that cGMP participates in DNP-induced relaxing of circular smooth muscle, by using a pharmacologic approach. Thus, the aim of this study was to systematically investigate if the cGMP-PKG or cAMP signaling pathway may participate in DNP-induced relaxation using pharmacologic, radioimmunoassay and patch-clamp technique in gastric circular smooth muscle of guinea pigs.

MATERIALS AND METHODS

Preparation of muscle strips

Guinea pigs of either sex, weighing 250-350 g, were purchased from the Experimental Animal Center, Dalian Medical University. The guinea pigs were housed in plastic cages containing corn-chip bedding with free access to food and water for 1 d before they were used for experiments. The care and use of the animals were followed strictly in accordance with the National Institutions of Health Guide for the Care and Use of Laboratory Animals. Guinea pigs were euthanized by a lethal intravenous dose of pentobarbital sodium (50 mg/kg). The abdomen of each guinea pig was opened along the midline, and the stomach was removed and placed in pre-oxygenated Tyrode's solution at room temperature. After the mucous layer was removed, strips (approximately 2.0 mm × 15.0 mm) of gastric antral circular muscles were prepared. The muscle strips were placed in a bath chamber (2 mL volume). One end of the strip was fixed on the lid of the chamber through a glass claw, and the other end was attached to an isometric force transducer (TD-112S, Nihon Kohden-Kogyo Japan). The chamber was constantly perfused with pre-oxygenated Tyrode's solution at 1 mL/min. The temperature was maintained at 37.0 ± 0.5℃ with a water bath thermostat (WC/09-05, Chongqing, China). The muscle strips were allowed to incubate for at least 40 min before the experiments were started.

Isolated cell preparation and electrophysiological recording

The longitudinal layer of muscle was dissected from the other muscle layers using fine scissors and then cut into small segments (1 mm × 4 mm). These segments were kept in modified Kraft-Bruhe (K-B) medium at 4℃ for 15 min. They were then incubated at 36℃ in 4 mL of digestion medium [Ca-free physiologic salt solution (Ca-free PSS)] containing 0.1% collagenase Ⅱ, 0.1% dithioerythritol, 0.15% trypsin inhibitor, and 0.2% BSA for 25-35 min. The digested muscle segments were transferred into the modified K-B medium, and the single cells were dispersed by gentle disruption with a wide-bore, fire-polished glass pipette. The isolated gastric myocytes were kept in modified K-B medium at 4℃ prior to use. Isolated cells were transferred to a 0.1 mL chamber on the stage of an inverted microscope (IX-70 Olympus, Tokyo, Japan) and allowed to settle for 10-15 min. The cells were continuously perfused with an isosmotic PSS at a rate of 0.9-1.0 mL/min. An 8-channel perfusion system (L/M-sps-8, List Electronics, Berlin, Germany) was used to exchange different solutions. The Ca^{2+}-activated K^+ currents ($I_{K(ca)}$) were recorded using the conventional whole-cell patch-clamp technique. Patch-clamp pipettes were manufactured from borosilicate glass capillaries (GC 150T-7.5, Clark Electromedical Instruments, London, UK) using a 2-stage puller (PP-83, Narishige, Tokyo, Japan). The resistance of the patch pipette was 3-5 MΩ when filled with pipette solution. Liquid junction potentials were canceled prior to the seal formation. Whole-cell currents were recorded using an Axopatch 1-D patch-clamp amplifier (Axon Instruments, Foster City California, USA), and data were filtered at 1 KHz. Command pulses, data acquisition, and storage were applied using the IBM-compatible, 486-grade computer and pCLAMP 6.02 software (Axon Foster City, California, USA). Spontaneous transient outward currents (STOCs) were recorded simultaneously by an EPC-10-HEAKA amplifier (HEAKA Instruments, Berlin, Germany). All experiments were performed at room temperature (20-25℃).

Radioimmunoassay

Radioimmunoassay was performed as described elsewhere[14].

Drugs and solutions

Tyrode solution contained (in mmol/L): NaCl 147, KCl 4, $MgCl_2{\cdot}6H_2O$ 1.05, $CaCl_2{\cdot}2H_2O$ 0.42, $Na_2PO_4{\cdot}2H_2O$ 1.81, and 5.5 mmol/L glucose. Ca^{2+}-free PSS was composed of (in mmol/L): NaCl 134.8, KCl 4.5, glucose 5, and N-(2-hydroxyethyl) piperazine-N-(2-ethanesulphonic acid) (HEPES; pH was adjusted to 7.4 with Tris (hydroxymethyl aminomethane). Modified K-B solution contained (in mmol/L): *L*-glutamate 50, KCl 50, taurine 20, KH_2PO_4 20, $MgCl_2{\cdot}6H_2O$ 3, glucose 10, HEPES 10, and egtazic acid 0.5 (pH 7.40 with KOH). PSS contained (in mmol/L): NaCl 134.8, KCl 4.5, $MgCl_2{\cdot}6H_2O$ 1, $CaCl_2{\cdot}2H_2O$ 2, glucose 5, HEPES 10, and sucrose 110 (pH 7.4 with Tris). In order to eliminate delayed rectifier K^+ currents ($I_{K(V)}$), external solution contained 4-aminopyridine (10 mmol/L), a selective inhibitor of $I_{K(V)}$. The pipette solution for recording $I_{K(Ca)}$

contained (in mmol/L): K^+-aspartic acid 110, Mg-ATP 5, HEPES 5, $MgCl_2{\cdot}6H_2O$ 1.0, KCl 20, egtazic acid 0.1, di-tris-creatine phosphate 2.5, and disodium-creatine phosphate 2.5 (pH 7.3 with KOH). Tetraethylammonium (TEA), DNP, LY83583, zaprinast, KT5823 and KT5720 were made up as stock solutions. All chemicals in this experiment were purchased from Sigma (St Louis, MO, USA).

Statistical analysis

All data was expressed as mean ± SD. Statistical significance was evaluated using Student *t*-test. Differences were considered to be significant when $P < 0.05$.

RESULTS

Effect of DNP on cGMP production

Our previous pharmacological study[15] suggests that DNP obviously inhibits spontaneous contraction in gastric antral circular smooth muscle through the cGMP-dependent signaling pathway. To directly confirm the involvement of cGMP on the effect of DNP, in the present study, we measured the content of cGMP in the smooth muscle tissue and in the perfusion solution using radioimmunoassay. The result indicated that cGMP in the smooth muscle tissue and perfusion solution was markedly increased after addition of 10 nmol/L DNP (Figure 1A and B). Pretreatment with 10 nmol/L LY83583 significantly diminished DNP-induced increase in the content of cGMP (Figure 1A and B).

Effect of cGMP-dependent protein kinase on DNP-induced relaxation

Because cGMP activates PKG, we tested the effect of KT-5823 (1 μmol/L), a membrane-permeable PKG-specific inhibitor, on DNP-induced relaxation in the gastric antral circular smooth muscle to determine the potential involvement of PKG. The result indicated that KT-5823 could markedly diminish, although not completely abolish the inhibitory effect of DNP on spontaneous contraction (Figure 1C).

Effect of cGMP on DNP-induced increase of $I_{K(ca)}$

Considering our previous finding that DNP relaxed smooth muscle by increasing $I_{K(ca)}$[15], here we further investigated the relationship between cGMP and DNP-induced increase of $I_{K(ca)}$, and found that the effect of DNP on $I_{K(ca)}$ was observed in the presence of LY83583, an inhibitor of guanylate cyclase to change the production of cGMP. LY83583 (10 nmol/L) significantly blocked DNP-induced increase of $I_{K(ca).}$ The percentage of DNP-induced increase was diminished from 63.24% ± 4.32% to 28.53% ± 3.31% at 60 mV (Figure 2).

Effect of cGMP-dependent protein kinase on DNP-induced increase of $I_{K(ca)}$

To extend our understanding of the role of DNP in the regulation of $I_{K(ca)}$ through the cGMP/PKG pathway, the effect of KT-5823 (a membrane-permeable PKG-specific inhibitor) on channel activity was tested. As the results show in Figure 3, the addition of KT-5823 (1 μmol/L) completely inhibited DNP-induced increase of $I_{K(ca)}$. This data suggests the involvement of PKG-mediated phosphorylation in DNP-mediated regulation of $I_{K(ca)}$.

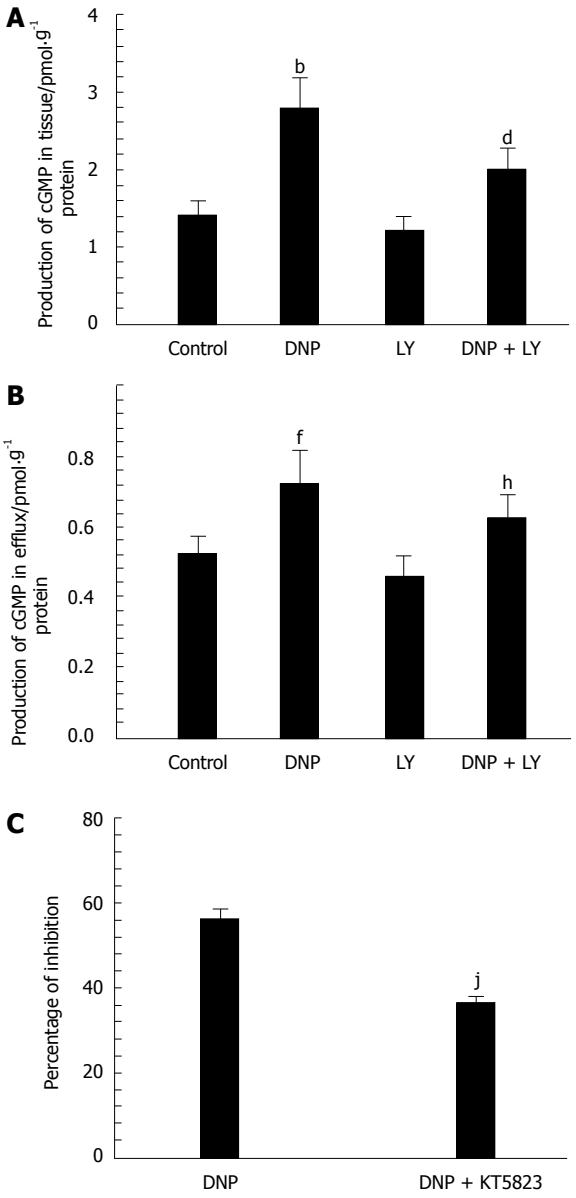


Figure 1 **A**: Effect of 10 nmol/L LY83583 on cGMP production of in tissue ($n = 8$), $^bP < 0.01$ *vs* control group, $^dP < 0.01$ *vs* DNP group; **B**: Effect of 10 nmol/L LY83583 on cGMP production of in efflux ($n = 8$), $^fP < 0.01$ *vs* control group, $^hP < 0.01$ *vs* DNP group; **C**: Effect of a membrane-permeable PKG-specific inhibitor (KT5823) on DNP-induced inhibition of spontaneous contraction in gastric antral circular smooth muscle ($n = 8$), $^jP < 0.01$ *vs* DNP group.

Effect of cGMP-dependent protein kinase on DNP-induced increase in STOCs

STOCs, which can be activated by extracellular Ca^{2+} influx and intracellular Ca^{2+} release, were recorded at -20 mV. The currents were sensitive to TEA (a nonselective K^+ channel blocker) and CHTX (a selective Ca^{2+}-activated K^+ channel blocker). As described in our previous study, DNP increased STOCs in gastric circular myocytes. To

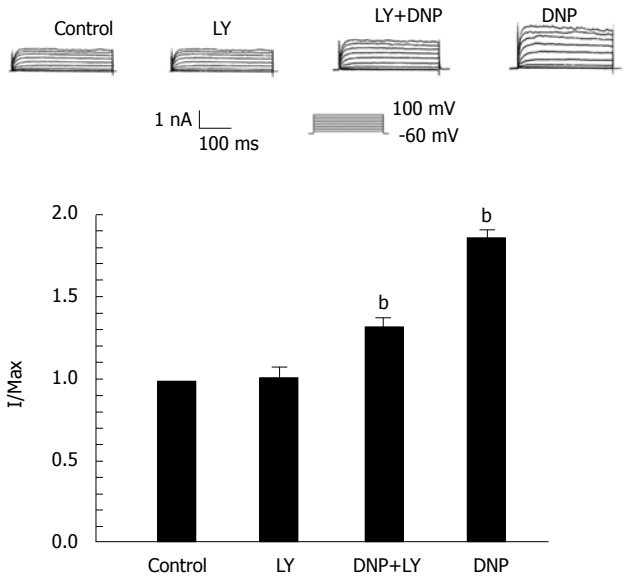


Figure 2 Effect of LY83583 on DNP-induced increase of $I_{K(Ca)}$, $^{b}P < 0.01$ *vs* control group.

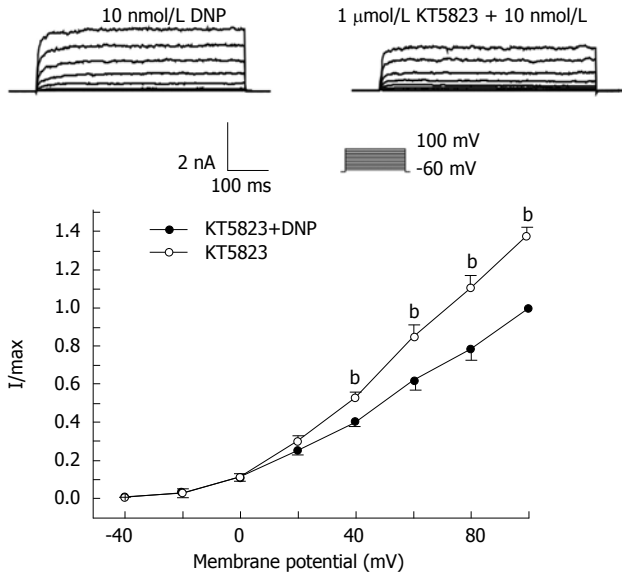


Figure 3 Effect of a membrane-permeable PKG-specific inhibitor, KT-5823, on DNP-induced increase of $I_{K(Ca)}$ (n = 8), $^{b}P < 0.01$ *vs* control group.

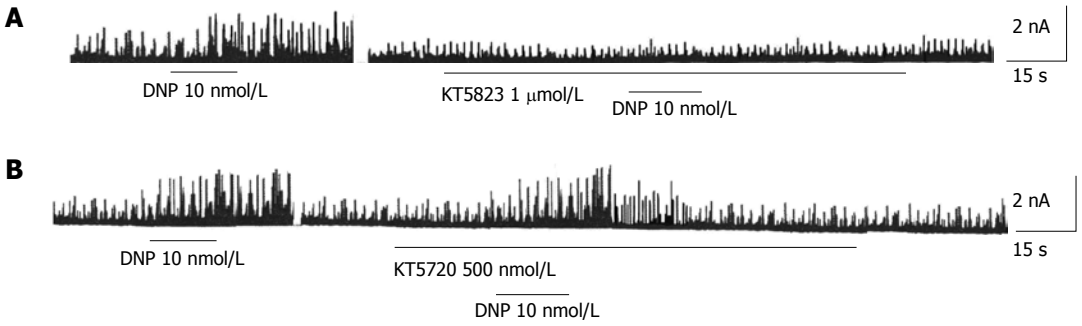


Figure 4 **A**: Effect of a membrane-permeable PKG-specific inhibitor, KT-5823, on STOCs (n = 10); **B**: Effect of KT-5720 (500 nmol/L), a PKA-specific inhibitor, on STOCs (n = 10).

further investigate the relationship between cGMP/PKG pathway and DNP-induced increase in STOCs, we examined the effect of KT5823 on DNP-induced increase in STOCs. The result indicated that KT5823 almost completely abolished DNP-induced increase of STOCs (Figure 4A). However, KT-5720 (500 nmol/L), a PKA-specific inhibitor, could not suppress DNP-induced increase in STOCs (Figure 4B).

DISCUSSION

In the present study, the patch clamp technique, radioimmunoassay and specific pharmacological inhibitors were used to determine involvement of the pGC-cGMP-PKG pathway in DNP-mediated relaxation in guinea-pig gastric antral circular smooth muscle.

In this study, we found that cGMP in the smooth muscle tissue and perfusion solution both were markedly increased after the addition of DNP. The effect of DNP was diminished after treatment with LY83583, an inhibitor of guanylate cyclase to change the production of cGMP. These data indicate that DNP may inhibit spontaneous contraction by increasing cGMP levels. Consistent with this view, KT5823, a PKG inhibitor, markedly diminished the inhibitory effect of DNP on spontaneous contraction. In an attempt to understand how DNP relaxes smooth muscle by affecting the cGMP-dependant pathway, patch clamp experiments were carried out. We observed that KT5823 inhibited DNP-induced increase of $I_{K(ca)}$, and almost completely abolished the DNP-induced increase of STOCs. However, KT-5720 (500 nmol/L), a PKA-specific inhibitor, had no effect on DNP-induced increase in STOCs.

NPs, similar to NO, can increase the generation of cGMP and cAMP, and play important physiological functions in a variety of cell types. In smooth muscle cells, NPs exhibited an inhibitory effect on motility *via* the cGMP pathway. For example, ANP increases intracellular cGMP levels and mediates the role of endothelium- and cardiac-derived NO in regulating sympathetic control functions of the heart and the microvasculature in conscious rats by affecting cGMP-dependent release of catecholamines[16]. Additionally, Kedia *et al*[17] observed that CNP is involved in the cGMP-dependent control of the normal function of human prostatic smooth muscle. Our previous study also found that CNP inhibited spontaneous contraction

by increasing cGMP in gastric antral circular smooth muscle[18]. All these previous reports are consistent with our current findings that DNP-induced relaxation is related to cGMP in gastric circular smooth muscle.

It has been reported that intracellular cGMP may not only result in activation of PKG, but also inhibits activity of PDE3[19]. The latter action would lead to an increase in cAMP, and hence may stimulate another cyclic nucleotide-dependent protein kinase, PKA. NPs exert some physiological functions by affecting PKA. Birukova *et al*[20] have found that Epac/Rap and PKA are novel mechanisms of ANP-induced Rac-mediated pulmonary endothelial barrier protection. However, our present study indicates that DNP-induced increase of $I_{K(ca)}$ and STOCs were significantly blocked by LY83583 and KT-5823. DNP stimulated STOCs even in the presence of a PKA-specific inhibitor (KT-5720), suggesting that the DNP-induced increase of STOCs was due to stimulation of PKG, rather than PKA. The direct effect of cGMP on the activity of ion channels has been reported previously. Yao *et al*[21] showed that a cGMP-gated K^+ channel is expressed in the kidney. Nakamura *et al*[22] revealed that protein kinase G activates inwardly rectifying K^+ channels in cultured human proximal tubule cells. Hirsch *et al*[23] demonstrated the existence of cGMP-regulated K^+ channels that were inhibited by cGMP without PKG-mediated phosphorylation. In our current study, however, DNP-induced relaxation in gastric antral myocytes was inhibited by KT5823. This indicates that PKG-mediated phosphorylation participates in DNP-induced relaxation. Consistent with our data, a previous report has shown that CNP can inhibit L-type Ca^{2+} channel currents, and the inhibitory effect is mediated by pGC-cGMP-PKG-dependent signal pathway in gastric antral myocytes of guinea pigs[24]. However, it should be pointed out that the results of our current study can not determine whether a direct or indirect activation of Ca^{2+}-activated K^+ channels by PKG participates in DNP-induced relaxation of in gastric antral smooth muscle cells. As such, further experiments are necessary to decode this intriguing question.

Taken together, it can be concluded that DNP relaxes gastric circular smooth muscle by activating Ca^{2+}-activated K^+ channels, mediated by a pGC-cGMP-PKG-dependent signal pathway. cAMP did not participate in the process.

COMMENTS

Background

Dendroaspis natriuretic peptide (DNP) is a recently isolated peptide that contains 38 amino residues and shares structural and functional properties with the other members of the natriuretic peptide (NP) family. Studies about its physiologic functions mainly focus on cardiovascular, nervous, and urinary systems. In a previous study, these authors found that DNP inhibited spontaneous contraction in gastric circular smooth muscle. NP plays important physiological functions by affecting the activity of cGMP and cAMP. However, it is unclear whether cGMP or cAMP participates in regulating DNP-induced inhibition of gastric motility.

Research frontiers

Studies about the physiologic functions of DNP mainly focus on cardiovascular, nervous, and urinary systems. There are few reports about the relationship between DNP and gastrointestinal functions. Studies have demonstrated that the DNP system is present in the rat colon and regulates colonic motility as a local regulator. The relationship between DNP and gastrointestinal function has become a focus of study. A previous study has indicated that DNP inhibits gastric motility, which is the first report about DNP regulating gastric motility. However, the mechanism on how DNP regulates gastric motility is still unclear and is the focus of the author's study.

Innovations and breakthroughs

The author's of this paper have shown that, for the fist time, DNP activates $I_{K(Ca)}$ and relaxes guinea-pig gastric antral circular smooth muscle *via* the cGMP/PKG-dependent signaling axis, instead of the cAMP/PKA pathway. The combined use of pharmacologic, radioimmunoassy and patch-clamp techniques can sufficiently demonstrate the mechanism involved in DNP regulation of gastric motility.

Applications

This work enhanced the understanding of the mechanism on how DNP regulates gastric motility.

Peer review

This is a very interesting study. The authors demonstrated that DNP activates $I_{K(Ca)}$ and relaxes guinea-pig gastric antral circular smooth muscle *via* the cGMP/PKG-dependent signaling axis instead of the cAMP/PKA pathway. This study is well designed, and the analysis is reasonable.

REFERENCES

1 **Pandey KN**. Biology of natriuretic peptides and their receptors. *Peptides* 2005; **26**: 901-932
2 **Lisy O**, Jougasaki M, Heublein DM, Schirger JA, Chen HH, Wennberg PW, Burnett JC. Renal actions of synthetic dendroaspis natriuretic peptide. *Kidney Int* 1999; **56**: 502-508
3 **Singh G**, Maguire JJ, Kuc RE, Skepper JN, Fidock M, Davenport AP. Characterization of the snake venom ligand [125I]-DNP binding to natriuretic peptide receptor-A in human artery and potent DNP mediated vasodilatation. *Br J Pharmacol* 2006; **149**: 838-844
4 **Singh G**, Kuc RE, Maguire JJ, Fidock M, Davenport AP. Novel snake venom ligand dendroaspis natriuretic peptide is selective for natriuretic peptide receptor-A in human heart: downregulation of natriuretic peptide receptor-A in heart failure. *Circ Res* 2006; **99**: 183-190
5 **Ha KC**, Piao CS, Chae HJ, Kim HR, Chae SW. Dendroaspis natriuretic peptide protects the post-ischemic myocardial injury. *Regul Pept* 2006; **133**: 13-19
6 **Piao FL**, Park SH, Han JH, Cao C, Kim SZ, Kim SH. Dendroaspis natriuretic peptide and its functions in pig ovarian granulosa cells. *Regul Pept* 2004; **118**: 193-198
7 **Lee S**, Park SK, Kang KP, Kang SK, Kim SZ, Kim W. Relationship of plasma Dendroaspis natriuretic peptide-like immunoreactivity and echocardiographic parameters in chronic haemodialysis patients. *Nephrology* (Carlton) 2004; **9**: 171-175
8 **Kim JH**, Yang SH, Yu MY, Lee HK, Kim SY, Kim SH. Dendroaspis natriuretic peptide system and its paracrine function in rat colon. *Regul Pept* 2004; **120**: 93-98
9 **Guo HS**, Cai ZX, Wu TH, Xu J, Qiu Y, Xu WX. Inhibitory effect of dendroaspis natriuretic peptide on spontaneous contraction in gastric antral circular smooth muscles of guinea pigs. *Acta Pharmacol Sin* 2007; **28**: 1797-1802
10 **Sabbatini ME**, Rodríguez M, di Carlo MB, Davio CA, Vatta MS, Bianciotti LG. C-type natriuretic peptide enhances amylase release through NPR-C receptors in the exocrine pancreas. *Am J Physiol Gastrointest Liver Physiol* 2007; **293**: G987-G994
11 **Borán MS**, Baltrons MA, García A. The ANP-cGMP-protein kinase G pathway induces a phagocytic phenotype but decreases inflammatory gene expression in microglial cells. *Glia* 2008; **56**: 394-411
12 **Moro C**, Klimcakova E, Lafontan M, Berlan M, Galitzky J. Phosphodiesterase-5A and neutral endopeptidase activities in human adipocytes do not control atrial natriuretic peptide-

mediated lipolysis. *Br J Pharmacol* 2007; **152**: 1102-1110
13 **Wen JF**, Quan HX, Zhou GH, Cho KW. Altered role of C-type natriuretic peptide-activated pGC-cGMP-PDE3-cAMP signaling in hyperthyroid beating rabbit atria. *Regul Pept* 2007; **142**: 123-130
14 **Cui X**, Lee SJ, Kim SZ, Kim SH, Cho KW. Effects of pituitary adenylate cyclase activating polypeptide27 on cyclic AMP efflux and atrial dynamics in perfused beating atria. *Eur J Pharmacol* 2000; **402**: 129-137
15 **Guo HS**, Yang YZ, Zou Y, Xu J, Cai ZX, Qi QH. Effects of dendroaspis natriuretic peptide on calcium-activated potassium current and its mechanism. *J Physiol Sci* 2008; **58**: 1-6
16 **Whalen EJ**, Saurer TB, Johnson AK, Lewis SJ. Intracellular cGMP may promote Ca2+-dependent and Ca2+-independent release of catecholamines from sympathetic nerve terminals. *Vascul Pharmacol* 2006; **45**: 102-111
17 **Kedia G**, Uckert S, Scheller F, Chigogidze T, Managadze L, Jonas U, Truss MC. In vitro functional responses of isolated normal human prostatic tissue to compounds interacting with the cyclic guanosine monophosphate pathway. *Urology* 2006; **67**: 1292-1297
18 **Guo HS**, Cui X, Cui YG, Kim SZ, Cho KW, Li ZL, Xu WX. Inhibitory effect of C-type natriuretic peptide on spontaneous contraction in gastric antral circular smooth muscle of rat. *Acta Pharmacol Sin* 2003; **24**: 1021-1026
19 **Degerman E**, Belfrage P, Manganiello VC. Structure, localization, and regulation of cGMP-inhibited phosphodiesterase (PDE3). *J Biol Chem* 1997; **272**: 6823-6826
20 **Birukova AA**, Zagranichnaya T, Alekseeva E, Bokoch GM, Birukov KG. Epac/Rap and PKA are novel mechanisms of ANP-induced Rac-mediated pulmonary endothelial barrier protection. *J Cell Physiol* 2008; **215**: 715-724
21 **Yao X**, Segal AS, Welling P, Zhang X, McNicholas CM, Engel D, Boulpaep EL, Desir GV. Primary structure and functional expression of a cGMP-gated potassium channel. *Proc Natl Acad Sci USA* 1995; **92**: 11711-11715
22 **Nakamura K**, Hirano J, Itazawa S, Kubokawa M. Protein kinase G activates inwardly rectifying K(+) channel in cultured human proximal tubule cells. *Am J Physiol Renal Physiol* 2002; **283**: F784-F791
23 **Hirsch JR**, Weber G, Kleta I, Schlatter E. A novel cGMP-regulated K+ channel in immortalized human kidney epitheliall cells (IHKE-1). *J Physiol* 1999; **519** Pt 3: 645-655
24 **Sun JB**, Huang X, Xu HY, Li XL, Gao L, Kim YC, Xu WX. Inhibitory effect of C-type natriuretic peptide on L-type calcium channel currents in gastric antral myocytes of guinea pigs. *Gen Physiol Biophys* 2006; **25**: 365-377

S- Editor Li DL **L- Editor** Rippe RA **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5467

World J Gastroenterol 2008 September 21; 14(35): 5467-5470
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Recovery from respiratory failure after decompression laparotomy for severe acute pancreatitis

Sylvia Siebig, Igors Iesalnieks, Tanja Bruennler, Christine Dierkes, Julia Langgartner, Juergen Schoelmerich, Christian E Wrede

Sylvia Siebig, Tanja Bruennler, Christine Dierkes, Julia Langgartner, Juergen Schoelmerich, Christian E Wrede, Department of Internal Medicine I, University of Regensburg, Regensburg D-93042, Germany
Igors Iesalnieks, Department of Surgery, University of Regensburg, Regensburg D-93042, Germany
Author contributions: Siebig S and Wrede CE contributed equally to this work; Siebig S, Wrede CE, Bruennler T, Dierkes C, Langgartner J, Iesalnieks I performed the treatment for the three patients and read the manuscript; Siebig S and Wrede CE wrote the paper and analyzed the data.
Correspondence to: Sylvia Siebig, MD, Department of Internal Medicine I, University of Regensburg, Regensburg D-93042, Germany. sylvia.siebig@klinik.uni-r.de
Telephone: +49-941-9447079 Fax: +49-941-9447021
Received: March 18, 2008 Revised: June 30, 2008
Accepted: July 7, 2008
Published online: September 21, 2008

Abstract

We present three cases of patients (at the age of 56 years, 49 years and 74 years respectively) with severe acute pancreatitis (SAP), complicated by intra-abdominal compartment syndrome (ACS) and respiratory insufficiency with limitations of mechanical ventilation. The respiratory situation of the patients was significantly improved after decompression laparotomy (DL) and lung protective ventilation was re-achieved. ACS was discussed followed by a short review of the literature. Our cases show that DL may help patients with SAP to recover from severe respiratory failure.

Key words: Severe acute pancreatitis; Intra-abdominal compartment syndrome; Decompression laparotomy; Intensive care Unit; Respiratory failure

Peer reviewer: Capecomorin S Pitchumoni, Professor, Robert Wood Johnson School of Medicine, Robert Wood Johnson School of Medicine, New Brunswiuc NJ D8903, United States

Siebig S, Iesalnieks I, Bruennler T, Dierkes C, Langgartner J, Schoelmerich J, Wrede CE. Recovery from respiratory failure after decompression laparotomy for severe acute pancreatitis. *World J Gastroenterol* 2008; 14(35): 5467-5470 Available from: URL: http://www.wjgnet.com/1007-9327/14/5467.asp DOI: http://dx.doi.org/10.3748/wjg.14.5467

INTRODUCTION

Severe acute pancreatitis (SAP) is associated with organ failure leading to a mortality rate of 10%-20% that is often related to respiratory failure. Approximately one third of SAP patients develop respiratory complications[1,2]. In the majority of cases, this lung injury is characterized by an increased permeability of pulmonary microvasculature and subsequent leakage of protein-rich exudates into the alveolar spaces[1]. Furthermore, concomitant diseases, such as intra-abdominal hypertension (IAH) defined by intra-abdominal pressure (IAP) greater than 12 mmHg[3], may result in restrictive ventilation disorders and deteriorate the pulmonary situation.

Surgical debridement was the preferred treatment to control necrotizing pancreatitis in the past. However, management of necrotizing pancreatitis has changed since the last decade. The first approach now tends to be non-surgical and relies on conservative strategies including early transfer of patients to intensive care units at specialized centres. Indication for necrosectomy is still given in cases of infected necrosis as well as intestinal infarction, perforation or bleeding, but there is a clear trend towards surgical treatment as late and as rare as possible[2]. In contrast, more and more studies are published promoting decompression laparotomy (DL) for SAP patients developing abdominal compartment syndrome (ACS) defined by IAP greater than 20 mmHg associated with new organ failure[3-7]. This procedure can not only prevent critical decrement of intestinal and renal perfusion, but may lead to improvement in the respiratory situation.

We present three patients with SAP and abdominal compartment syndrome, who developed respiratory insufficiency with limitations of mechanical ventilation associated with high peak pressure levels, low tidal volumes and poor Horowitz-indices (pO_2/FiO_2; HI). The three patients showed a benefit from decompression laparotomy so that ventilation with adequate oxygenation could be re-achieved. Surgery was performed at the Intensive Care Unit of our hospital without transportation of the patients to the operating room.

CASE REPORT

Patient 1

A 56-year-old male electrician was admitted to the

A

	After intubation	Before 1 DL	Before 2 DL
Ventilation mode	Pressure controlled ventilation (PCV)		
FiO_2[4]	0.97	1.0	1.0
Minute volume (L)	10.5	9.0	19.7
Tidal volume (mL)	629	410	310
Ventilation frequency (/min)	16	20	60
Peep (mmHg)	12	20	20
Peak ventilator pressure (mmHg)	14	39	44
Mean ventilator pressure (mmHg)	19	27	32
I:E ratio	1:2	1:1	1:1
pO_2 (mmHg)	67	67	61
pCO_2 (mmHg)	35	53	25
pH	7.44	7.35	7.55

B

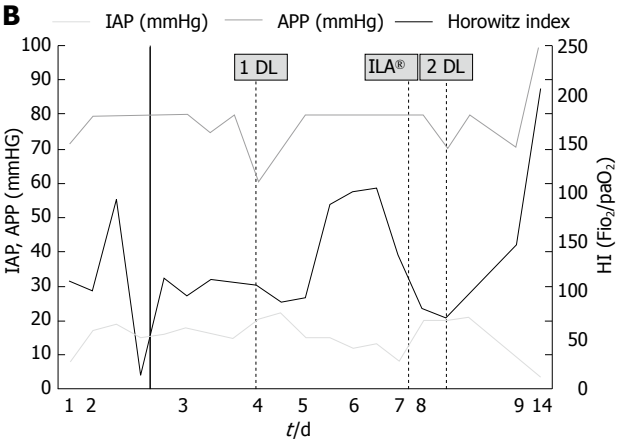


Figure 1 Ventilation adjustment of patient 1 after intubation, before 1 DL and 2 DL (**A**), and development of IAP, APP and HI in this patient over 14 d of ICU hospitalisation (**B**). The spotted lines refer time points of the first and second DL as well as installation of ILA®.

Intensive Care Unit of our hospital because of necrotizing pancreatitis. One week prior to admission, he underwent an ambulant gastroduodenoscopy in the referring hospital and resection of pancreatic papilla minor due to misinterpretation as a polyp. Following endoscopy, progredient upper abdominal pain developed with rising serum lipase and CRP levels. Diagnostic procedures including abdominal sonography, CT-scan and endoscopic-retrograde-cholangio-pancreaticography (ERCP) confirmed pancreatitis. At admission, intubation was performed followed by mechanical ventilation because of respiratory insufficiency (Figure 1A). In the next days, fluid resuscitation was performed, and increasing peak inspiratory and mean airway pressures were needed to achieve sufficient oxygenation. Meanwhile, IAP determined by an indwelling transurethral bladder catheter increased. On day 4, criteria for an ACS were fulfilled (Figure 1B). Simultaneously, Horowitz index decreased (75 mmHg) with 100% oxygen, a positive end expiratory pressure of 20 mmHg and a peak inspiratory airway pressure of 39 mmHg. Sonography revealed an elevation of the diaphragm without any movement. On day five, we decided to perform decompression laparotomy to improve pulmonary gas exchange. A median laparotomy of approximately 30 cm was performed. The abdominal cavity was closed with absorbable Vicryl®-Mesh. Postoperative oxygenation improved with decreasing ventilation pressures and chest wall compliance. Nevertheless the respiratory situation deteriorated again with low oxygenation values and hypercapnia, while IAP equally rose. A pumpless extracorporeal lung assist system (ILA®) was installed on day eight, resulting in a marked decrease in pCO_2, but the Horowitz index did not improve. Since the IAP values increased again (> 20 mmHg), the laparotomy was extended from xiphoid process to symphysis, thereby leading to a significant improvement in the respiratory situation. Again, the abdominal cavity was closed with Vicryl®-Mesh. During the next days, CT scan-guided retroperitoneal drainage was performed for debridement of pancreatic necrosis. As a result, IAP further decreased (Figure 1B). The patient was successfully weaned, therapy with ILA® system could be quickly terminated and the abdomen healed by secondary wound healing.

Patient 2

A 49-year-old man with morbid obesity (172 cm, 150 kg, BMI = 50.7 kg/m^2) presented in the Emergency Department of our hospital for colicky abdominal pain. His past medical history included a limited cardiac function due to ischemic heart disease and recurrent ventricular tachycardia. Laboratory and radiological findings as well as endoscopy revealed biliary pancreatitis. Three days after admission, his respiratory situation worsened and he was transferred to the Intensive Care Unit. Mechanical ventilation had to be initiated. On the second day of intensive care treatment, intra-abdominal inflammation, massive fluid resuscitation (fluid intake about 10 L/d, urine output 1 L/d the first day, up to 7 L/d the next days) and pre-existing obesity led to ACS with a measured IAP above 25 mmHg. Limitations of ventilation therapy with decreased lung and chest compliance prompted us to perform decompression laparotomy. IAP, APP, Horowitz index as well as compliance values significantly improved after surgical therapy and lung protective ventilation could be re-achieved. Secondary wound healing of the open abdomen was improved after vacuum-assisted closure (VAC) therapy (Figure 2B). Unfortunately, the patient developed cardiovascular complications and died of fulminant lung bleeding two weeks later.

Patient 3

A 74-year-old woman was admitted with post-ERCP pancreatitis after an ERCP was performed for symptomatic cholecystolithiasis. She was transferred to our hospital from another hospital due to aggravation of her condition with rising inflammation parameters. On the day of admission, she had to be intubated, mechanical ventilation was started and fluid resuscitation was performed. Within 3 d the HI dramatically dropped with simultaneously increasing peak pressure levels (Figure 3). Under 100% oxygen ventilation, inverse ventilation ratio (2:1), peak pressure of 45 mmHg and positive end-expiratory pressure (PEEP) of 22 mmHg, and PO_2 of 70 mmHg could be achieved. Clinically, she presented with massive abdominal tenderness. IAP-values above 20 mmHg were measured

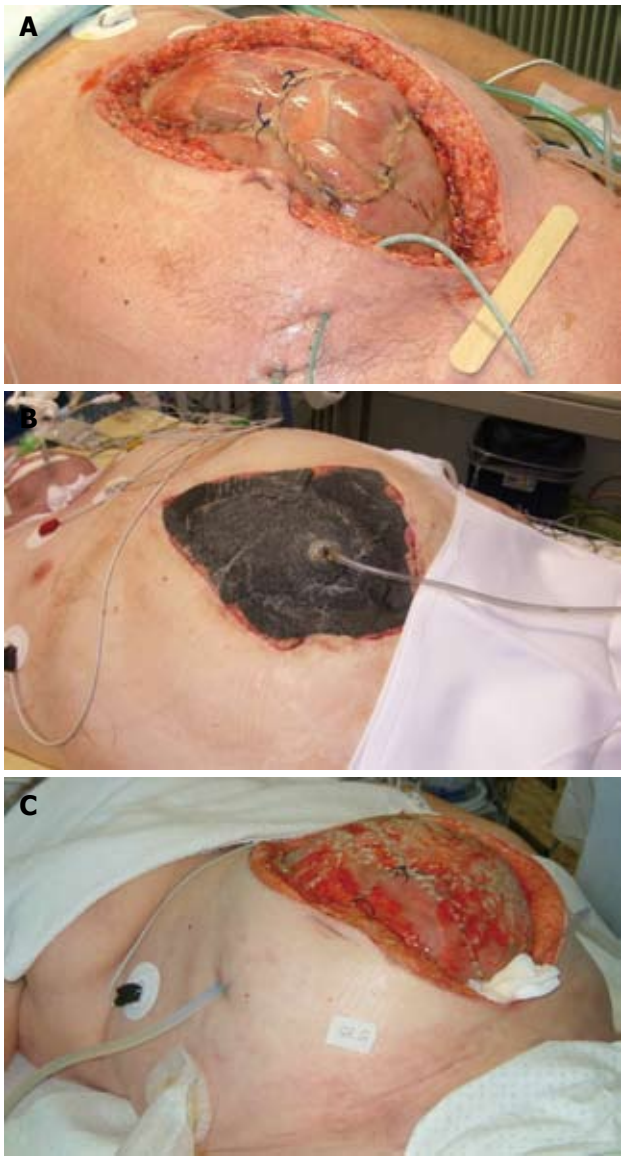


Figure 2 Open abdomen after surgical decompression. **A** and **C**: Intra-abdominal drains further reduce intra-abdominal tension; **B**: Vacuum bondage improves wound healing. **A**: Patient No. 1; **B**: Patient No. 2; **C**: Patient No. 3.

and decompression laparotomy was performed due to respiratory failure. Immediately after the intervention, her respiratory situation improved significantly. During the next days, interventional CT-guided drainage therapy was performed and led to a further decrease in intra-abdominal pressure. Weaning was successful and spontaneouse breathing was re-achieved after dilatation tracheotomy and prolonged respiratory therapy due to septic complications. Three months after recovery, the abdominal laparotomy wound was closed along with elective cholecystectomy.

DISCUSSION

We present three cases of patients with SAP developing ACS leading to severe limitations of respiratory therapy. In each of these patients, decompression laparotomy caused immediate improvement in pulmonary function and led to definite survival in two cases.

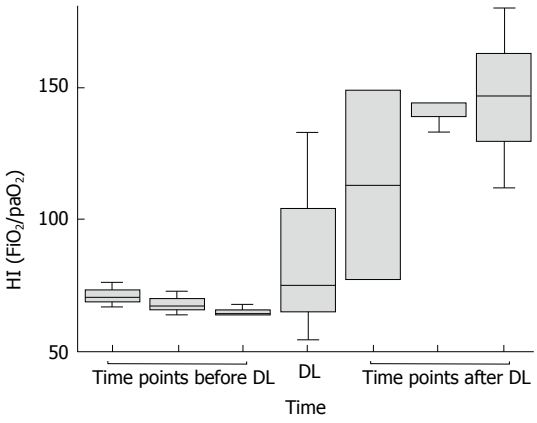


Figure 3 Development of Horowitz-indices (HI). In the Box blots, the HI 3, 2 and 1 d before, at and 1, 2 and 3 d after DL of all 3 patients is shown. The line refers to the median, whereas the boxes refer to the interquartile ranges. The whiskers represent the 5th and 95th percentiles, respectively.

Approximately 30%-40% of SAP patients develop ACS because of pancreatic-retroperitoneal inflammation, edema of peripancreatic tissue, fluid formation or abdominal distension, subsequently leading to intestinal ischemia with ileus and renal failure[5]. Besides, additional fluid resuscitation is known to further increase IAP. It was reported that ACS generally affects cardiac, pulmonary and renal function, and contributes to multi-organ dysfunction with a mortality rate ranging 10%-50% within two weeks[6,8-11]. The degree of IAP in patients with SAP seems to correlate with the degree of organ dysfunction, the severity of disease, the length of intensive care unit stay and mortality[6,8-11].

Pulmonary side effects mediated by IAH, such as atelectasis, edema, decreased oxygen transport and increased intrapulmonary shunt fraction, are caused by compression of pulmonary parenchyma. IAP is transmitted to the thorax through the elevated diaphragm and causes pulmonary parenchyma compression. The abdominal pressure on lung parenchyma is aggravated in mechanically ventilated patients due to high positive airway pressure, thereby leading to an elevated risk of alveolar barotraumas[12]. Furthermore, a study in ACS trauma patients demonstrated an increased rate of pulmonary infections[13].

Animal and human studies showed that abdominal decompression laparotomy can reverse the cardiopulmonary and abdominal effects of ACS[9,10]. In our patients, decompression laparotomy led to decreasd IAP levels, improved Horowitz indices and increased lung compliance (Figure 3). Ventilation pressures could be reduced in order to re-achieve a lung protective ventilation regime. In addition, all three patients benefited from improving renal perfusion (rising urine volume and decreasing creatinine and urea levels), mesenterial perfusion (declining lactate levels) and cardiac output (less need for catecholamines). Moreover, patients with SAP may specifically profit from decompression laparotomy since elevated IAP influences pancreatic and intestinal perfusion and might therefore contribute to pancreatic necrosis[14].

Although decompression laparotomy can lead to

recovery of patients with SAP from respiratory failure, its complications, such as massive intra-abdominal bleeding, need to be taken into account. Moreover, not only surgical complications but also persisting open abdomen is associated with risks and subsequent extensive abdominal wall reconstruction. New surgical concepts like subcutaneous anterior abdominal fasciotomy[15] may minimize complications and reach comparable clinical improvement in patients with SAP and ACS. Randomized studies are few so far, and the use of decompression laparotomy for ACS has been criticized by several authors since the mortality remains high in these patients[5,16].

Abdominal decompression laparotomy may help to overcome respiratory failure in patients with abdominal compartment syndrome and severe acute pancreatitis. Therefore, serial indirect measurement of IAP through an indwelling transurethral bladder catheter should be used routinely in critically ill patients with SAP in order to detect ACS. We hold that decompression laparotomy should be performed in patients with SAP and IAH/ACS with severe limitation of mechanical ventilation. However, prospective randomized studies are needed to further define the role of surgical decompression in SAP.

REFERENCES

1 **De Campos T**, Deree J, Coimbra R. From acute pancreatitis to end-organ injury: mechanisms of acute lung injury. *Surg Infect* (Larchmt) 2007; **8**: 107-120

2 **Werner J**, Feuerbach S, Uhl W, Buchler MW. Management of acute pancreatitis: from surgery to interventional intensive care. *Gut* 2005; **54**: 426-436

3 **Cheatham ML**, Malbrain ML, Kirkpatrick A, Sugrue M, Parr M, De Waele J, Balogh Z, Leppaniemi A, Olvera C, Ivatury R, D'Amours S, Wendon J, Hillman K, Wilmer A. Results from the International Conference of Experts on Intra-abdominal Hypertension and Abdominal Compartment Syndrome. II. Recommendations. *Intensive Care Med* 2007; **33**: 951-962

4 **Gecelter G**, Fahoum B, Gardezi S, Schein M. Abdominal compartment syndrome in severe acute pancreatitis: an indication for a decompressing laparotomy? *Dig Surg* 2002; **19**: 402-404; discussion 404-405

5 **De Waele JJ**, Hesse UJ. Life saving abdominal decompression in a patient with severe acute pancreatitis. *Acta Chir Belg* 2005; **105**: 96-98

6 **Keskinen P**, Leppaniemi A, Pettila V, Piilonen A, Kemppainen E, Hynninen M. Intra-abdominal pressure in severe acute pancreatitis. *World J Emerg Surg* 2007; **2**: 2

7 **Wong K**, Summerhays CF. Abdominal compartment syndrome: a new indication for operative intervention in severe acute pancreatitis. *Int J Clin Pract* 2005; **59**: 1479-1481

8 **Zhang WF**, Ni YL, Cai L, Li T, Fang XL, Zhang YT. Intra-abdominal pressure monitoring in predicting outcome of patients with severe acute pancreatitis. *Hepatobiliary Pancreat Dis Int* 2007; **6**: 420-423

9 **Chang MC**, Miller PR, D'Agostino R Jr, Meredith JW. Effects of abdominal decompression on cardiopulmonary function and visceral perfusion in patients with intra-abdominal hypertension. *J Trauma* 1998; **44**: 440-445

10 **Obeid F**, Saba A, Fath J, Guslits B, Chung R, Sorensen V, Buck J, Horst M. Increases in intra-abdominal pressure affect pulmonary compliance. *Arch Surg* 1995; **130**: 544-547; discussion 547-548

11 **Sugerman HJ**, Bloomfield GL, Saggi BW. Multisystem organ failure secondary to increased intraabdominal pressure. *Infection* 1999; **27**: 61-66

12 **Quintel M**, Pelosi P, Caironi P, Meinhardt JP, Luecke T, Herrmann P, Taccone P, Rylander C, Valenza F, Carlesso E, Gattinoni L. An increase of abdominal pressure increases pulmonary edema in oleic acid-induced lung injury. *Am J Respir Crit Care Med* 2004; **169**: 534-541

13 **Aprahamian C**, Wittmann DH, Bergstein JM, Quebbeman EJ. Temporary abdominal closure (TAC) for planned relaparotomy (etappenlavage) in trauma. *J Trauma* 1990; **30**: 719-723

14 **Kotzampassi K**, Grosomanidis B, Dadoukis D, Eleftheriadis E. Retroperitoneal compartment pressure elevation impairs pancreatic tissue blood flow. *Pancreas* 2007; **35**: 169-172

15 **Leppaniemi AK**, Hienonen PA, Siren JE, Kuitunen AH, Lindstrom OK, Kemppainen EA. Treatment of abdominal compartment syndrome with subcutaneous anterior abdominal fasciotomy in severe acute pancreatitis. *World J Surg* 2006; **30**: 1922-1924

16 **De Waele JJ**, Hoste EA, Malbrain ML. Decompressive laparotomy for abdominal compartment syndrome--a critical analysis. *Crit Care* 2006; **10**: R51

S- Editor Zhong XY **L- Editor** Wang XL **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5471

World J Gastroenterol 2008 September 21; 14(35): 5471-5473
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Atypical presentation of pioderma gangrenosum complicating ulcerative colitis: Rapid disappearance with methylprednisolone

Paolo Aseni, Stefano Di Sandro, Plamen Mihaylov, Luca Lamperti, Luciano Gregorio De Carlis

Paolo Aseni, Stefano Di Sandro, Plamen Mihaylov, Luca Lamperti, Luciano Gregorio De Carlis, Liver Transplantation Center, Niguarda Ca' Granda Hospital, Milan 20162, Italy
Author contributions: Aseni P, Di Sandro S, Mihaylov P, Lamperti L, De Carlis LG contributed equally to this work.
Correspondence to: Paolo Aseni, MD, Liver Transplantation Center, Niguarda Ca' Granda Hospital, P.za Ospedale Maggiore 3, Milan 20162, Italy. paoloaseni@gmail.com
Telephone: +38-2-64442252 Fax: +39-2-64442893
Received: June 3, 2008 Revised: July 28, 2008
Accepted: August 3, 2008
Published online: September 21, 2008

Abstract

Piodermal gangrenosum (PG) is an uncommon ulcerative cutaneous dermatosis associated with a variety of systemic diseases, including inflammatory bowel disease (IBD), arthritis, leukaemia, hepatitis, and primary billiary cirrhosis. Other cutaneous ulceration resembling PG had been described in literature. There has been neither laboratory finding nor histological feature diagnostic of PG, and diagnosis of PG is mainly made based on the exclusion criteria. We present here a patient, with ulcerative colitis (UC) who was referred to the emergency section with a large and rapidly evolving cutaneous ulceration. Laboratory and microbiological investigation associated with histological findings of the ulcer specimen allowed us to exclude autoimmune and systemic diseases as well as immuno-proliferative disorders. An atypical presentation of PG with UC was diagnosed. Pulse boluses of i.v. methyl-prednisolone were started, and after tapering steroids, complete resolution of the skin lesion was achieved in 3 wk. The unusual rapid healing of the skin ulceration with steroid mono-therapy and the atypical cutaneous presentation in this patient as well as the risk of misdiagnosis of PG in the clinical practice were discussed.

Key words: Ulcerative colitis; Pioderma gangrenosum; Steroids; Cutaneous lesion; Immunosuppression

Peer reviewer: Alastair JM Watson, Professor, Department of Gastroenterology, University of Liverpool, the Henry Wellcome Laboratory, Nuffield Bldg, Crown St, Liverpool L69 3GE, United Kingdom

Aseni P, Di Sandro S, Mihaylov P, Lamperti L, De Carlis LG. Atypical presentation of pioderma gangrenosum complicating ulcerative colitis: Rapid disappearance with methylprednisolone. *World J Gastroenterol* 2008; 14(35): 5471-5473 Available from: URL: http://www.wjgnet.com/1007-9327/14/5471.asp DOI: http://dx.doi.org/10.3748/wjg.14.5471

INTRODUCTION

Piodermal gangrenosum (PG) is an uncommon ulcerative cutaneous dermatosis associated with a variety of systemic conditions including inflammatory bowel disease (IBD), arthritis, haematological malignancies, paraproteinemia and hepatitis[1-5]. Many other cutaneous ulcerations resembling PG have been described in literature[6-10]. There has been neither laboratory finding nor histological feature diagnostic of PG, and diagnosis of PG is mainly established by exclusion criteria. We described here a patient with ulcerative colitis (UC) who manifested atypical presentation of PG. After diagnosis, a rapid healing of the large and painful skin ulceration was obtained by high doses of i.v. steroid therapy.

CASE REPORT

An 82-year-old man was referred to the emergency section with a round painful cutaneous ulcer of 15 cm in diameter in the left mammary region. The edges were undermined and presented with granulated tissues, crusts, and purulent exudates (Figure 1A). One month before a lesion appeared in the same skin area presenting as a small red plaque with surrounding erythema. This was supposed to be a consequence of a mosquito bite according to his family doctor. The lesion rapidly progressed to a wider and painful cutaneous ulceration over the past month. Antimicrobial treatment with amoxicillin and ciprofloxacin was totally ineffective and the patient required paracetamol and codeine every 6 h for pain relief.

The patient was admitted to our hospital 3 years before due to rectal bleeding and anaemia. UC was

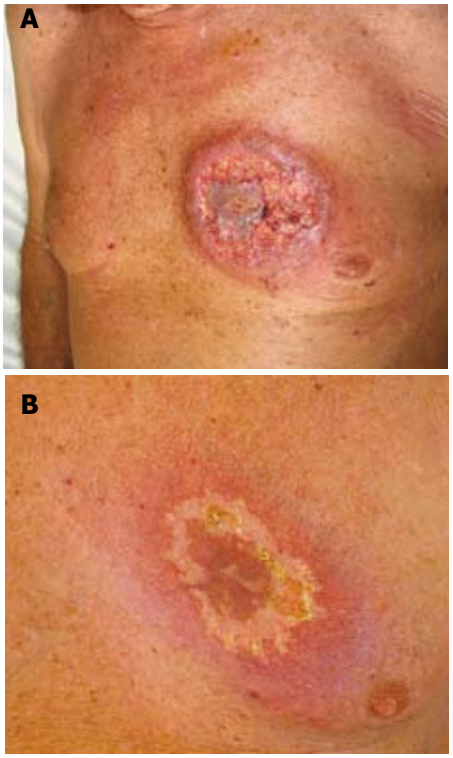


Figure 1 **A**: Patient with chronic ulcerative colitis presenting a round and painful cutaneous ulcer of 15 cm in diameter in the left mammary region. The edges were undermined. Granulated tissue, crusts, and purulent exudates are evident; **B**: Resolution of the skin lesion after 20 d of metilprednisolone therapy.

diagnosed. Therefore, the patient received prednisone and mesalazine therapy (10 mg/d and 800 mg thrice/d, respectively). Prednisone was tapered and stopped after 3 mo, whereas mesalazine administration was continued.

On examination, the patient presented with mild hyperthermia (37.5-38℃). He complained of 3-5 daily episodes of diarrhoea but without rectal bleeding. No lymphoadenopathy was observed. The lesion was very painful. A swab and microbiological examination of the specimen from the ulcer was negative for bacteria and fungi. Routine laboratory investigations revealed white cell count of 13.8×10^9/L with neutrophilia. The erythrocyte sedimentation rate was 32 mm/h. Liver and kidney function tests, immunoglobulin, protein electrophoresis, anticoagulation panel were normal. Venereal Disease Research Laboratory (VDRL) test, HIV test, anti-neutrophilic cytoplasmic, antinuclear and anti-DNA antibodies, rheumatoid factor, LE test, were all negative, and cryoglobulins were absent.

Chest X-ray, venous and arterial functional studies were normal. A skin biopsy of the lesion was performed under local anaesthesia. Histological analysis showed focal necrotizing flogosis associated with ulceration and peripheral lymphocytic and neutrophilic infiltration extending through the dermis and subcutaneous tissue; extravascular red blood cell infiltration was also present.

Necrotizing vasculitis was not observed and the histological changes were consistent with pioderma gangrenosum. Methylprednisolone pulse boluses (500 mg/d for 3 d) were given i.v. Steroid was reduced to 80 mg/d and then tapered to 20 mg/d for 3 wk. The patient healed from skin lesion 20 d after beginning of steroid therapy (Figure 1B).

DISCUSSION

Brunsting *et al*[11] in 1930 first described five patients with rapidly progressive and painful suppurative skin ulceration with necrotic and undermined borders that were called PG. This lesion is a neutrophilic dermatosis associated with a variety of systemic diseases, such as paraproteinemia, arthritis, and myeloproliferative diseases, and IBD. In about 50% of the cases, UC is the underlying condition and PG may parallel the severity of the disease[1,9,12]. The pathogenesis of PG is poorly understood and over-expression of interleukin (IL)-8 and IL-16 has been reported, suggesting an over-reactive inflammatory response to a traumatic process. Although the lesion can occur in any surface it is more common on the legs in perineal, vulvar, penile and neck region. Atypical presentations are considered on the arms or in the chest. Weenig *et al*[6] reported two cases of livedoid vasculopathy, a rare thrombo-occlusive disease of post-capillary venules, which may occur with cutaneous ulcers of the legs characterized by a very similar macroscopic and histological pattern. These lesions may be confused with PG. However, livedoid vasculopathy is not responsive to steroid therapy. Therefore, PG is an excluded diagnosis on the basis of laboratory findings and histology, associated with a high rate of clinical suspicion. The good and rapid clinical responses to steroids associated with other immunosuppressive therapy such as cyclosporine, azathioprine and ciclophosphamide are also important "*ex-adiuvantibus*" criteria.

Patients with vasculitis associated with or not associated with cryoglobulinemia or those with antiphospholipid-antibody syndrome, and those with Wegener granulomatosis and poliartrite nodosa, may present lesions resembling PG[5,6,8,9,13]. These lesions may be misdiagnosed with PG due to initial response to steroid therapy, but without evidence of complete healing. The clinical pattern of a patient with very painful skin lesion, suffering from IBD should raise the suspicion of PG; however laboratory findings and functional and radiologic analysis to rule out other systemic disease are mandatory for a correct diagnosis.

Other rare malignant lesions, such as lymphoma, leukaemia cutis and Langherans cell hystiocytosis can be ruled out according to the histological studies of the specimen.

PG is a diagnosis of exclusion and its misdiagnosis can result in serious clinical consequences.

The chronic UC in our patient based on the exclusion criteria, convinced us to start therapy with a high dose of corticosteroid. The rapid healing of such a large skin lesion is unusual. Some patients refractory to steroid treatment can benefit from the combination of steroid with cyclosporine[14,15]. At the moment, our patient is disease free at 12 mo after diagnosis without

clinical symptoms related to UC under a maintenance therapy of 7.5 mg/d prednisone.

ACKNOWLEDGEMENT

The Authors thank Giuliana Fantini, MD, for valuable technical suggestions.

REFERENCES

1 **Callen JP**. Pyoderma gangrenosum. *The Lancet* 1998; **351**: 581-585
2 **Powell FC**, Su WP, Perry HO. Pyoderma gangrenosum: classification and management. *J Am Acad Dermatol* 1996; **34**: 395-409; quiz 410-412
3 **Levitt MD**, Ritchie JK, Lennard-Jones JE, Phillips RK. Pyoderma gangrenosum in inflammatory bowel disease. *Br J Surg* 1991; **78**: 676-678
4 **Neesse A**, Michl P, Kunsch S, Ellenrieder V, Gress TM, Steinkamp M. Simultaneous onset of ulcerative colitis and disseminated pyoderma gangrenosum. *Case Rep Gastroenterol* 2007; **1**: 110-115
5 **Smith JB**, Shenefelt PD, Soto O, Valeriano J. Pyoderma gangrenosum in a patient with cryoglobulinemia and hepatitis C successfully treated with interferon alfa. *J Am Acad Dermatol* 1996; **34**: 901-903
6 **Weenig RH**, Davis MD, Dahl PR, Su WP. Skin ulcers misdiagnosed as pyoderma gangrenosum. *N Engl J Med* 2002; **347**: 1412-1418
7 **Hay CR**, Messenger AG, Cotton DW, Bleehen SS, Winfield DA. Atypical bullous pyoderma gangrenosum associated with myeloid malignancies. *J Clin Pathol* 1987; **40**: 387-392
8 **Schlesinger IH**, Farber GA. Cutaneous ulceration resembling pyoderma gangrenosum in the primary antiphospholipid syndrome: a report of two additional cases and review of the literature. *J La State Med Soc* 1995; **147**: 357-361
9 **Norris JF**, Marshall TL, Byrne JP. Histiocytosis X in an adult mimicking pyoderma gangrenosum. *Clin Exp Dermatol* 1984; **9**: 388-392
10 **Papi M**, Didona B, De Pita O, Frezzolini A, Di Giulio S, De Matteis W, Del Principe D, Cavalieri R. Livedo vasculopathy vs small vessel cutaneous vasculitis: cytokine and platelet P-selectin studies. *Arch Dermatol* 1998; **134**: 447-452
11 **Brunsting LA**, Undewood LJ. Pyoderma vegetans in association with chronic ulcerative colitis. *Arch Derm Syphilol* 1949; **60**: 161-172
12 **Gibson LE**, Daoud MS, Muller SA, Perry HO. Malignant pyodermas revisited. *Mayo Clin Proc* 1997; **72**: 734-736
13 **Nguyen KH**, Miller JJ, Helm KF. Case reports and a review of the literature on ulcers mimicking pyoderma gangrenosum. *Int J Dermatol* 2003; **42**: 84-94
14 **Futami H**, Kodaira M, Furuta T, Hanai H, Kaneko E. Pyoderma gangrenosum complicating ulcerative colitis: Successful treatment with methylprednisolone pulse therapy and cyclosporine. *J Gastroenterol* 1998; **33**: 408-411
15 **Reichrath J**, Bens G, Bonowitz A, Tilgen W. Treatment recommendations for pyoderma gangrenosum: an evidence-based review of the literature based on more than 350 patients. *J Am Acad Dermatol* 2005; **53**: 273-283

S- Editor Li DL **L- Editor** Ma JY **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5474

World J Gastroenterol 2008 September 21; 14(35): 5474-5477
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Development of autoimmune hepatitis type 1 after pulsed methylprednisolone therapy for multiple sclerosis: A case report

Atsushi Takahashi, Yukiko Kanno, Yuta Takahashi, Natsumi Sakamoto, Kyoko Monoe, Hironobu Saito, Kazumichi Abe, Junko Yokokawa, Atsushi Irisawa, Hiromasa Ohira

Atsushi Takahashi, Yukiko Kanno, Yuta Takahashi, Natsumi Sakamoto, Kyoko Monoe, Hironobu Saito, Kazumichi Abe, Junko Yokokawa, Atsushi Irisawa, Hiromasa Ohira, Department of Internal Medicine Ⅱ, Fukushima Medical University School of Medicine, Fukushima, Japan
Author contributions: Takahashi A treated the patient and wrote the manuscript; Kanno Y, Takahashi Y, Sakamoto N, Monoe K, Saito H, Abe K and Yokokawa J treated the patient; Ohira H and Irisawa A revised the manuscript; Ohira H approved the final manuscripts.
Correspondence to: Atsushi Takahashi, Department of Internal Medicine Ⅱ, Fukushima Medical University School of Medicine, 1 Hikarigaoka, Fukushima 960-1295, Japan. junior@fmu.ac.jp
Telephone: +81-24-547-1202 Fax: +81-24-547-2055
Received: July 14, 2008 Revised: August 26, 2008
Accepted: September 3, 2008
Published online: September 21, 2008

Abstract

A 43-year-old woman with multiple sclerosis (MS) was treated with pulsed methylprednisolone and interferon β at a hospital. Four weeks after initiating treatment, liver dysfunction occurred and she was referred and admitted to our hospital. Clinical and laboratory findings were consistent with and fulfilled the criteria for drug-induced hepatitis, but not for autoimmune hepatitis (AIH). She was successfully treated with corticosteroids. As ataxia developed after 1 year, she was treated with pulsed methylprednisolone for 3 d, then readmitted to our hospital when liver dysfunction occurred. Clinical and laboratory findings led to the diagnosis of AIH. To the best of our knowledge, this is the second case of AIH developed after pulsed methylprednisolone for MS.

Key words: Multiple sclerosis; Autoimmune hepatitis; Pulsed methylprednisolone

Peer reviewer: Dr. Stefan Wirth, Professor, Children's Hospital, Heusnerstt. 40, Wuppertal 42349, Germany

Takahashi A, Kanno Y, Takahashi Y, Sakamoto N, Monoe K, Saito H, Abe K, Yokokawa J, Irisawa A, Ohira H. Development of autoimmune hepatitis type 1 after pulsed methylprednisolone therapy for multiple sclerosis: A case report. *World J Gastroenterol* 2008; 14(35): 5474-5477 Available from: URL: http://www.wjgnet.com/1007-9327/14/5474.asp DOI: http://dx.doi.org/10.3748/wjg.14.5474

INTRODUCTION

Intravenous methylprednisolone pulse therapy is the standard treatment for relapsing multiple sclerosis (MS). Interferon (IFN)-β is the most commonly used drug in the treatment of MS, and has been proven to reduce the disease activity, progression and relapse rate[1,2]. IFN-β is associated with hepatotoxicity, although it rarely induces severe liver injury. It was reported that autoimmune hepatitis (AIH) occurs during IFN-β therapy for MS[3,4], but only one report has described the occurrence of AIH after intravenous methylprednisolone pulse therapy for MS[5]. We describe herein a case of a MS patient who developed AIH after treatment with IFN-β and pulsed methylprednisolone.

CASE REPORT

A 43-year-old woman with abdominal discomfort and nausea was referred to our hospital on August 7, 2006. She was diagnosed with MS on the basis of clinical and laboratory findings 7 years ago. Three years ago, she was treated with pulsed methylprednisolone (1000 mg/day for 3 d) followed by 50 mg/day of oral prednisolone because of ataxia. Although oral prednisolone was tapered and stopped for 1 month, she remained healthy until June 2006, when ataxia developed again. On June 28, 2006, she was treated with pulsed methylprednisolone (1000 mg/day for 3 d) followed by 50 mg/day of oral prednisolone. Despite pulsed methylprednisolone therapy, symptoms did not improve. She was therefore retreated with pulsed methylprednisolone (1000 mg/day) for 3 d from July 5, 2006. Moreover, she was treated with IFN-β 8 at MU every other day from July 11 to 26, 2006. After pulsed methylprednisolone, oral prednisolone was not administered. On August 3, 2006, the patient

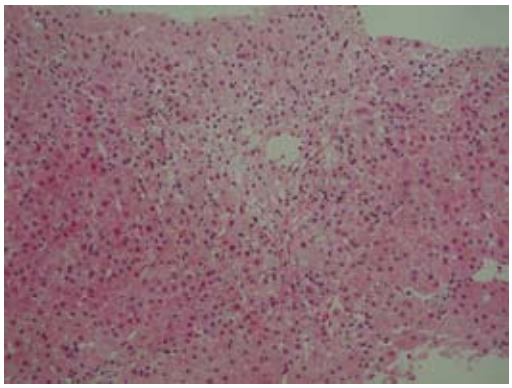


Figure 1 Histological examination of a liver biopsy specimen showing bridging perivenular necrosis and infiltration of inflammatory cells including eosinophils (hematoxylin-eosin staining × 100).

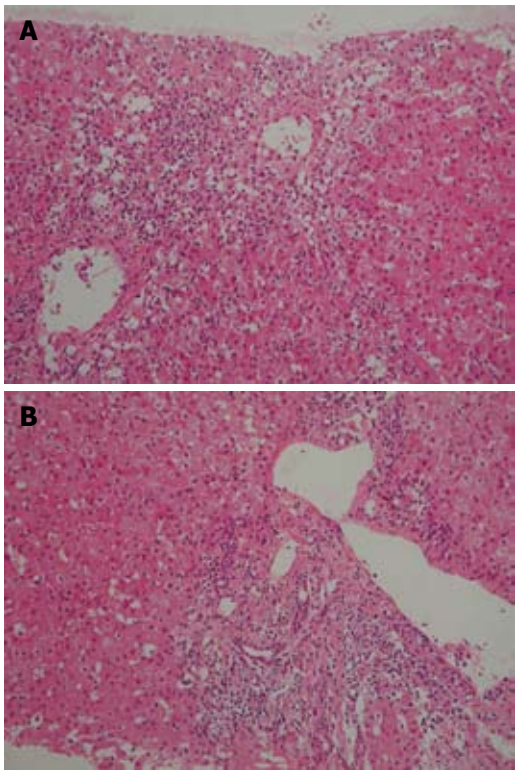


Figure 2 Histological examination of a liver biopsy specimen showing bridging perivenular necrosis (**A**) and interface hepatitis (**B**) (HE staining × 100).

became nauseous and vomited, and these symptoms did not improve. On August 7, 2006, she was referred to our hospital and admitted after blood testing revealed severe liver dysfunction. Three years ago, she developed acute hepatitis due to Epstein-Barr (EB) virus after treatment with pulsed methylprednisolone. Since then, she had been free of liver dysfunction.

On admission, her blood pressure was 156/89 mmHg and heart rate was 102 beats/min, body temperature was 37.3℃, and the areas of skin at sites of IFN-β injection became welts. Her conjunctivae were not jaundiced, heart and respiratory sounds were normal. No abnormalities were noted in the chest or abdomen. The liver and spleen were not palpable. Neurological examination showed no abnormalities suggestive of MS. Laboratory findings were as follows: 1102 IU/L aspartate aminotransferase (AST) (normal, 10-35 IU/L), 1067 IU/L alanine aminotransferase (ALT) (normal, 12-33 IU/L), 377 IU/L alkaline phosphatase (ALP) (normal, 300-500 IU/L), 3.4 mg/dL total bilirubin (TB) (normal, < 1.1 mg/dL), 2.2 mg/dL direct bilirubin (DB) (normal, 0.2-0.4 mg/dL), 26 IU/L γ-glutamyl transpeptidase (γGTP) (normal, 10-47 IU/L), 6.4 g/dL total protein (TP) (normal, 6.0-8.5 g/dL), 3.7 g/dL albumin (normal, 4.0-5.3 g/dL), 1370 mg/dL serum immunoglobulin (Ig)G, 147 mg/dL IgA, 272 mg/dL IgM, and 71.4% prothrombin time (PT). Anti-nuclear antibody (ANA), anti-smooth muscle antibody and anti-LKM-1 antibody were all negative. HBs antigens, IgM-HA and HCV antibodies were negative. Other viral infections including EB virus and cytomegalovirus infection were excluded by serological testing. Abdominal computed tomography showed no abnormalities. Biopsy specimen of the liver showed bridging perivenular necrosis with infiltration of inflammatory cells including eosinophils (Figure 1). A lymphocyte-stimulation test for IFN-β yielded negative results, but the patient displayed a score of 9 according to the criteria for drug-induced liver injuries[5], indicating a high probability of drug-induced liver injury. All these findings led to the diagnosis of drug-induced liver injury caused by IFN-β. Despite intravenous administration of stronger neo-minophagen C (60 mL/day) and prostaglandin, jaundice developed with a serum TB level of 19.1 mg/dL. Methylprednisolone (125 mg/day for 3 d) and ursodeoxycholic acid (UDCA, 600 mg/day) were therefore administered. Symptoms subsequently improved and serum TB level normalized. Prednisolone was decreased gradually and stopped on April 10, 2007. UDCA was stopped on May 10, 2007. Liver function remained normal even after withdrawal of prednisolone and UDCA.

However, ataxia developed and the patient was again treated with pulsed methylprednisolone (1000 mg/day) for 3 d from October 1, 2007. After pulsed methylprednisolone, oral prednisolone was not administered. Two weeks later, she was readmitted to our hospital due to fatigue and liver dysfunction. Laboratory findings on admission were as follows: 566 IU/L AST, 875 IU/L ALT, 214 IU/L ALP, 1.7 mg/dL TB, 12 IU/L γGTP, 1785 mg/dL IgG, and 71.4% PT. Anti-nuclear antibody (ANA) titer was × 80 with a homogeneous pattern, positive results were obtained for anti-smooth muscle antibody, and HLA DR was 4. Viral infections were excluded by serological testing. Biopsy specimen from the liver revealed bridging perivenular necrosis and interface hepatitis (Figure 2A and B). In this case, IgG was not elevated, which is atypical for AIH. However, according to the criteria for AIH[6], the patient had a score of 16 on the second admission, indicating definite AIH, compared to a score of 9 on the first admission. Conversely, according to the criteria for drug-induced liver injury[7], our patient displayed a score of 2, indicating a low possibility that this case represented drug-induced liver injury. Moreover,

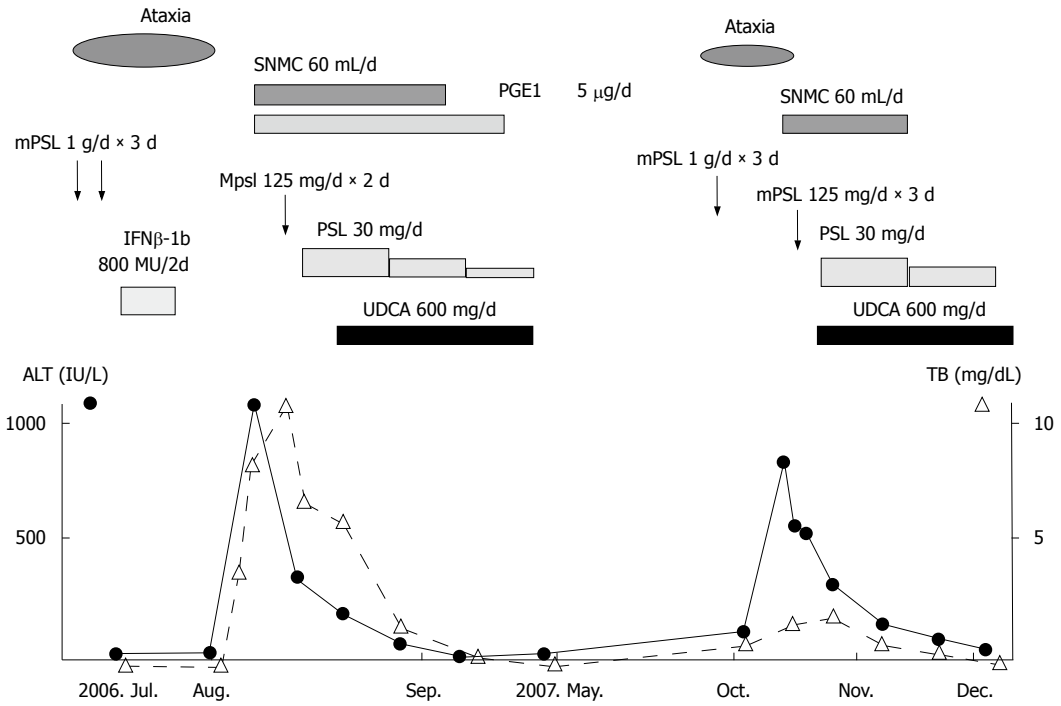


Figure 3 Clinical course of the disease. SNMC: Stronger neo-minophagen C, PGE1: Prostaglandin, mPSL: Methylprednisolone, PSL: Prednisolone, IFN-β: Interferon β.

lymphocyte-stimulation testing for methylprednisolone yielded negative results.

These clinical and laboratory findings supported the diagnosis of AIH. After administration of prednisolone and UDCA, symptoms and liver function improved. The charts for the overall clinical course are shown in Figure 3. Her condition is now under control with prednisolone, 10 mg/day.

DISCUSSION

MS is an inflammatory demyelinating disease of the central nervous system. Liver dysfunction is not always caused by MS itself, but can result from many factors, such as drug toxicity, fatty infiltration and viral infection. Liver dysfunction in patients with MS is most commonly caused by drugs. IFN-β, which raises serum ALT level as a side effect, is one of the drugs well known to cause liver injury in patients with MS.

Tremlett *et al*[8] reported that 36.9% of patients with MS develop new elevations of ALT, although only 1.4% reach grade 3 hepatotoxicity (> 5-20 upper limit of normal). In patients with MS receiving IFN-β, if *de novo* elevation of aminotransferases is mild, IFN-β treatment is often continued, and elevated aminotransferases return to almost normal[4]. However, severe liver dysfunction does not resolve simply after stopping IFN-β, and prompt treatment is needed. A case of fulminant liver failure occurring during IFN-β treatment has been reported[9]. Our patient satisfied the criteria for drug-induced hepatitis, but not for AIH on the first admission. Byrnes *et al*[10] have also reported drug-induced liver injury secondary to IFN-β in patients with MS. However, the precise mechanisms underlying IFN-β-induced hepatotoxicity remain unclear.

IFN-β may cause autoimmune complications including thyroiditis, lupus erythematosus and rheumatoid arthritis[11]. Duchini *et al*[3] have reported a case of AIH occurring during treatment with IFN-β. Conversely, Reuβ *et al*[5] have reported a case of AIH that developed after high-dose intravenous methylprednisolone pulse in MS and speculated that AIH may occur in patients with multiple autoimmunity as an immune rebound phenomenon after immunosuppressive regimens.

The typical histological pattern of AIH is chronic active hepatitis that shows portal inflammation with fibrosis, interface hepatitis and rosette formation of hepatocytes. However, few cases of AIH with centrilobular necrosis (CN) as the dominant finding have been reported[12]. Recently, some cases of CN with autoimmune features have been confirmed as early-stage AIH[13,14]. Acute-onset AIH sometimes does not satisfy AIH criteria serologically and shows CN histologically[14-16]. Although our patient showed a typical pattern of AIH at the second admission, liver dysfunction at the first admission may have been due to early-stage AIH.

The cause of AIH in this patient was an immune rebound phenomenon after pulsed methylprednisolone, because the second episode of liver dysfunction occurred after pulsed methylprednisolone therapy rather than after IFN-β therapy. In fact, some reports have described AIH occurring in patients with multiple autoimmunity after pulsed methylprednisolone therapy[5,17-18]. In particular, withdrawal of glucocorticoids after pulsed methylprednisolone therapy might have induced immune rebound phenomenon in the present

case. However, we cannot deny the possibility that AIH was induced by IFN-β in this patient. She received IFN-β treatment before the first admission. Moreover, Misdraji *et al*[12] reported that AIH with CN occurs after IFN-β therapy in patients with MS.

In conclusion, the prevalence of AIH seems to be about 10-fold higher in patients with MS than in the general population[19]. Attention should be paid to the development of AIH after pulsed methylprednisolone or IFN-β treatment in patients with MS, and if AIH develops, immediate treatment with corticosteroids or azathioprine should be initiated. Moreover, administration of corticosteroids or azathioprine after pulsed methylprednisolone might be effective for preventing the development of AIH.

REFERENCES

1 **The IFNB Multiple Sclerosis Study Group**. Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. I. Clinical results of a multicenter, randomized, double-blind, placebo-controlled trial. *Neurology* 1993; **43**: 655-661

2 **Saida T**, Tashiro K, Itoyama Y, Sato T, Ohashi Y, Zhao Z. Interferon beta-1b is effective in Japanese RRMS patients: a randomized, multicenter study. *Neurology* 2005; **64**: 621-630

3 **Duchini A**. Autoimmune hepatitis and interferon beta-1a for multiple sclerosis. *Am J Gastroenterol* 2002; **97**: 767-768

4 **Durelli L**, Bongioanni MR, Ferrero B, Oggero A, Marzano A, Rizzetto M. Interferon treatment for multiple sclerosis: autoimmune complications may be lethal. *Neurology* 1998; **50**: 570-571

5 **Reuβ R**, Retzlaff K, Vogel S, Franke FE, Oschmann P. Autoimmune hepatitis after high-dose intravenous methylprednisolone pulse in RR-MS. *CEJMed* 2007; **2**: 356-359

6 **Alvarez F**, Berg PA, Bianchi FB, Bianchi L, Burroughs AK, Cancado EL, Chapman RW, Cooksley WG, Czaja AJ, Desmet VJ, Donaldson PT, Eddleston AL, Fainboim L, Heathcote J, Homberg JC, Hoofnagle JH, Kakumu S, Krawitt EL, Mackay IR, MacSween RN, Maddrey WC, Manns MP, McFarlane IG, Meyer zum Buschenfelde KH, Zeniya M. International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. *J Hepatol* 1999; **31**: 929-938

7 **Benichou C**, Danan G, Flahault A. Causality assessment of adverse reactions to drugs--II. An original model for validation of drug causality assessment methods: case reports with positive rechallenge. *J Clin Epidemiol* 1993; **46**: 1331-1336

8 **Tremlett HL**, Yoshida EM, Oger J. Liver injury associated with the beta-interferons for MS: a comparison between the three products. *Neurology* 2004; **62**: 628-631

9 **Yoshida EM**, Rasmussen SL, Steinbrecher UP, Erb SR, Scudamore CH, Chung SW, Oger JJ, Hashimoto SA. Fulminant liver failure during interferon beta treatment of multiple sclerosis. *Neurology* 2001; **56**: 1416

10 **Byrnes V**, Afdhal N, Challies T, Greenstein PE. Drug induced liver injury secondary to interferon-beta (IFN-beta) in multiple sclerosis. *Ann Hepatol* 2006; **5**: 56-59

11 **Walther EU**, Hohlfeld R. Multiple sclerosis: side effects of interferon beta therapy and their management. *Neurology* 1999; **53**: 1622-1627

12 **Misdraji J**, Thiim M, Graeme-Cook FM. Autoimmune hepatitis with centrilobular necrosis. *Am J Surg Pathol* 2004; **28**: 471-478

13 **Zen Y**, Notsumata K, Tanaka N, Nakanuma Y. Hepatic centrilobular zonal necrosis with positive antinuclear antibody: a unique subtype or early disease of autoimmune hepatitis? *Hum Pathol* 2007; **38**: 1669-1675

14 **Hofer H**, Oesterreicher C, Wrba F, Ferenci P, Penner E. Centrilobular necrosis in autoimmune hepatitis: a histological feature associated with acute clinical presentation. *J Clin Pathol* 2006; **59**: 246-249

15 **Abe M**, Hiasa Y, Masumoto T, Kumagi T, Akbar SM, Ninomiya T, Matsui H, Michitaka K, Horiike N, Onji M. Clinical characteristics of autoimmune hepatitis with histological features of acute hepatitis. *Hepatol Res* 2001; **21**: 213-219

16 **Okano N**, Yamamoto K, Sakaguchi K, Miyake Y, Shimada N, Hakoda T, Terada R, Baba S, Suzuki T, Tsuji T. Clinicopathological features of acute-onset autoimmune hepatitis. *Hepatol Res* 2003; **25**: 263-270

17 **Salvi M**, Vannucchi G, Sbrozzi F, Del Castello AB, Carnevali A, Fargion S, Beck-Peccoz P. Onset of autoimmune hepatitis during intravenous steroid therapy for thyroid-associated ophthalmopathy in a patient with Hashimoto's thyroiditis: case report. *Thyroid* 2004; **14**: 631-634

18 **Marino M**, Morabito E, Altea MA, Ambrogini E, Oliveri F, Brunetto MR, Pollina LE, Campani D, Vitti P, Bartalena L, Pincheral A, Marcocci C. Autoimmune hepatitis during intravenous glucocorticoid pulse therapy for Graves' ophthalmopathy treated successfully with glucocorticoids themselves. *J Endocrinol Invest* 2005; **28**: 280-284

19 **de Seze J**, Canva-Delcambre V, Fajardy I, Delalande S, Stojkovic T, Godet E, Vermersch P. Autoimmune hepatitis and multiple sclerosis: a coincidental association? *Mult Scler* 2005; **11**: 691-693

S- Editor Xiao LL **L- Editor** Wang XL **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5478

World J Gastroenterol 2008 September 21; 14(35): 5478-5480
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Acute pancreatitis successfully diagnosed by diffusion-weighted imaging: A case report

Satoshi Shinya, Takamitsu Sasaki, Yoshifumi Nakagawa, Zhang Guiquing, Fumio Yamamoto, Yuichi Yamashita

Satoshi Shinya, Takamitsu Sasaki, Yoshifumi Nakagawa, Zhang Guiquing, Fumio Yamamoto, Department of Gastroenterological Surgery, Yamamoto Memorial Hospital, 88-4 Hachiyagarami, Nirimachi, Imari, Saga 848-0031, Japan
Satoshi Shinya, Takamitsu Sasaki, Yuichi Yamashita, Department of Gastroenterological Surgery, Fukuoka University School of Medicine, Nanakuma 7-45-1, Jonan-ku, Fukuoka 814-0180, Japan
Author contributions: Shinya S wrote the paper and organized the figures and patient data; Sasaki T, Nakagawa Y, Guiquing Z and Yamamoto F helped write, organize and correct the paper; Yamashita Y supervised the writing and organization process.
Correspondence to: Satoshi Shinya, MD, Department of Gastroenterological Surgery, Yamamoto Memorial Hospital, 88-4 Hachiyagarami, Nirimachi, Imari, Saga 848-0031, Japan. shinya-satoshi-rie@lake.ocn.ne.jp
Telephone: +81-955-23-2166 Fax: +81-955-22-4702
Received: October 10, 2007 Revised: January 12, 2008
Accepted: January 19, 2008
Published online: September 21, 2008

Abstract

Diffusion-weighted imaging (DWI) is an established diagnostic method of acute stroke. The latest advances in magnetic resonance imaging (MRI) technology have greatly expanded the utility of DWI in the examination of various organs. Recent studies have revealed the usefulness of DWI for imaging of the liver, kidney, ovary, and breast. We report a patient with acute pancreatitis detected by DWI and discussed the efficacy of DWI in diagnosing acute pancreatitis. A 50-year old man presented with a primary complaint of abdominal pain. We performed both DWI and computed tomography (CT) for this patient. The signal intensity in a series of DWI was measured and the apparent diffusion coefficient (ADC) values were calculated to differentiate inflammation from normal tissue. Two experienced radiologists evaluated the grade of acute pancreatitis by comparing the CT findings. Initially, the pancreas and multiple ascites around the pancreas produced a bright signal and ADC values were reduced on DWI. As the inflammation decreased, the bright signal faded to an iso-signal and the ADC values returned to their normal level. There was no difference in the abilities of DWI and CT images to detect acute pancreatitis. However, our case indicates that DWI can evaluate the manifestations of acute pancreatitis using no enhancement material and has the potential to replace CT as a primary diagnostic strategy for acute pancreatitis.

Key words: Diffusion-weighted imaging; Apparent diffusion coefficients; Magnetic resonance imaging; Acute pancreatitis

Peer reviewers: Dr. Aydin Karabacakoglu, Assistant Professor, Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey; Patrick Veit-Haibach, MD, Department of Diagnostic and Interventional Radiology and Neuroradiology University Hospital Essen Hufelandstrasse 55 45121 Essen, Germany

Shinya S, Sasaki T, Nakagawa Y, Guiquing Z, Yamamoto F, Yamashita Y. Acute pancreatitis successfully diagnosed by diffusion-weighted imaging: A case report. *World J Gastroenterol* 2008; 14(35): 5478-5480 Available from: URL: http://www.wjgnet.com/1007-9327/14/5478.asp DOI: http://dx.doi.org/10.3748/wjg.14.5478

INTRODUCTION

Acute pancreatitis is a potentially fatal disease with an overall mortality rate of 7%-11%[1-3]. Patients suffering from acute pancreatitis (AP) often have additional complications such as sepsis, systemic inflammatory syndrome (SIRS) and multiple organ failure (MOF), resulting in a life-threatening condition[4-6]. Therefore, it is important to accurately evaluate the grade of inflammation and absence of necrotizing pancreatitis to improve its prognosis. Diffusion-weighted imaging (DWI) is an established diagnostic tool of acute stroke and brain tumors[7-11]. Due to the latest technical advances in magnetic resonance imaging (MRI), DWI has also been applied in detecting various disorders of abdominal organs[12-16]. This report describes the efficacy of DWI in evaluation of acute pancreatitis.

CASE REPORT

A 50-year-old man presented with a primary complaint of epigastric pain after drinking alcohol. Laboratory tests upon admission revealed slightly higher levels of white blood cells (108 × 10^2/mL), C-reactive protein (53.3 mg/L),

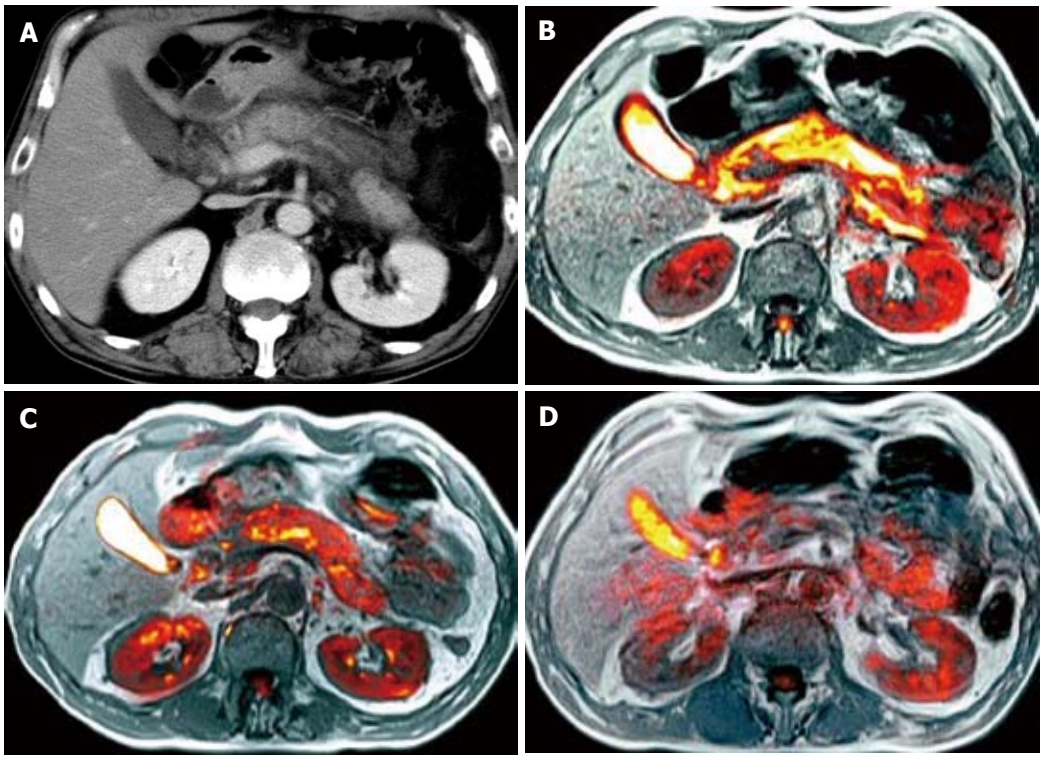


Figure 1 A CT scan at admission revealing enlarged pancreas complicated by acute multiple ascites (**A**), a fusion image at admission (**B**), on days 10 (**C**) and 50 (**D**) showing bright signals in the whole pancreas and ascites around it, diminished pancreatic enlargement and slightly decreased signal-intensity, as well as disappearance of all signs of acute pancreatitis, respectively.

serum amylase (276 IU/L). His APACHI Ⅱ score and Ranson score were 2 and 0, respectively. An enhanced abdominal computed tomography (CT) scan revealed an enlarged pancreas complicated by multiple acute ascites (Figure 1A). An abdominal DWI at 1.5 T (Toshiba; Excelart vantage AGV, screw ratio 130 mT/m per ms) showed bright signals in the whole pancreas and multiple ascites around it (Figure 1B). Furthermore, the apparent diffusion coefficient (ADC) map in that area revealed a reduced ADC value. Following admission, the patient received drip infusion of 300 mg gabexate per day. Seven days after admission, laboratory tests revealed almost normal levels of WBC (54×10^2/mL), CRP (4.1 mg/L) and serum amylase (93 IU/L). Ten days after admission, DWI revealed diminished pancreatic enlargement, slightly decreased signal-intensity (Figure 1C), slightly increased ADC values and disappearance of ascites. His symptoms improved significantly at that time. After 50 d, DWI showed complete disappearance of the manifestations of acute pancreatitis (Figure 1D).

DISCUSSION

Severe acute pancreatitis is often associated with pancreatic necrosis and has a rather high mortality rate. It was reported that necrotizing pancreatitis and inflammatory changes are related to its various complications and prognosis[4-6]. To improve its mortality, it is essential to accurately evaluate the grade of inflammation and the absence of necrotizing pancreatitis. Plane CT can show the changes in inflammation around the pancreas, but cannot detect necrotizing pancreatitis without the use of enhancement material[17]. However, enhancement material has been reported to aggravate acute pancreatitis[18] and it is hard to employ enhanced CT in patients with renal failure due to severe acute pancreatitis. At present, CT is the only available diagnostic imaging method of acute pancreatitis. Clearly, it is urgent to develop new diagnostic strategies for this condition. DWI is a MR imaging technique that provides information about the diffusion of water protons, such as brownian motion in living tissues. DWI has been applied in the diagnosis of brain ischemia and brain tumors[7-11]. Recent technical development in MRI has expanded the utility of DWI in examinations of the liver, kidney, breast, *etc*[12-16]. The apparent diffusion coefficient (ADC) is a quantitative parameter, which reflects the microenvironment of diffusing water molecules. It was reported that reduced ADC is observed in most malignant tumors[8,12,13,15,16]. This present study demonstrated that DWI could detect acute pancreatitis with reduced ADC values at the time of diagnosis. As serum WBC, CRP, and amylase became normal, the signal-intensity and ADC values returned to their normal levels. The decreased ADC value is thought to result from the increased number and size of cells. Therefore, intercellular spaces become smaller, restricting the movement of water molecules[15,16]. If a malignancy is found in abnorm, the ADC value would remain low. These results suggest that inflammation may be closely related to the bright signal. In addition, changed ADC values are useful in differentiating malignant from benign tumors. DWI has a potential to evaluate the manifestations

of acute pancreatitis. Furthermore, the greatest advantage of DWI in diagnosing this condition is that no enhancing material is needed.

In conclusion, DWI is a powerful tool for evaluating acute pancreatitis and has a potential to replace CT as a primary diagnostic strategy for acute pancreatitis.

REFERENCES

1 **Takahashi Y**, Fukushima J, Fukusato T, Shiga J, Tanaka F, Imamura T, Fukayama M, Inoue T, Shimizu S, Mori S. Prevalence of ischemic enterocolitis in patients with acute pancreatitis. *J Gastroenterol* 2005; **40**: 827-832

2 **Brisinda G**, Maria G, Ferrante A, Civello IM. Evaluation of prognostic factors in patients with acute pancreatitis. *Hepatogastroenterology* 1999; **46**: 1990-1997

3 **Lankisch PG**, Assmus C, Pflichthofer D, Struckmann K, Lehnick D. Which etiology causes the most severe acute pancreatitis? *Int J Pancreatol* 1999; **26**: 55-57

4 **Sugimoto M**, Takada T, Yasuda H, Nagashima I, Amano H, Yoshida M, Miura F, Uchida T, Isaka T, Toyota N, Wada K, Takagi K, Kato K. The lethal toxicity of pancreatic ascites fluid in severe acute necrotizing pancreatitis. *Hepatogastroenterology* 2006; **53**: 442-446

5 **Sawa H**, Ueda T, Takeyama Y, Yasuda T, Matsumura N, Nakajima T, Ajiki T, Fujino Y, Suzuki Y, Kuroda Y. Elevation of plasma tissue factor levels in patients with severe acute pancreatitis. *J Gastroenterol* 2006; **41**: 575-581

6 **Ueda T**, Takeyama Y, Yasuda T, Matsumura N, Sawa H, Nakajima T, Ajiki T, Fujino Y, Suzuki Y, Kuroda Y. Significant elevation of serum interleukin-18 levels in patients with acute pancreatitis. *J Gastroenterol* 2006; **41**: 158-165

7 **Seitz RJ**, Meisel S, Weller P, Junghans U, Wittsack HJ, Siebler M. Initial ischemic event: perfusion-weighted MR imaging and apparent diffusion coefficient for stroke evolution. *Radiology* 2005; **237**: 1020-1028

8 **Yamasaki F**, Kurisu K, Satoh K, Arita K, Sugiyama K, Ohtaki M, Takaba J, Tominaga A, Hanaya R, Yoshioka H, Hama S, Ito Y, Kajiwara Y, Yahara K, Saito T, Thohar MA. Apparent diffusion coefficient of human brain tumors at MR imaging. *Radiology* 2005; **235**: 985-991

9 **Stadlbauer A**, Ganslandt O, Buslei R, Hammen T, Gruber S, Moser E, Buchfelder M, Salomonowitz E, Nimsky C. Gliomas: histopathologic evaluation of changes in directionality and magnitude of water diffusion at diffusion-tensor MR imaging. *Radiology* 2006; **240**: 803-810

10 **Provenzale JM**, Mukundan S, Barboriak DP. Diffusion-weighted and perfusion MR imaging for brain tumor characterization and assessment of treatment response. *Radiology* 2006; **239**: 632-649

11 **Higano S**, Yun X, Kumabe T, Watanabe M, Mugikura S, Umetsu A, Sato A, Yamada T, Takahashi S. Malignant astrocytic tumors: clinical importance of apparent diffusion coefficient in prediction of grade and prognosis. *Radiology* 2006; **241**: 839-846

12 **Naganawa S**, Kawai H, Fukatsu H, Sakurai Y, Aoki I, Miura S, Mimura T, Kanazawa H, Ishigaki T. Diffusion-weighted imaging of the liver: technical challenges and prospects for the future. *Magn Reson Med Sci* 2005; **4**: 175-186

13 **Woodhams R**, Matsunaga K, Kan S, Hata H, Ozaki M, Iwabuchi K, Kuranami M, Watanabe M, Hayakawa K. ADC mapping of benign and malignant breast tumors. *Magn Reson Med Sci* 2005; **4**: 35-42

14 **Thoeny HC**, De Keyzer F, Oyen RH, Peeters RR. Diffusion-weighted MR imaging of kidneys in healthy volunteers and patients with parenchymal diseases: initial experience. *Radiology* 2005; **235**: 911-917

15 **Hosonuma T**, Tozaki M, Ichiba N, Sakuma T, Hayashi D, Yanaga K, Fukuda K. Clinical usefulness of diffusion-weighted imaging using low and high b-values to detect rectal cancer. *Magn Reson Med Sci* 2006; **5**: 173-177

16 **Kuroki-Suzuki S**, Kuroki Y, Nasu K, Nawano S, Moriyama N, Okazaki M. Detecting breast cancer with non-contrast MR imaging: combining diffusion-weighted and STIR imaging. *Magn Reson Med Sci* 2007; **6**: 21-27

17 **Larvin M**, Chalmers AG, McMahon MJ. Dynamic contrast enhanced computed tomography: a precise technique for identifying and localising pancreatic necrosis. *BMJ* 1990; **300**: 1425-1428

18 **Carmona-Sanchez R**, Uscanga L, Bezaury-Rivas P, Robles-Diaz G, Suazo-Barahona J, Vargas-Vorackova F. Potential harmful effect of iodinated intravenous contrast medium on the clinical course of mild acute pancreatitis. *Arch Surg* 2000; **135**: 1280-1284

S- Editor Sun YL **L- Editor** Wang XL **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5481

World J Gastroenterol 2008 September 21; 14(35): 5481-5483
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Asymptomatic colonic metastases from primary squamous cell carcinoma of the lung with a positive fecal occult blood test

Shoji Hirasaki, Seiyuu Suzuki, Shigeki Umemura, Haruhito Kamei, Masato Okuda, Kenichiro Kudo

Shoji Hirasaki, Seiyuu Suzuki, Shigeki Umemura, Haruhito Kamei, Masato Okuda, Kenichiro Kudo, Department of Internal Medicine, Sumitomo Besshi Hospital, Niihama 7928543, Japan

Author contributions: Hirasaki S, Suzuki S, Umemura S, Kamei H, Okuda M, Kudo K were involved in the care of the patient; Hirasaki S wrote the paper.

Correspondence to: Shoji Hirasaki, Department of Internal Medicine, Sumitomo Besshi Hospital, 3-1 Ohji-cho, Niihama 7928543, Japan. shoji_hirasaki@ni.sbh.gr.jp

Telephone: +81-897-377111 Fax: +81-897-377121

Received: May 13, 2008 Revised: August 1, 2008
Accepted: August 8, 2008
Published online: September 21, 2008

Abstract

We describe a 74-year-old man with a colonic metastatic squamous cell carcinoma (SCC) from the lung. His chest X-ray revealed an abnormal shadow in the right upper lobe. Computed tomography (CT) of the chest demonstrated a large lung tumor in the right upper lobe obstructing the right upper bronchus. Bronchoscopy revealed an easy-bleeding tumor in the right upper bronchus that was diagnosed as poorly differentiated squamous cell lung carcinoma. He underwent colonoscopy because he had a positive fecal occult blood test. Colonoscopy revealed a large protruding lesion with central ulceration in the descending colon. Histological examination of the biopsy specimen obtained from the colonic lesion revealed SCC. The lesion was diagnosed as metastatic colonic SCC. He had no abdominal symptoms. He underwent chemotherapy with an infusion of cisplatin 130 mg i.v. day 1, and docetaxel hydrate 100 mg i.v. day 1, repeated every 4 wk, followed by 4 courses of chemotherapy. The primary lesion shrank by less than 10% and was judged to be "Partial Response" (PR) after 3 courses of treatment. The patient still lived 23 wk after the diagnosis of metastatic colonic SCC. Colonic metastasis of primary SCC of the lung is rare.

Key words: Gastrointestinal metastatic tumor; Colonic metastasis; Large intestine; Colonoscopy; Chemotherapy

Peer reviewer: Zvi Fireman, Professor, Department of Gastroenterology, Hillel-yaffe Med. Ctr., POB 169, Gastroenterology Department, Hadera 38100, Israel

Hirasaki S, Suzuki S, Umemura S, Kamei H, Okuda M, Kudo K. Asymptomatic colonic metastases from primary squamous cell carcinoma of the lung with a positive fecal occult blood test. *World J Gastroenterol* 2008; 14(35): 5481-5483 Available from: URL: http://www.wjgnet.com/1007-9327/14/5481.asp DOI: http://dx.doi.org/10.3748/wjg.14.5481

INTRODUCTION

Primary lung cancer is a common neoplasm, and frequently metastasizes to internal organs such as the lung, liver and adrenal gland; however, it is relatively rare for lung cancer to metastasize to the gastrointestinal (GI) tract. Colonic metastasis from primary carcinoma of the lung has rarely been described. Clinically, patients may present with symptoms of colonic obstruction, lower GI bleeding, bowel perforation, or GI fistula[1-4]. Herein, we describe a rare case of metastatic colonic squamous cell carcinoma (SCC) from the lung.

CASE REPORT

A 74-year-old man presented with the symptom of shoulder pain. His chest X-ray revealed an abnormal shadow in the right upper lobe (Figure 1A). He was in good health with no specific family or past medical history. His body temperature was 36.7℃, blood pressure was 148/82 mmHg, radial pulse rate was 72 beats/min and regular. He had anemia, but no jaundice. Neurological examination revealed no abnormal findings or lymphadenopathy. Abdominal palpation revealed tenderness in the left lower quadrant. Laboratory tests showed a red blood cell count of $318 \times 10^4/\mu L$ [normal range (NR), $430\text{-}570 \times 10^4/\mu L$], a white blood cell count of 7600 /μL, and a platelet count of $30.8 \times 10^4/\mu L$, and a hemoglobin concentration of 8.9 g/dL (NR, 14-18 g/dL). Liver function tests were normal except for lactate dehydrogenase (LDH) of 340 IU/L (NR, 106-211 IU/L). A test for C reactive protein revealed a level of 0.8 mg/dL (NR, < 0.5 mg/dL). Renal function tests showed that blood urea nitrogen

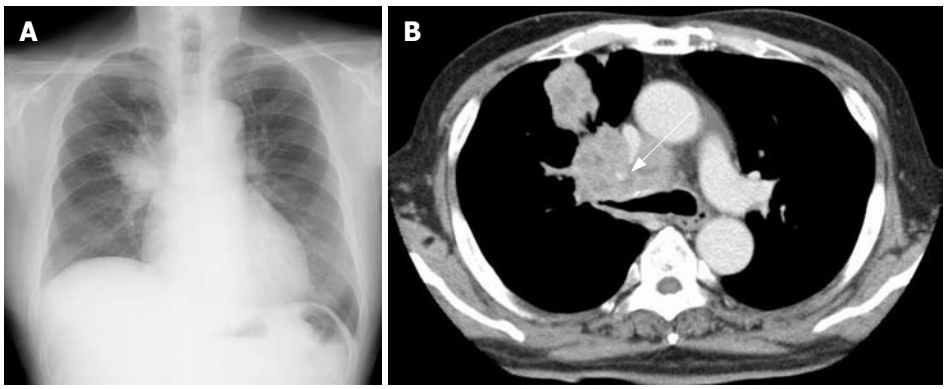


Figure 1 **A**: Chest X-ray revealed a large abnormal shadow in the right upper lobe; **B**: A computed tomography (CT) of the chest demonstrated a large lung tumor in the right upper lobe obstructing the right upper bronchus.

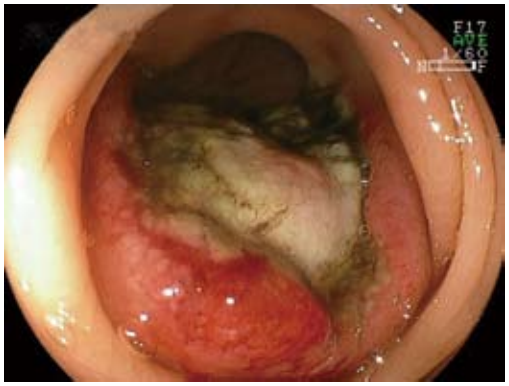


Figure 2 Endoscopic appearance of the descending colon. A large protruding lesion with central ulceration, about 40 mm in diameter, was seen.

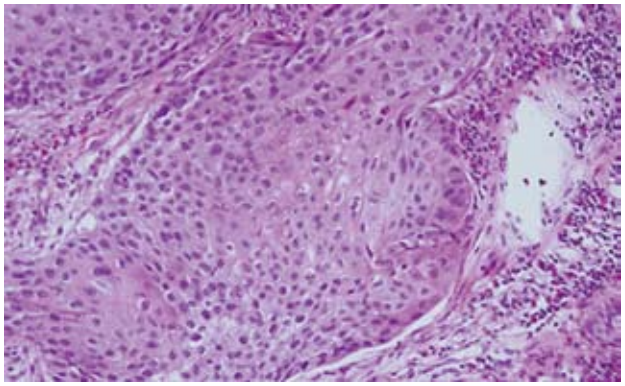


Figure 3 Histological examination of the biopsy specimen obtained from the colonic protruding lesion revealed tumor cells diagnosed as SCC (× 50).

and creatinine were normal. Computed tomography (CT) of the chest demonstrated a large lung tumor in the right upper lobe obstructing the right upper bronchus (Figure 1B). Bronchoscopy revealed an easy-bleeding tumor in the right upper bronchus. Poorly differentiated SCC was diagnosed by punch biopsy.

Colonoscopy revealed a large protruding lesion with central ulceration in the descending colon (Figure 2). This finding suggested a clinical diagnosis of metastatic colonic tumor. Biopsy specimens obtained from the lesion showed SCC (Figure 3). Based on these findings, the patient was diagnosed with metastatic colonic SCC from primary lung cancer. Subsequent positron emission tomography (PET) with radiolabeled-18 fluorine fluorodeoxyglucose (FDG) imaging revealed an abnormal lesion in the descending colon. The patient underwent chemotherapy with an infusion of cisplatin 130 mg i.v. day 1, and docetaxel hydrate 100 mg i.v. day 1, repeated every 4 wk, followed by 4 courses of chemotherapy. The primary lesion shrank by less than 10% and was judged to be "Partial Response" (PR) after 3 courses of treatment. The patient still lived 23 wk after the diagnosis of metastatic colonic SCC.

DISCUSSION

GI metastasis from lung cancer is considered to be rare, although there is about 4.7%-14% prevalence at autopsy[5-7]. A recent report described by Kim *et al*[8] revealed that GI metastases were detected in 10 (0.19%) of 5239 lung cancer patients. In an autopsy study from Japan, the rate of GI tract metastasis, excluding the esophagus, was 1.8% and the colonic metastasis rate was only 0.5%[6]. The histological type of lung cancer that causes colonic metastasis varies according to different studies. The most common types were large cell or SCC[5,9,10]. We believe that it is hard to pinpoint the type of primary lung cancer causing colon metastasis because of the small number of cases. Reviewing the English literature, there are several reports on colonic metastasis[11-14]. Patients with GI metastasis of lung cancer are often asymptomatic, as in the present case. Yang *et al*[14] reported that the clinical prevalence of symptomatic GI metastasis of lung cancer is 1.77% (6/339). Habesoglu *et al* described that about 1/3 of colonic metastases from lung cancer are asymptomatic and the diagnosis is made at autopsy[4]. The most common symptoms are abdominal pain, nausea, vomiting, anemia, and weight loss[1,5]. Other symptoms of colonic obstruction, lower GI bleeding, bowel perforation, or GI fistula may occur[1-4]. These findings generally present after the diagnosis of primary disease but can occur synchronously or before diagnosis of the primary lesion[1,4,10].

Lung cancer with intestinal metastasis has been reported to have a poor prognosis of less than 16 wk in several studies[4,6,12,14]; however, because of advanced improvement in chemotherapy, supportive care for lung cancer, and extending life expectancy, we may come across an increasing number of GI metastasis in the future.

Thus, we should pay attention to GI metastatic signs such as GI bleeding, abdominal pain, nausea, vomiting, or less commonly, ileus. Development of GI symptoms after chemotherapy should be carefully managed in patients with GI metastasis because of the possibility of chemotherapy-induced perforation. The present patient underwent colonoscopy for further evaluation of a positive fecal occult blood test, and a metastatic colonic lesion was detected before chemotherapy. He has been receiving chemotherapy without chemotherapy-induced perforation or abdominal symptoms. If we aggressively examine lung cancer patients with a positive fecal occult blood test by colonoscopy, more latent patients with metastatic colonic tumor from the lung might be discovered in the future.

Recently, PET-FDG imaging has become useful to diagnose the extent of GI metastasis[13]. Cases of small intestinal[15] and large intestinal metastasis[13] from lung cancer have been reported. In the present case, we could also detect colonic metastatic lesions with PET-FDG; however, the definite role of PET-FDG in the diagnosis of GI metastasis from primary lung cancer is still controversial because of the few cases and lack of clinical data. To clarify the usefulness of PET-FDG for the diagnosis of GI metastasis from lung cancer, we should accumulate and analyze more cases of GI metastasis from primary lung cancer.

In conclusion, we reported a rare case of metastatic colonic SCC from the lung with a positive fecal occult blood test. It is necessary to be aware that primary SCC of the lung may cause colonic metastasis, although lung SCC rarely causes colonic metastasis.

REFERENCES

1 **John AK**, Kotru A, Pearson HJ. Colonic metastasis from bronchogenic carcinoma presenting as pancolitis. *J Postgrad Med* 2002; **48**: 199-200

2 **Burbige EJ**, Radigan JJ, Belber JP. Metastatic lung carcinoma involving the gastrointestinal tract. *Am J Gastroenterol* 1980; **74**: 504-506

3 **Gateley CA**, Lewis WG, Sturdy DE. Massive lower gastrointestinal haemorrhage secondary to metastatic squamous cell carcinoma of the lung. *Br J Clin Pract* 1993; **47**: 276-277

4 **Habesoglu MA**, Oguzulgen KI, Ozturk C, Akyurek N, Memis L. A case of bronchogenic carcinoma presenting with acute abdomen. *Tuberk Toraks* 2005; **53**: 280-283

5 **Antler AS**, Ough Y, Pitchumoni CS, Davidian M, Thelmo W. Gastrointestinal metastases from malignant tumors of the lung. *Cancer* 1982; **49**: 170-172

6 **McNeill PM**, Wagman LD, Neifeld JP. Small bowel metastases from primary carcinoma of the lung. *Cancer* 1987; **59**: 1486-1489

7 **Rossi G**, Marchioni A, Romagnani E, Bertolini F, Longo L, Cavazza A, Barbieri F. Primary lung cancer presenting with gastrointestinal tract involvement: clinicopathologic and immunohistochemical features in a series of 18 consecutive cases. *J Thorac Oncol* 2007; **2**: 115-120

8 **Kim MS**, Kook EH, Ahn SH, Jeon SY, Yoon JH, Han MS, Kim CH, Lee JC. Gastrointestinal metastasis of lung cancer with special emphasis on a long-term survivor after operation. *J Cancer Res Clin Oncol* 2008; In press

9 **Ryo H**, Sakai H, Ikeda T, Hibino S, Goto I, Yoneda S, Noguchi Y. [Gastrointestinal metastasis from lung cancer] *Nihon Kyobu Shikkan Gakkai Zasshi* 1996; **34**: 968-972

10 **Gitt SM**, Flint P, Fredell CH, Schmitz GL. Bowel perforation due to metastatic lung cancer. *J Surg Oncol* 1992; **51**: 287-291

11 **Carroll D**, Rajesh PB. Colonic metastases from primary squamous cell carcinoma of the lung. *Eur J Cardiothorac Surg* 2001; **19**: 719-720

12 **Rouhanimanesh Y**, Vanderstighelen Y, Vanderputte S, Cools P, Wassenaar H, Vallaeys J. Intra-abdominal metastases from primary carcinoma of the lung. *Acta Chir Belg* 2001; **101**: 300-303

13 **Stinchcombe TE**, Socinski MA, Gangarosa LM, Khandani AH. Lung cancer presenting with a solitary colon metastasis detected on positron emission tomography scan. *J Clin Oncol* 2006; **24**: 4939-4940

14 **Yang CJ**, Hwang JJ, Kang WY, Chong IW, Wang TH, Sheu CC, Tsai JR, Huang MS. Gastro-intestinal metastasis of primary lung carcinoma: clinical presentations and outcome. *Lung Cancer* 2006; **54**: 319-323

15 **Shiono S**, Masaoka T, Sato T, Yanagawa N. [Positron emission tomography (PET)-computed tomography (CT) suggesting small intestinal metastasis from lung cancer; report of a case] *Kyobu Geka* 2006; **59**: 426-429

S- Editor Li DL **L- Editor** Ma JY **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2008 September 21; 14(35): 5484
World Journal of Gastroenterology ISSN 1007-9327

ACKNOWLEDGMENTS

Acknowledgments to Reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

Kyoichi Adachi, MD
Department of Gastroenterology and Hepatology, Shimane University, School of Medicine Shimane, 89-1 Enya-cho, Izumo-shi Shimane 693-8501, Japan

Minoti V Apte, Associate Professor
Pancreatic Research Group, South Western Sydney Clinical School, The University of New South Wales. Liverpool, NSW 2170, Australia

Masahiro Arai, MD, PhD
Department of Gastroenterology, Toshiba General Hospital, 6-3-22 Higashi-ooi, Shinagawa-ku,Tokyo 140-8522, Japan

Einar S Björnsson, Professor
Department of Internal Medicine, Section of Gastroenterology and Hepatology, Sahlgrenska University hospital, Med pol Ⅱ, SE-413 45 Gothenburg, Sweden

Herbert L Bonkovsky, MD, Vice president for Research
Research and The hepato-Biliary-Pancreatic Ce, Carolinas Medical Center, Cannon Research Bldg, Suite 201, 1000 Blythe Blvd, Charlotte, NC 28203, United States

Dr. Yogesh K Chawla, Professor
Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

Dario Conte, Professor
GI Unit-IRCCS Osp. Maggiore, Chair of Gastroenterology, Via F. Sforza, 35, Milano 20122, Italy

Dr. Adrian G Cummins
Department of Gastroenterology and Hepatology, (DX 465384), 28 Woodville Road, Woodville South, 5011, South Australia, Australia

Arno J Dormann, PD, MED, Habil
Medizinische Klinik, Krankenhaus Holweide, Kliniken der Stadt Köln gGmbH, Neufelder St. 32, 51067 Köln, Germany

Hugh J Freeman, Professor
Department of Medicine, University of British Columbia, UBC Hospital2211 Wesbrook Mall, Vancouver, BC V6T 1W5, Canada

Xin-Yuan Guan, Professor
Department of Clinical Oncology, The University of Hong Kong, Room L10-56, 10/F, Laboratory Block, 21 Sassoon Road, Pokfulam, Hong Kong, China

Maria Concepción Gutiérrez-Ruiz, PhD
Departamento de Ciencias de la Salud, Universidad Autónoma Metropolitana-Iztapalapa, DCBS, Av San Rafael Atlixco 186, Colonia Vicentina, México, DF 09340, México

Eva Herrmann, Professor
Department of Internal Medicine, Biomathematics Saarland University, Faculty of Medicine, Kirrberger Str., 66421 Homburg/Saar, Germany

Yik-Hong Ho, Professor
Department of Surgery, School of Medicine, James Cook University, Townsville 4811, Australia

Dr. Milan Jirsa
Laboratory of Experimental Medicine-building Z1,Institute for Clinical and Experimental Medicine, Videnska 1958/9, Praha 4, 14000, Czech

Ioannis E Koutroubakis, MD, PhD, Assistant Professor of Medicine
University Hospital Heraklion, Department of Gastroenterology, PO Box 1352, 71110 Heraklion, Crete, Greece

James David Luketich, MD, Professor and Chief
Division of Thoracic and Foregut Surgery University of Pittsburgh Medical Center Pittsburgh, PA 15213, United States

Wendy M Mars, PhD
Department of Pathology, University of Pittsburgh, S-411B South Biomedical Science Tower Pittsburgh, PA 15261, United States

Nahum Méndez-Sánchez, MD, PhD
Departments of Biomedical Research, Gastroenterology & Liver Unit, Medica Sur Clinic & Foundation, Puente de Piedra 150, Col. Toriello Guerra, Tlalpan 14050, México, City, México

Ibrahim A Al Mofleh, Professor
Deaprtment of Medicine, College of Medicine, King Saud University, PO Box 2925, Riyadh 11461, Saudi Arabia

Kostas Pantopoulos, Associate Professor
Department of Medicine, McGill University, Lady Davis Institute for Medical Research, 3755 Cote Ste-Catherine Road, Montreal, Quebec, H3T 1E2, Canada

Maurizio Parola, Professor
Department Medicina e Oncologia Sperimentale University of Torino Corso Raffaello 30, 10125 Torino, Italy

Dr. Richard A Rippe
Department of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7038, United States

Ian C Roberts-Thomson, Professor
Department of Gastroenterology and Hepatology, The Queen Elizabeth Hospital, 28 Woodville Road, Woodville South 5011, Australia

Dr. Shawn David Safford
Department of Surgery, Duke University Medical Center, 994 West Ocean View Avenue, Norfolk VA23503, United States

Riina Salupere, MD, PhD
Division of Endocrinology and Gastroenterology, University of Tartu, L. Puusepa street 6, Tartu 51014, Estonia

Henning Schulze-Bergkamen, MD
Henning Schulze-Bergkamen, First Medical Department, University of Mainz, Langenbeckstr, 1, 55101 Mainz, Germany

Steven D, MD Wexner, Professor of Surgery
The Cleveland Clinic Foundation Health Sciences Center of the Ohio State University, and Clinical Professor, Department of Surgery, Division of General Surgery, University of South Florida College of Medicine, 21st Century Oncology Chair in Colorectal Surgery, Chairman Department of Colorectal Surgery, Chief of Staff, Cleveland Clinic Florida, 2950 Cleveland Clinic Boulevard, Weston, Florida 33331, United States

Dr. Stefan Wirth, Professor
Children's Hospital, Heusnerstt. 40, Wuppertal 42349, Germany

Jia-Yu Xu, Professor
Ruijin Hospital, Shanghai Jiaotong University School of Medicine, 197 Rui Jin Er Road, Shanghai 200025, China

Özlem Yilmaz, PhD, Associate Professor of Microbiology
Dokuz Eylul University, School of Medicine, Department of Microbiology and Clinical Microbiology, Inciralti 35340, Izmir, Turkey

Yuan Yuan, Professor
Cancer Institute of China Medical University, 155 North Nanjing Street, Heping District, Shenyang 110001, Liaoning Province, China

Silvana Zanlungo, Professor
Departamento de Gastroenterología, Pontificia Universidad Católica de Chile, Marcoleta 367, Casilla 114-D, Santiago, Chile

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2008 September 21; 14(35): 5485
World Journal of Gastroenterology ISSN 1007-9327

Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
January 24-25, Frankfurt, Germany
Falk Workshop: Perspectives in Liver
Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
E-mail: education@asge.org

June 4-7, Helsinki, Finland
The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
ESGAR 2008 19th Annual Meeting and Postgraduate Course
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden
16th International Congress of the European Association for Endoscopic Surgery
E-mail: info@eaes-eur.org

June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
10th World Congress on Gastrointestinal Cancer
Imedex and ESMO
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland
Joint Meeting of the European Pancreatic Club (EPC)
and the International Association of Pancreatology (IAP)
E-mail: office@epc-iap2008.org
www.e-p-c.org
www.pancreatology.org

June 26-28, Bratislava, Slovakia
5th Central European Gastroenterology Meeting
www.ceurgem2008.cz

July 9-12, Paris, France
ILTS 14th Annual International Congress
www.ilts.org

September 10-13, Budapest, Hungary
11th World Congress of the International Society for Diseases of the Esophagus
E-mail: isde@isde.net

September 13-16, New Delhi, India
Asia Pacific Digestive Week
E-mail: apdw@apdw2008.net

APDW 2008
September 13-16, New Delhi, Indian
Organized: Indian Society of Gastroenterology

III FALK GASTRO-CONFERENCE
September 17, Mainz, Germany
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
Falk Symposium 166:
GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
Falk Symposium 167:
Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
Third Annual Meeting
European Society of Coloproctology
www.escp.eu.com

October 8-11, Istanbul, Turkey
18th World Congress of the International Association of Surgeons,
Gastroenterologists and Oncologists
E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria
16th United European Gastroenterology Week
www.negf.org
www.acv.at

October 22-25, Minnesota, USA
Anstralian Gastroenterology Week 2008
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia
71st Annual Colon and Rectal Surgery Conference
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA
59th AASLD Annual Meeting and Postgraduate Course
The Liver Meeting
Information: www.aasld.org

November 6-9, Lucerne, Switzerland
Neurogastroenterology & Motility Joint International Meeting 2008
E-mail: ngm2008@mci-group.com
www.ngm2008.com

November 12, Santiago de Chile, Chile
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt
1st Hepatology and Gastroenterology Post Graduate Course
www.egyptgastrohep.com

December 7-9, Seoul, Korea
6th International Meeting
Hepatocellular Carcinoma: Eastern and Western Experiences
E-mail: sglee@amc.seoul.kr

INFORMATION FOR ALL
FALK FOUNDATION e.V.
E-mail: symposia@falkfoundation.de
www.falkfoundation.de

Advanced Courses - European Institute of Telesurgery EITS - 2008
Strasbourg, France
January 18-19, March 28-29, June 6-7, October 3-4

N.O.T.E.S
April 3-5, November 27-29
Laparoscopic Digestive Surgery

June 27-28, November 7-8
Laparoscopic Colorectal Surgery

July 3-5
Interventional GI Endoscopy Techniques
Contact address for all courses:
E-mail: info@eits.fr

International Gastroenterological Congresses 2009
March 23-26, Glasgow, Scotland
Meeting of the British Society of Gastroenterology (BSG)
E-mail: bsg@mailbox.ulcc.ac.uk

May 17-20, Denver, Colorado, USA
Digestive Disease Week 2009

November 21-25, London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2008 September 21; 14(35): 5486-5488
World Journal of Gastroenterology ISSN 1007-9327

Instructions to authors

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol* ISSN 1007-9327 CN 14-1219/R) is a weekly open access peer-reviewed journal supported by an editorial board consisting of 1208 experts in gastroenterology and hepatology from 60 countries. The aim of the journal is to deliver the most clinically relevant original and commentary articles to readers, and to make the full text publicly available to all clinicians, scientists, patients and biomedical students on an unrestricted platform, so that they can access and learn about the most recent key advances in the field.

In addition to the open access nature, another key characteristic of *WJG* is its reading guidance for each article which includes background, research frontier, related reports, breakthroughs, applications, terminology, and comments of peer reviewers for the general readers.

WJG publishes articles on esophageal, gastrointestinal, hepatobiliary and pancreatic tumors, and other esophageal, gastrointestinal, hepatic-biliary and pancreatic diseases in relation to epidermiology, immunology, microbiology, motility & nerve-gut interaction, endocrinology, nutrition & obesity, endoscopy, imaging and advanced hi-technology.

The main goal of *WJG* is to publish high quality commentary articles contributed by leading experts in gastroenterology and hepatology and original articles that combine the clinical practice and advanced basic research, to provide an interactive platform for clinicians and researchers in internal medicine, surgery, infectious diseases, traditional Chinese medicine, oncology, integrated Chinese and Western medicine, imaging, endoscopy, interventional therapy, pathology and other basic medical specialities, and thus eventually improving the clinical practice and healthcare for patients.

Indexed and abstracted in

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, Index Medicus, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology* and *Hepatology*, CAB Abstracts and Global Health. ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

Published by

The WJG Press

SUBMISSION OF MANUSCRIPTS

Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of The WJG Press, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the International Committee of Medical Journal Editors to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now available, to our knowledge, is http://www. clinicaltrials.gov sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

Authors should retain one copy of the text, tables, photographs and illustrations because rejected manuscripts will not be returned to the author(s) and the editors will not be responsible for loss or damage to photographs and illustrations sustained during mailing.

Online submissions

Manuscripts should be submitted through the Online Submission System at: http://wjg.wjgnet.com/wjg. Authors are highly recommended to consult the ONLINE INSTRUCTIONS TO AUTHORS (http://www.wjgnet.com/wjg/help/instructions.jsp) before attempting to submit online. For assistance, authors encountering problems with the Online Submission System may send an email describing the problem to submission@wjgnet.com, or by telephone: +86-10-85381892. If you submit your manuscript online, do not make a postal contribution. Repeated online submission for the same manuscript is strictly prohibited.

MANUSCRIPT PREPARATION

All contributions should be written in English. All articles must be submitted using word-processing software. All submissions must be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Style should conform to our house format. Required information for each of the manuscript sections is as follows:

Title page

Full manuscript title, running title, all author(s) name(s), affiliations, institution(s) and/or department(s) where the work was carried out; author contributions; disclosure of any financial support for the research; and the name, full address, telephone and fax numbers and email address of the corresponding author should be included. Titles should be concise and informative (remove all unnecessary words), emphasize what is new, and avoid abbreviations. A short running title of less than 40 letters should be provided. List the author(s)' name(s) as follows: initial and/or first name, middle name or initial(s), and full family name.

Author controbutions: The format of this section should be like this: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed research; Wang CL, Zou CC, Hong F and Wu XM performed research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed data; and Wang CL, Liang L and Fu JF wrote the paper.

Peer reviewers: All articles received are subject to peer review. Normally, three experts are invited for each article. Decision for acceptance is made only when at least two experts recommend an article for publication. Reviewers for accepted manuscripts are acknowledged in each manuscript, and reviewers of articles which were not accepted will be acknowledged at the end of each issue. To ensure the quality of the articles published in *WJG*, reviewers of accepted manuscripts will be announced by publishing the name, title/position and institution of the reviewer in the footnote accompanying the printed article. For example, reviewers: Professor Jing-Yuan Fang, Shanghai Institute of Digestive Disease, Shanghai, Affiliated Renji Hospital, Medical Faculty, Shanghai Jiaotong University, Shanghai, China; Professor Xin-Wei Han, Department of Radiology, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan Province, China; and Professor Anren Kuang, Department of Nuclear Medicine, Huaxi Hospital, Sichuan University, Chengdu, Sichuan Province, China.

Abstract

An informative, structured abstract of no more than 350 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections: AIM: Only the purpose should be included. METHODS: The materials, techniques, instruments and equipment, and the experimental procedures should be included. RESULTS: The observed and experimental results, including data, effects, outcome, *etc.* should be included. Authors should present *P* value where necessary, and also include any significant data. CONCLUSION: Accurate view and the value of the results should be included.

The format for structured abstracts can be found at: http://www.wjgnet.com/wjg/help/11.doc.

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles, rapid communication

and case reports, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the body text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, should be found at: http://www.wjgnet.com/wjg/help/instructions.jsp.

Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: …*etc.* It is our principle to publish high resolution-figures for the printed and E-versions.

Tables

Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of P values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscripts and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

Coding system

The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability[1,2]". If references are cited directly in the text, they should be put together within the text, for example, "From references[19,22-24], we know that..."

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

PMID requirement

PMID roots in the abstract serial number indexed by PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed). The author should supply the PMID for journal citation. For those references that have not been indexed by PubMed, a printed copy of the first page of the full reference should be submitted.

The accuracy of the information for journal citations is very important. Using the reference testing system, the authors and editor should check the authors name, title, journal title, publication date, volume number, start page, and end page. We will interlink all references with PubMed in an ASP file so that the readers can immediately access the abstract of the citations online.

DOI requirement

A CrossRef DOI® (Digital Object Identifier) name is a unique string created to identify a piece of scholarly content in the online environment. The author should supply the DOIs for journal citation(doi:10.3748/wjg.13.6458). This link (http://www.crossref.org/SimpleTextQuery/) allows you to retrieve Digital Object Identifiers (DOIs) for journal articles, books, and chapters by simply cutting and pasting the reference list into the box. You may use the form with any reference style, although the tool works most reliably if references are formatted in a standard style such as shown in this example: Assimakopoulos SF, Scopa CD, Vagianos CE. Pathophysiology of increased intestinal permeability in obstructive jaundice. *World J Gastroenterol* 2007; 13(48): 6458-6464

The accuracy of the information of journal citations is very important. We will interlink all references with DOI in ASP file so that readers can access the abstracts of cited articles online immediately.

Style for journal references

Authors: the name of the first author should be typed in bold-faced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

Style for book references

Authors: the name of the first author should be typed in bold-faced letters. The surname of all authors should be typed with the initial letter capitalized, followed by their abbreviated middle and first initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR) Book title. Publication number. Publication place: Publication press, Year: start page and end page.

Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Inappropriate references

Authors should always cite references that are relevant to their article, and avoid any inappropriate references. Inappropriate references include those linked with a hyphen when the difference between the two numbers is greater than five. For example, [1-6], [2-14] and [1, 3, 4-10, 22] are all considered inappropriate references. Authors should not cite their own unrelated published articles.

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/wjg/help/15.doc.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H pylori*, *E coli*, *etc.*

SUBMISSION OF THE REVISED MANUSCRIPTS AFTER ACCEPTED

Please revise your article according to the revision policies of *WJG*. The revised version including manuscript and high-resolution image figures (if any) should be copied on a floppy or compact disk. The author should send the revised manuscript, along with printed high-resolution color or black and white photos, copyright transfer letter, and responses to the reviewers by courier (such as EMS/DHL).

Editorial Office

World Journal of Gastroenterology
Editorial Department: Room 903
Ocean International Center, Building D
No. 62 Dongsihuan Zhonglu
Chaoyang District, Beijing 100025, China
E-mail: wjg@wjgnet.com
http://www.wjgnet.com
Telephone: +86-10-59080039
Fax: +86-10-85381893

Language evaluation

The language of a manuscript will be graded before it is sent for revision. (1) Grade A: priority publishing; (2) Grade B: minor language polishing; (3) Grade C: a great deal of language polishing needed; (4) Grade D: rejected. Revised articles should reach Grade A or B.

Copyright assignment form

Please download a Copyright assignment form from http://www.wjgnet.com/wjg/help/9.doc.

Responses to reviewers

Please revise your article according to the comments/suggestions provided by the reviewers. The format for responses to the reviewers' comments can be found at: http://www.wjgnet.com/wjg/help/10.doc.

Proof of financial support

For paper supported by a foundation, authors should provide a copy of the document and serial number of the foundation.

Links to documents related to the manuscript

WJG will be initiating a platform to promote dynamic interactions between the editors, peer reviewers, readers and authors. After a manuscript is published online, links to the PDF version of the submitted manuscript, the peer-reviewers' report and the revised manuscript will be put on-line. Readers can make comments on the peer reviewer's report, authors' responses to peer reviewers, and the revised manuscript. We hope that authors will benefit from this feedback and be able to revise the manuscript accordingly in a timely manner.

Science news releases

Authors of accepted manuscripts are suggested to write a science news item to promote their articles. The news will be released rapidly at EurekAlert/AAAS (http://www.eurekalert.org). The title for news items should be less than 90 characters; the summary should be less than 75 words; and main body less than 500 words. Science news items should be lawful, ethical, and strictly based on your original content with an attractive title and interesting pictures.

Publication fee

Authors of accepted articles must pay a publication fee.
EDITORIAL, TOPIC HIGHLIGHTS, BOOK REVIEWS and LETTERS TO THE EDITOR are published free of charge.

ISSN 1007-9327
CN 14-1219/R

World Journal of Gastroenterology®

Indexed and Abstracted in:

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, *Index Medicus*, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology and Hepatology*, CAB Abstracts and Global Health.
ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

Volume 14 Number 36
September 28, 2008
World J Gastroenterol
2008 September 28; 14(36): 5489-5616

Online Submissions
wjg.wjgnet.com
www.wjgnet.com
Printed on Acid-free Paper
世界胃肠病学杂志

A Weekly Journal of Gastroenterology and Hepatology

World Journal of Gastroenterology®

Editorial Board

2007-2009

Published by The WJG Press and Baishideng
Room 903, Ocean International Center, Building D
No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Fax: +86-10-8538-1893 E-mail: wjg@wjgnet.com http://www.wjgnet.com

The World Journal of Gastroenterology Editorial Board consists of 1208 members, representing a team of worldwide experts in gastroenterology and hepatology. They are from 60 countries, including Albania (1), Argentina (4), Australia (39), Austria (10), Belarus (1), Belgium (15), Brazil (2), Bulgaria (1), Canada (28), Chile (1), China (60), Croatia (2), Cuba (1), Czech (2), Denmark (7), Egypt (4), Estonia (1), Finland (4), France (44), Germany (108), Greece (9), Hungary (2), Iceland (1), India (12), Iran (3), Ireland (4), Israel (8), Italy (96), Japan (176), Lebanon (3), Lithuania (1), Macedonia (1), Malaysia (3), Mexico (6), Monaco (1), Morocco (1), The Netherlands (26), New Zealand (1), Nigeria (1), Norway (3), Pakistan (2), Peru (1), Poland (6), Portugal (1), Russia (3), Saudi Arabia (2), Serbia (1), Singapore (4), Slovakia (2), Slovenia (1), South Africa (2), South Korea (14), Spain (38), Sweden (15), Switzerland (13), Turkey (8), United Arab Emirates (1), United Kingdom (83), United States (316) and Uruguay (2).

Andrew V Biankin, *Sydney*
Filip Braet, *Sydney*
Andrew D Clouston, *Sydney*
Graham Cooksley, *Queensland*
Darrell HG Crawford, *Brisbane*
Adrian G Cummins, *Woodville South*
Guy D Eslick, *Sydney*
Michael A Fink, *Melbourne*
Robert JL Fraser, *Daw Park*
Peter Raymond Gibson, *Victoria*
Jacob George, *Westmead*
Mark D Gorrell, *Sydney*
Yik-Hong Ho, *Townsville*
Gerald J Holtmann, *Adelaide*
Michael Horowitz, *Adelaide*
John E Kellow, *Sydney*
Rupert Leong, *Concord*
Geoffrey W McCaughan, *Sydney*
Finlay A Macrae, *Victoria*
Daniel Markovich, *Brisbane*
Phillip S Oates, *Perth*
Jacqui Richmond, *Victoria*
Stephen M Riordan, *Sydney*
Ian C Roberts-Thomson, *Adelaide*
Devanshi Seth, *Camperdown*
Arthur Shulkes, *Melbourne*
Ross C Smith, *Sydney*
Kevin J Spring, *Brisbane*
Huy A Tran, *New South Wales*
Debbie Trinder, *Fremantle*
Martin J Veysey, *Gosford*
Daniel L Worthley, *Bedford*

Austria

Peter Ferenci, *Vienna*
Valentin Fuhrmann, *Vienna*
Alfred Gangl, *Vienna*
Christoph Gasche, *Vienna*
Kurt Lenz, *Linz*
Markus Peck-Radosavljevic, *Vienna*
Rudolf E Stauber, *Auenbruggerplatz*
Herbert Tilg, *Innsbruck*
Michael Trauner, *Graz*
Harald Vogelsang, *Vienna*
Guenter Weiss, *Innsbruck*

Belarus

Yury K Marakhouski, *Minsk*

Belgium

Rudi Beyaert, *Gent*
Bart Rik De Geest, *Leuven*
Inge I Depoortere, *Leuven*
Olivier Detry, *Liège*
Benedicte Y De Winter, *Antwerp*
Karel Geboes, *Leuven*
Thierry Gustot, *Brussels*
Yves J Horsmans, *Brussels*
Geert G Leroux-Roels, *Ghent*
Louis Libbrecht, *Leuven*
Etienne M Sokal, *Brussels*
Marc Peeters, *De Pintelaan*
Gert A Van Assche, *Leuven*
Yvan Vandenplas, *Brussels*
Eddie Wisse, *Keerbergen*

Brazil

Heitor Rosa, *Goiania*
Ana Cristina Simões e Silva, *Belo Horizonte*

Bulgaria

Zahariy Krastev, *Sofia*

Canada

Fernando Alvarez, *Québec*
David Armstrong, *Ontario*
Jeffrey P Baker, *Toronto*
Olivier Barbier, *Québec*
Nancy Baxter, *Toronto*
Matthew Bjerknes, *Toronto*
Frank J Burczynski, *Manitoba*
Michael F Byrne, *Vancouver*
Wang-Xue Chen, *Ottawa*
Chantal Guillemette, *Québec*
Samuel S Lee, *Calgary*
Gary A Levy, *Toronto*
Andrew L Mason, *Alberta*
John K Marshall, *Ontario*
Donna-Marie McCafferty, *Calgary*
Thomas I Michalak, *St. John's*
Gerald Y Minuk, *Manitoba*
Paul Moayyedi, *Hamilton*
Kostas Pantopoulos, *Québec*
William G Paterson, *Kingston*
Eldon Shaffer, *Calgary*
Morris Sherman, *Toronto*
Martin Storr, *Calgary*
Elena F Verdu, *Ontario*
John L Wallace, *Calgary*
Eric M Yoshida, *Vancouver*

Chile

Silvana Zanlungo, *Santiago*

China

Henry LY Chan, *Hongkong*
Xiao-Ping Chen, *Wuhan*
Zong-Jie Cui, *Beijing*
Da-Jun Deng, *Beijing*
Er-Dan Dong, *Beijing*
Sheung-Tat Fan, *Hong Kong*
Jin Gu, *Beijing*
Xin-Yuan Guan, *Pokfulam*
De-Wu Han, *Taiyuan*
Ming-Liang He, *Hong Kong*
Wayne HC Hu, *Hong Kong*
Chee-Kin Hui, *Hong Kong*
Ching-Lung Lai, *Hong Kong*
Kam Chuen Lai, *Hong Kong*
James YW Lau, *Hong Kong*
Yuk-Tong Lee, *Hong Kong*
Suet-Yi Leung, *Hong Kong*
Wai-Keung Leung, *Hong Kong*
John M Luk, *Pokfulam*
Chung-Mau Lo, *Hong Kong*
Jing-Yun Ma, *Beijing*
Ronnie Tung Ping Poon, *Hong Kong*
Lun-Xiu Qin, *Shanghai*
Yu-Gang Song, *Guangzhou*
Qin Su, *Beijing*
Wai-Man Wong, *Hong Kong*
Hong Xiao, *Shanghai*
Dong-Liang Yang, *Wuhan*
Winnie Yeo, *Hong Kong*
Yuan Yuan, *Shenyang*
Man-Fung Yuen, *Hong Kong*
Jian-Zhong Zhang, *Beijing*
Xin-Xin Zhang, *Shanghai*
Bo-Jian Zheng, *Hong Kong*
Shu Zheng, *Hangzhou*

Croatia

Tamara Cacev, *Zagreb*
Marko Duvnjak, *Zagreb*

Cuba

Damian C Rodriguez, *Havana*

Czech

Milan Jirsa, *Praha*
Pavel Trunečka, *Prague*

Denmark

Peter Bytzer, *Copenhagen*
Asbjørn M Drewes, *Aalborg*
Hans Gregersen, *Aalborg*
Jens H Henriksen, *Hvidovre*
Claus P Hovendal, *Odense*
Fin S Larsen, *Copenhagen*
Søren Møller, *Hvidovre*

Egypt

Abdel-Rahman El-Zayadi, *Giza*
Amr M Helmy, *Cairo*
Ayman Yosry, *Cairo*

Estonia

Riina Salupere, *Tartu*

Finland

Irma E Jarvela, *Helsinki*
Katri M Kaukinen, *Tampere*
Minna Nyström, *Helsinki*
Pentti Sipponen, *Espoo*

France

Bettaieb Ali, *Dijon*
Corlu Anne, *Rennes*
Denis Ardid, *Clermont-Ferrand*
Charles P Balabaud, *Bordeaux*
Soumeya Bekri, *Rouen*
Jacques Belghiti, *Clichy*
Jacques Bernuau, *Clichy Cedex*
Pierre Brissot, *Rennes*
Patrice P Cacoub, *Paris*
Franck Carbonnel, *Besancon*
Laurent Castera, *Pessac*
Bruno Clément, *Rennes*
Benoit Coffin, *Colombes*
Jacques Cosnes, *Paris*
Thomas Decaens, *Cedex*

Francoise L Fabiani, *Angers*
Gérard Feldmann, *Paris*
Jean Fioramonti, *Toulouse*
Jean-Noël Freund, *Strasbourg*
Jean-Paul Galmiche, *Nantes*
Catherine Guettier, *Villejuif*
Chantal Housset, *Paris*
Juan L Iovanna, *Marseille*
Rene Lambert, *Lyon*
Patrick Marcellin, *Paris*
Philippe Mathurin, *Lille*
Tamara Matysiak–Budnik, *Paris*
Francis Mégraud, *Bordeaux*
Richard Moreau, *Clichy*
Thierry Piche, *Nice*
Raoul Poupon, *Paris*
Jean Rosenbaum, *Bordeaux*
Dominique Marie Roulot, *Bobigny*
Thierry Poynard, *Paris*
Jean-Philippe Salier, *Rouen*
Didier Samuel, *Villejuif*
Jean-Yves Scoazec, *Lyon*
Khalid A Tazi, *Clichy*
Emmanuel Tiret, *Paris*
Baumert F Thomas, *Strasbourg*
Marie-Catherine Vozenin-brotons, *Villejuif*
Jean-Pierre H Zarski, *Grenoble*
Jessica Zucman-Rossi, *Paris*

Germany

Hans-Dieter Allescher, *G-Partenkirchen*
Martin Anlauf, *Kiel*
Rudolf Arnold, *Marburg*
Max G Bachem, *Ulm*
Thomas F Baumert, *Freiburg*
Daniel C Baumgart, *Berlin*
Hubert Blum, *Freiburg*
Thomas Bock, *Tuebingen*
Katja Breitkopf, *Mannheim*
Dunja Bruder, *Braunschweig*
Markus W Büchler, *Heidelberg*
Christa Buechler, *Regensburg*
Reinhard Buettner, *Bonn*
Elke Cario, *Essen*
Uta Dahmen, *Essen*
Christoph F Dietrich, *Bad Mergentheim*
Arno J Dormann, *Koeln*
Rainer J Duchmann, *Berlin*
Volker F Eckardt, *Wiesbaden*
Paul Enck, *Tuebingen*
Fred Fändrich, *Kiel*
Ulrich R Fölsch, *Kiel*
Helmut Friess, *Heidelberg*
Peter R Galle, *Mainz*
Nikolaus Gassler, *Aachen*
Andreas Geier, *Aachen*
Markus Gerhard, *Munich*
Wolfram H Gerlich, *Giessen*
Dieter Glebe, *Giessen*
Burkhard Göke, *Munich*
Florian Graepler, *Tuebingen*
Axel M Gressner, *Aachen*
Veit Gülberg, *Munich*
Rainer Haas, *Munich*
Eckhart G Hahn, *Erlangen*
Stephan Hellmig, *Kiel*
Martin Hennenberg, *Bonn*
Johannes Herkel, *Hamburg*
Klaus R Herrlinger, *Stuttgart*
Eva Herrmann, *Homburg/Saar*
Eberhard Hildt, *Berlin*
Joerg C Hoffmann, *Berlin*
Ferdinand Hofstaedter, *Regensburg*
Werner Hohenberger, *Erlangen*
Jörg C Kalff, *Bonn*
Ralf Jakobs, *Ludwigshafen*
Jutta Keller, *Hamburg*
Andrej Khandoga, *Munich*
Sibylle Koletzko, *München*
Stefan Kubicka, *Hannover*
Joachim Labenz, *Siegen*
Frank Lammert, *Bonn*
Thomas Langmann, *Regensburg*
Christian Liedtke, *Aachen*
Matthias Löhr, *Mannheim*
Christian Maaser, *Muenster*
Ahmed Madisch, *Dresden*
Peter Malfertheiner, *Magdeburg*
Michael P Manns, *Hannover*
Helmut Messmann, *Augsburg*
Stephan Miehlke, *Dresden*
Sabine Mihm, *Göttingen*
Silvio Nadalin, *Essen*
Markus F Neurath, *Mainz*
Johann Ockenga, *Berlin*
Florian Obermeier, *Regensburg*
Gustav Paumgartner, *Munich*
Ulrich KS Peitz, *Magdeburg*
Markus Reiser, *Bochum*
Emil C Reisinger, *Rostock*
Steffen Rickes, *Magdeburg*
Tilman Sauerbruch, *Bonn*
Dieter Saur, *Munich*
Hans Scherubl, *Berlin*
Joerg Schirra, *Munich*
Roland M Schmid, *München*
Volker Schmitz, *Bonn*
Andreas G Schreyer, *Regensburg*
Tobias Schroeder, *Essen*
Henning Schulze-Bergkamen, *Mainz*
Hans Seifert, *Oldenburg*
Norbert Senninger, *Muenster*
Manfred V Singer, *Mannheim*
Gisela Sparmann, *Rostock*
Christian J Steib, *München*
Jurgen M Stein, *Frankfurt*
Ulrike S Stein, *Berlin*
Manfred Stolte, *Bayreuth*
Christian P Strassburg, *Hannover*
Wolfgang R Stremmel, *Heidelberg*
Harald F Teutsch, *Ulm*
Robert Thimme, *Freiburg*
Hans L Tillmann, *Leipzig*
Tung-Yu Tsui, *Regensburg*
Axel Ulsenheimer, *Munich*
Patrick Veit-Haibach, *Essen*
Claudia Veltkamp, *Heidelberg*
Siegfried Wagner, *Deggendorf*
Henning Walczak, *Heidelberg*
Heiner Wedemeyer, *Hannover*
Fritz von Weizsacker, *Berlin*
Jens Werner, *Heidelberg*
Bertram Wiedenmann, *Berlin*
Reiner Wiest, *Regensburg*
Stefan Wirth, *Wuppertal*
Stefan JP Zeuzem, *Homburg*

Greece

Alexandra A Alexopoulou, *Athens*
George N Dalekos, *Larissa*
Christos Dervenis, *Athens*
Melanie Maria Deutsch, *Athens*
Tsianos Epameinondas, *Ioannina*
Elias A Kouroumalis, *Heraklion*
George Papatheodoridis, *Athens*
Spiros Sgouros, *Athens*

Hungary

Peter L Lakatos, *Budapest*
Zsuzsa Szondy, *Debrecen*

Iceland

Hallgrimur Gudjonsson, *Reykjavik*

India

Philip Abraham, *Mumbai*
Rakesh Aggarwal, *Lucknow*
Kunissery A Balasubramanian, *Vellore*
Deepak Kumar Bhasin, *Chandigarh*
Sujit K Bhattacharya, *Kolkata*
Yogesh K Chawla, *Chandigarh*
Radha K Dhiman, *Chandigarh*
Sri Prakash Misra, *Allahabad*
Ramesh Roop Rai, *Jaipur*
Nageshwar D Reddy, *Hyderabad*
Rakesh Kumar Tandon, *New Delhi*

Iran

Seyed-Moayed Alavian, *Tehran*
Reza Malekzadeh, *Tehran*
Seyed A Taghavi, *Shiraz*

Ireland

Billy Bourke, *Dublin*
Ronan A Cahill, *Cork*
Anthony P Moran, *Galway*

Israel

Simon Bar-Meir, *Hashomer*
Abraham R Eliakim, *Haifa*
Zvi Fireman, *Hadera*
Yaron Ilan, *Jerusalem*
Avidan U Neumann, *Ramat-Gan*
Yaron Niv, *Pardesia*
Ran Oren, *Tel Aviv*
Ami D Sperber, *Beer-Sheva*

Italy

Giovanni Addolorato, *Roma*
Luigi E Adinolfi, *Naples*
Domenico Alvaro, *Rome*
Mario Angelico, *Rome*
Vito Annese, *San Giovanni Rotond*
Filippo Ansaldi, *Genoa*
Adolfo F Attili, *Roma*
Giovanni Barbara, *Bologna*
Claudio Bassi, *Verona*
Gabrio Bassotti, *Perugia*
Pier M Battezzati, *Milan*
Stefano Bellentani, *Carpi*
Antomio Benedetti, *Ancona*
Mauro Bernardi, *Bologna*
Livia Biancone, *Rome*
Luigi Bonavina, *Milano*
Flavia Bortolotti, *Padova*
Giuseppe Brisinda, *Rome*
Elisabetta Buscarini, *Crema*
Giovanni Cammarota, *Roma*

Antonino Cavallari, *Bologna*
Giuseppe Chiarioni, *Valeggio*
Michele Cicala, *Rome*
Massimo Colombo, *Milan*
Amedeo Columbano, *Cagliari*
Massimo Conio, *Sanremo*
Dario Conte, *Milano*
Gino R Corazza, *Pavia*
Francesco Costa, *Pisa*
Antonio Craxi, *Palermo*
Silvio Danese, *Milan*
Roberto de Franchis, *Milano*
Roberto De Giorgio, *Bologna*
Maria Stella De Mitri, *Bologna*
Giovanni D De Palma, *Naples*
Fabio Farinati, *Padua*
Giammarco Fava, *Ancona*
Francesco Feo, *Sassari*
Fiorucci Stefano, *Perugia*
Andrea Galli, *Firenze*
Valeria Ghisett, *Turin*
Gianluigi Giannelli, *Bari*
Edoardo G Giannini, *Genoa*
Paolo Gionchetti, *Bologna*
Fabio Grizzi, *Milan*
Salvatore Gruttadauria, *Palermo*
Mario Guslandi, *Milano*
Pietro Invernizzi, *Milan*
Ezio Laconi, *Cagliari*
Giacomo Laffi, *Firenze*
Giovanni Maconi, *Milan*
Lucia Malaguarnera, *Catania*
Emanuele D Mangoni, *Napoli*
Paolo Manzoni, *Torino*
Giulio Marchesini, *Bologna*
Fabio Marra, *Florence*
Marco Marzioni, *Ancona*
Giuseppe Mazzella, *Bologna*
Mario U Mondelli, *Pavia*
Giuseppe Montalto, *Palermo*
Giovanni Monteleone, *Rome*
Giovanni Musso, *Torino*
Gerardo Nardone, *Napoli*
Valerio Nobili, *Rome*
Fabio Pace, *Milano*
Luisi Pagliaro, *Palermo*
Francesco Pallone, *Rome*
Fabrizio R Parente, *Milan*
Maurizio Parola, *Torino*
Francesco Perri, *San Giovanni Rotondo*
Raffaele Pezzilli, *Bologna*
Alberto Pilotto, *San Giovanni Rotondo*
Alberto Piperno, *Monza*
Mario Pirisi, *Novara*
Anna C Piscaglia, *Roma*
Paolo Del Poggio, *Treviglio*
Gabriele B Porro, *Milano*
Piero Portincasa, *Bari*
Cosimo Prantera, *Roma*
Bernardino Rampone, *Siena*
Oliviero Riggio, *Rome*
Claudio Romano, *Messina*
Marco Romano, *Napoli*
Gerardo Rosati, *Potenza*
Mario Del Tacca, *Pisa*
Gloria Taliani, *Rome*
Pier A Testoni, *Milan*
Enrico Roda, *Bologna*
Domenico Sansonno, *Bari*
Vincenzo Savarino, *Genova*
Vincenzo Stanghellini, *Bologna*
Giovanni Tarantino, *Naples*
Roberto Testa, *Genoa*
Dino Vaira, *Bologna*
Anna Linda Zignego, *Florence*

Japan

Kyoichi Adachi, *Izumo*
Yasushi Adachi, *Sapporo*
Taiji Akamatsu, *Matsumoto*
Sk Md Fazle Akbar, *Ehime*
Takafumi Ando, *Nagoya*
Akira Andoh, *Otsu*
Taku Aoki, *Tokyo*
Masahiro Arai, *Tokyo*
Tetsuo Arakawa, *Osaka*
Yasuji Arase, *Tokyo*
Masahiro Asaka, *Sapporo*
Hitoshi Asakura, *Tokyo*
Takeshi Azuma, *Fukui*
Yoichi Chida, *Fukuoka*
Takahiro Fujimori, *Tochigi*
Jiro Fujimoto, *Hyogo*
Kazuma Fujimoto, *Saga*
Mitsuhiro Fujishiro, *Tokyo*
Yoshihide Fujiyama, *Otsu*
Hiroyuki Fukui, *Tochigi*
Hiroyuki Hanai, *Hamamatsu*
Naohiko Harada, *Fukuoka*
Makoto Hashizume, *Fukuoka*
Tetsuo Hayakawa, *Nagoya*
Toru Hiyama, *Higashihiroshima*
Kazuhide Higuchi, *Osaka*
Keisuke Hino, *Ube*
Keiji Hirata, *Kitakyushu*
Yuji Iimuro, *Nishinomiya*
Kenji Ikeda, *Tokyo*
Toru Ikegami, *Fukuoka*
Kenichi Ikejima, *Bunkyo-ku*
Fumio Imazeki, *Chiba*
Yutaka Inagaki, *Kanagawa*
Yasuhiro Inokuchi, *Yokohama*
Haruhiro Inoue, *Yokohama*
Masayasu Inoue, *Osaka*
Hiromi Ishibashi, *Nagasaki*
Shunji Ishihara, *Izumo*
Toru Ishikawa, *Niigata*
Kei Ito, *Sendai*
Masayoshi Ito, *Tokyo*
Hiroaki Itoh, *Akita*
Ryuichi Iwakiri, *Saga*
Yoshiaki Iwasaki, *Okayama*
Terumi Kamisawa, *Tokyo*
Hiroshi Kaneko, *Aichi-Gun*
Shuichi Kaneko, *Kanazawa*
Takashi Kanematsu, *Nagasaki*
Mitsuo Katano, *Fukuoka*
Junji Kato, *Sapporo*
Mototsugu Kato, *Sapporo*
Shinzo Kato, *Tokyo*
Norifumi Kawada, *Osaka*
Sunao Kawano, *Osaka*
Mitsuhiro Kida, *Kanagawa*
Yoshikazu Kinoshita, *Izumo*
Tsuneo Kitamura, *Chiba*
Seigo Kitano, *Oita*
Kazuhiko Koike, *Tokyo*
Norihiro Kokudo, *Tokyo*
Satoshi Kondo, *Sapporo*
Shoji Kubo, *Osaka*
Shigeki Kuriyama, *Kagawa*[2]
Katsunori Iijima, *Sendai*
Masato Kusunoki, *Tsu Mie*
Shin Maeda, *Tokyo*
Shigeru Marubashi, *Suita*
Masatoshi Makuuchi, *Tokyo*
Osamu Matsui, *Kanazawa*
Yasuhiro Matsumura, *Chiba*
Yasushi Matsuzaki, *Tsukuba*
Kiyoshi Migita, *Omura*
Kenji Miki, *Tokyo*
Tetsuya Mine, *Kanagawa*
Hiroto Miwa, *Hyogo*
Masashi Mizokami, *Nagoya*
Yoshiaki Mizuguchi, *Tokyo*
Motowo Mizuno, *Hiroshima*
Morito Monden, *Suita*
Hisataka S Moriwaki, *Gifu*
Yasuaki Motomura, *Iizuka*
Yoshiharu Motoo, *Kanazawa*
Naofumi Mukaida, *Kanazawa*
Kazunari Murakami, *Oita*
Kunihiko Murase, *Tusima*
Hiroaki Nagano, *Suita*
Masahito Nagaki, *Gifu*
Masaki Nagaya, *Kawasaki*
Yujl Naito, *Kyoto*
Atsushi Nakajima, *Yokohama*
Hisato Nakajima, *Tokyo*
Hiroki Nakamura, *Yamaguchi*
Shotaro Nakamura, *Fukuoka*
Mikio Nishioka, *Niihama*
Shuji Nomoto, *Nagoya*
Susumu Ohmada, *Maebashi*
Hirohide Ohnishi, *Akita*
Masayuki Ohta, *Oita*
Tetsuo Ohta, *Kanazawa*
Kazuichi Okazaki, *Osaka*
Katsuhisa Omagari, *Nagasaki*
Saburo Onishi, *Nankoku*
Morikazu Onji, *Ehime*
Satoshi Osawa, *Hamamatsu*
Masanobu Oshima, *Kanazawa*
Hiromitsu Saisho, *Chiba*
Hidetsugu Saito, *Tokyo*
Yutaka Saito, *Tokyo*
Isao Sakaida, *Yamaguchi*
Michiie Sakamoto, *Tokyo*
Yasushi Sano, *Chiba*
Hiroki Sasaki, *Tokyo*
Iwao Sasaki, *Sendai*
Motoko Sasaki, *Kanazawa*
Chifumi Sato, *Tokyo*
Shuichi Seki, *Osaka*
Hiroshi Shimada, *Yokohama*
Mitsuo Shimada, *Tokushima*
Tomohiko Shimatan, *Hiroshima*
Hiroaki Shimizu, *Chiba*
Ichiro Shimizu, *Tokushima*
Yukihiro Shimizu, *Kyoto*
Shinji Shimoda, *Fukuoka*
Tooru Shimosegawa, *Sendai*
Tadashi Shimoyama, *Hirosaki*
Ken Shirabe, *Iizuka City*
Yoshio Shirai, *Niigata*
Katsuya Shiraki, *Mie*
Yasushi Shiratori, *Okayama*
Masayuki Sho, *Nara*
Yasuhiko Sugawara, *Tokyo*
Hidekazu Suzuki, *Tokyo*
Minoru Tada, *Tokyo*
Tadatoshi Takayama, *Tokyo*
Tadashi Takeda, *Osaka*
Koji Takeuchi, *Kyoto*
Kiichi Tamada, *Tochigi*
Akira Tanaka, *Kyoto*
Eiji Tanaka, *Matsumoto*
Noriaki Tanaka, *Okayama*
Shinji Tanaka, *Hiroshima*
Hideki Taniguchi, *Yokohama*
Kyuichi Tanikawa, *Kurume*
Akira Terano, *Shimotsugagun*
Hitoshi Togash, *Yamagata*
Shinji Togo, *Yokohama*
Kazunari Tominaga, *Osaka*
Takuji Torimura, *Fukuoka*

Minoru Toyota, *Sapporo*
Akihito Tsubota, *Chiba*
Takato Ueno, *Kurume*
Naomi Uemura, *Tokyo*
Shinichi Wada, *Tochigi*
Hiroyuki Watanabe, *Kanazawa*
Toshio Watanabe, *Osaka*
Yuji Watanabe, *Ehime*
Toshiaki Watanabe, *Tokyo*
Chun-Yang Wen, *Nagasaki*
Satoshi Yamagiwa, *Niigata*
Koji Yamaguchi, *Fukuoka*
Takayuki Yamamoto, *Yokkaichi*
Takashi Yao, *Fukuoka*
Masashi Yoneda, *Tochigi*
Hiroshi Yoshida, *Tokyo*
Masashi Yoshida, *Tokyo*
Norimasa Yoshida, *Kyoto*
Hitoshi Yoshiji, *Nara*
Kentaro Yoshika, *Toyoake*
Yasunobu Yoshikai, *Fukuoka*
Masahide Yoshikawa, *Kashihara*
Katsutoshi Yoshizato, *Higashihiroshima*

Lebanon

Bassam N Abboud, *Beirut*
Ala I Sharara, *Beirut*
Joseph D Boujaoude, *Beirut*

Lithuania

Limas Kupcinskas, *Kaunas*

Macedonia

Vladimir C Serafimoski, *Skopje*

Malaysia

Andrew Seng Boon Chua, *Ipoh*
Khean-Lee Goh, Kuala *Lumpur*
Jayaram Menon, *Sabah*

Mexico

Diego Garcia-Compean, *Monterrey*
Eduardo R Marin-Lopez, *Jesús García*
Nahum Méndez-Sánchez, *Mexico*
Saúl Villa-Treviño, *México*

Monaco

Patrick Rampal, *Monaco*

Morocco

Abdellah Essaid, *Rabat*

The Netherlands

Ulrich Beuers, *Amsterdam*
Gerd Bouma, *Amsterdam*
Lee Bouwman, *Leiden*
J Bart A Crusius, *Amsterdam*
NKH de Boer, *Amsterdam*
Koert P de Jong, *Groningen*
Henrike Hamer, *Maastricht*
Frank Hoentjen, *Haarlem*
Janine K Kruit, *Groningen*
Ernst J Kuipers, *Rotterdam*
CBHW Lamers, *Leiden*
Ton Lisman, *Utrecht*
Yi Liu, *Amsterdam*
Jeroen Maljaars, *Maastricht*
Servaas Morré, *Amsterdam*
Chris JJ Mulder, *Amsterdam*
Michael Müller, *Wageningen*
Amado S Peña, *Amsterdam*
Robert J Porte, *Groningen*
Ingrid B Renes, *Rotterdam*
Andreas Smout, *Utrecht*
Paul E Sijens, *Groningen*
Reinhold W Stockbrugger, *Maastricht*
Luc JW van der Laan, *Rotterdam*
Karel van Erpecum, *Utrecht*
Gerard P VanBerge-Henegouwen,*Utrecht*

New Zealand

Ian D Wallace, *Auckland*

Nigeria

Samuel B Olaleye, *Ibadan*

Norway

Trond Berg, *Oslo*
Tom H Karlsen, *Oslo*
Helge L Waldum, *Trondheim*

Pakistan

Muhammad S Khokhar, *Lahore*
Syed MW Jafri, *Karachi*

Peru

Hector H Garcia, *Lima*

Poland

Tomasz Brzozowski, *Cracow*
Robert Flisiak, *Bialystok*
Hanna Gregorek, *Warsaw*
Dariusz M Lebensztejn, *Bialystok*
Wojciech G Polak, *Wroclaw*
Marek Hartleb, *Katowice*

Portugal

Miguel C De Moura, *Lisbon*

Russia

Vladimir T Ivashkin, *Moscow*
Leonid Lazebnik, *Moscow*
Vasiliy I Reshetnyak, *Moscow*

Saudi Arabia

Ibrahim A Al Mofleh, *Riyadh*
Ahmed Helmy, *Riyadh*

Serbia

Dusan M Jovanovic, *Sremska Kamenica*

Singapore

Bow Ho, *Singapore*
Khek-Yu Ho, *Singapore*
Fock Kwong Ming, *Singapore*
Francis Seow-Choen, *Singapore*

Slovakia

Silvia Pastorekova, *Bratislava*
Anton Vavrecka, *Bratislava*

Slovenia

Sasa Markovic, *Ljubljana*

South Africa

Rosemar Joyce Burnett, *Pretoria*
Michael C Kew, *Parktown*

South Korea

Byung Ihn Choi, *Seoul*
Ho Soon Choi, *Seoul*
Marie Yeo, *Suwon*
Sun Pyo Hong, *Gyeonggi-do*
Jae J Kim, *Seoul*
Jin-Hong Kim, *Suwon*
Myung-Hwan Kim, *Seoul*
Chang Hong Lee, *Seoul*
Jong Kyun Lee, *Seoul*
Eun-Yi Moon, *Seoul*
Jae-Gahb Park, *Seoul*
Dong Wan Seo, *Seoul*
Dong Jin Suh, *Seoul*
Byung Chul Yoo, *Seoul*

Spain

Juan G Abraldes, *Barcelona*
Agustin Albillos, *Madrid*
Raul J Andrade, *Málaga*
Luis Aparisi, *Valencia*
Fernando Azpiroz, *Barcelona*
Ramon Bataller, *Barcelona*
Josep M Bordas, *Barcelona*
Xavier Calvet, *Sabadell*
Jordi Camps, *Catalunya*
Andres Cardenas, *Barcelona*
Vicente Carreño, *Madrid*
Jose Castellote, *Barcelona*
Antoni Castells, *Barcelona*
Vicente Felipo, *Valencia*
Juan C Garcia-Pagán, *Barcelona*
Jaime B Genover, *Barcelona*
Javier P Gisbert, *Madrid*
Jaime Guardia, *Barcelona*
Isabel Fabregat, *Barcelona*
Mercedes Fernandez, *Barcelona*
Angel Lanas, *Zaragoza*
Juan-Ramón Larrubia, *Guadalajara*
Laura Lladóa, *Barcelona*
María IT López, *Jaén*
Juan R Malagelada, *Barcelona*
José M Mato, *Derio*
Juan F Medina, *Pamplona*
Miguel A Muñoz-Navas, *Pamplona*
Julian Panes, *Barcelona*
Miguel M Perez, *Valencia*
Miguel Perez-Mateo, *Alicante*

Josep M Pique, *Barcelona*
Jesús M Prieto, *Pamplona*
Sabino Riestra, *Pola De Siero*
Luis Rodrigo, *Oviedo*
Manuel Romero-Gómez, *Sevilla*
Joan Roselló-Catafau, *Barcelona*

Sweden

Einar S Björnsson, *Gothenburg*
Curt Einarsson, *Huddinge*
Per M Hellström, *Stockholm*
Ulf Hindorf, *Lund*
Elisabeth Hultgren-Hörnquist, *Örebro*
Anders E Lehmann, *Mölndal*
Hanns-Ulrich Marschall, *Stockholm*
Lars C Olbe, *Molndal*
Lars A Pahlman, *Uppsala*
Matti Sallberg, *Stockholm*
Magnus Simrén, *Göteborg*
Xiao-Feng Sun, *Linköping*
Ervin Tóth, *Malmö*
Weimin Ye, *Stockholm*
Christer S von Holstein, *Lund*

Switzerland

Chrish Beglinger, *Basel*
Pierre A Clavien, *Zurich*
Jean-Francois Dufour, *Bern*
Franco Fortunato, *Zürich*
Jean L Frossard, *Geneva*
Gerd A Kullak-Ublick, *Zurich*
Pierre Michetti, *Lausanne*
Francesco Negro, *Genève*
Bruno Stieger, *Zurich*
Radu Tutuian, *Zurich*
Stephan R Vavricka, *Zurich*
Gerhard Rogler, *Zurich*
Arthur Zimmermann, *Berne*

Turkey

Yusuf Bayraktar, *Ankara*
Figen Gurakan, *Ankara*
Aydin Karabacakoglu, *Konya*
Serdar Karakose, *Konya*
Hizir Kurtel, *Istanbul*
Osman C Ozdogan, *Istanbul*
Özlem Yilmaz, *Izmir*
Cihan Yurdaydin, *Ankara*

United Arab Emirates

Sherif M Karam, *Al-Ain*

United Kingdom

David H Adams, *Birmingham*
Simon Afford, *Birmingham*
Navneet K Ahluwalia, *Stockport*
Ahmed Alzaraa, *Manchester*
Lesley A Anderson, *Belfast*
Charalambos G Antoniades, *London*
Anthony TR Axon, *Leeds*
Qasim Aziz, *Manchester*
Nicholas M Barnes, *Birmingham*
Jim D Bell, *London*
Mairi Brittan, *London*
Alastair D Burt, *Newcastle*
Simon S Campbell, *Manchester*
Simon R Carding, *Leeds*
Paul J Ciclitira, *London*
Eithne Costello, *Liverpool*
Tatjana Crnogorac-Jurcevic, *London*
Harry Dalton, *Truro*
Amar P Dhillon, *London*
William Dickey, *Londonderry*
James E East, *London*
Emad M El-Omar, *Aberdeen*
Ahmed M Elsharkawy, *Newcastle Upon Tyne*
Annette Fristscher-Ravens, *London*
Elizabeth Furrie, *Dundee*
Daniel R Gaya, *Edinburgh*
Subrata Ghosh, *London*
William Greenhalf, *Liverpool*
Indra N Guha, *Southampton*
Peter C Hayes, *Edinburgh*
Gwo-Tzer Ho, *Edinburgh*
Anthony R Hobson, *Salford*
Lesley A Houghton, *Manchester*
Stefan G Hübscher, *Birmingham*
Robin Hughes, *London*
Pali Hungin, *Stockton*
David P Hurlstone, *Sheffield*
Rajiv Jalan, *London*
Janusz AZ Jankowski, *Oxford*
Brian T Johnston, *Belfast*
David EJ Jones, *Newcastle*
Roger Jones, *London*
Michael A Kamm, *Harrow*
Peter Karayiannis, *London*
Laurens Kruidenier, *Harlow*
Patricia F Lalor, *Birmingham*
Chee Hooi Lim, *Midlands*
Hong-Xiang Liu, *Cambridge*
Yun Ma, *London*
Kenneth E L McColl, *Glasgow*
Stuart AC McDonald, *London*
Dermot P Mcgovern, *Oxford*
Giorgina Mieli-Vergani, *London*
Nikolai V Naoumov, *London*
John P Neoptolemos, *Liverpool*
James Neuberger, *Birmingham*
Philip Noel Newsome, *Birmingham*
Mark S Pearce, *Newcastle Upon Tyne*
Stephen P Pereira, *London*
D Mark Pritchard, *Liverpool*
Sakhawat Rahman, *London*
Stephen E Roberts, *Swansea*
Marco Senzolo, *Padova*
Soraya Shirazi-Beechey, *Liverpool*
Robert Sutton, *Liverpool*
Simon D Taylor-Robinson, *London*
Paris P Tekkis, *London*
Ulrich Thalheimer, *London*
David G Thompson, *Salford*
Nick P Thompson, *Newcastle*
David Tosh, *Bath*
Frank I Tovey, *London*
Chris Tselepis, *Birmingham*
Diego Vergani, *London*
Geoffrey Warhurst, *Salford*
Alastair John Watson, *Liverpool*
Peter J Whorwell, *Manchester*
Roger Williams, *London*
Karen L Wright, *Bath*
Min Zhao, *Foresterhill*

United States

Manal F Abdelmalek, *Durham*
Gary A Abrams, *Birmingham*
Maria T Abreu, *New York*
Reid B Adams, *Virginia*
Golo Ahlenstiel, *Bethesda*
BS Anand, *Houston*
Frank A Anania, *Atlanta*
M Ananthanarayanan, *New York*
Gavin E Arteel, *Louisville*
Jasmohan S Bajaj, *Milwaukee*
Subhas Banerjee, *Palo Alto*
Peter A Banks, *Boston*
Jamie S Barkin, *Miami Beach*
Kim E Barrett, *San Diego*
Marc D Basson, *Detroit*
Anthony J Bauer, *Pittsburgh*
Wallace F Berman, *Durham*
Timothy R Billiar, *Pittsburgh*
Edmund J Bini, *New York*
David G Binion, *Milwaukee*
Jennifer D Black, *Buffalo*
Herbert L Bonkovsky, *Charlotte*
Carla W Brady, *Durham*
Andrea D Branch, *New York*
Robert S Bresalier, *Houston*
Alan L Buchman, *Chicago*
Ronald W Busuttil, *Los Angeles*
Alan Cahill, *Philadelphia*
John M Carethers, *San Diego*
David L Carr-Locke, *Boston*
Maurice A Cerulli, *New York*
Ravi S Chari, *Nashville*
Jiande Chen, *Galveston*
Xian-Ming Chen, *Omaha*
Xin Chen, *San Francisco*
Ramsey Chi-man Cheung, *Palo Alto*
William D Chey, *Ann Arbor*
John Y Chiang, *Rootstown*
Parimal Chowdhury, *Arkansas*
Raymond T Chung, *Boston*
James M Church, *Cleveland*
Ram Chuttani, *Boston*
Mark G Clemens, *Charlotte*
Ana J Coito, *Los Angeles*
Vincent Coghlan, *Beaverton*
David Cronin II, *New Haven*
John Cuppoletti, *Cincinnati*
Mark J Czaja, *New York*
Peter V Danenberg, *Los Angeles*
Kiron M Das, New *Brunswick*
Conor P Delaney, *Cleveland*
Jose L del Pozo, *Rochester*
Sharon DeMorrow, *Temple*
Deborah L Diamond, *Seattle*
Douglas A Drossman, *Chapel Hill*
Katerina Dvorak, *Tucson*
Bijan Eghtesad, *Cleveland*
Hala El-Zimaity, *Houston*
Michelle Embree-Ku, *Providence*
Sukru Emre, New *Haven*
Douglas G Farmer, *Los Angeles*
Alessio Fasano, *Baltimore*
Mark A Feitelson, *Philadelphia*
Ariel E Feldstein, *Cleveland*
Alessandro Fichera, *Chicago*
Robert L Fine, *New York*
Magali Fontaine, *Stanford*
Chris E Forsmark, *Gainesville*
Glenn T Furuta, *Aurora*
Chandrashekhar R Gandhi, *Pittsburgh*
Susan L Gearhart, *Baltimore*
Xupeng Ge, *Boston*
Xin Geng, *New Brunswick*
M Eric Gershwin, *Suite*
Jean-Francois Geschwind, *Baltimore*
Ignacio Gil-Bazo, *New York*
Shannon S Glaser, *Temple*
Ajay Goel, *Dallas*
Richard M Green, *Chicago*
Julia B Greer, *Pittsburgh*

James H Grendell, *New York*
David R Gretch, *Seattle*
Stefano Guandalini, *Chicago*
Anna S Gukovskaya, *Los Angeles*
Sanjeev Gupta, *Bronx*
David J Hackam, *Pittsburgh*
Stephen B Hanauer, *Chicago*
Gavin Harewood, *Rochester*
Margaret M Heitkemper, *Washington*
Alan W Hemming, *Gainesville*
Samuel B Ho, *San Diego*
Peter R Holt, *New York*
Colin W Howden, *Chicago*
Hongjin Huang, *Alameda*
Jamal A Ibdah, *Columbia*
Atif Iqbal, *Omaha*
Hajime Isomoto, *Rochester*
Hartmut Jaeschke, *Tucson*
Dennis M Jensen, *Los Angeles*
Cheng Ji, *Los Angeles*
Leonard R Johnson, *Memphis*
Michael P Jones, *Chicago*
Peter J Kahrilas, *Chicago*
Anthony N Kalloo, *Baltimore*
Marshall M Kaplan, *Boston*
Neil Kaplowitz, *Los Angeles*
Serhan Karvar, *Los Angeles*
Rashmi Kaul, *Tulsa*
Jonathan D Kaunitz, *Los Angeles*
Ali Keshavarzian, *Chicago*
Miran Kim, *Providence*
Joseph B Kirsner, *Chicago*
Leonidas G Koniaris, *Miami*
Burton I Korelitz, *New York*
Robert J Korst, *New York*
Richard A Kozarek, *Seattle*
Alyssa M Krasinskas, *Pittsburgh*
Michael Kremer, *Chapel Hill*
Shiu-Ming Kuo, *Buffalo*
Paul Y Kwo, *Indianapolis*
Daryl Tan Yeung Lau, *Galvesto*
Stephen J Lanspa, *Omaha*
Joel E Lavine, *San Diego*
Bret Lashner, *Cleveland*
Dirk J van Leeuwen, *Lebanon*
Glen A Lehman, *Indianapolis*
Alex B Lentsch, *Cincinnati*
Andreas Leodolter, *La Jolla*
Gene LeSage, *Houston*
Josh Levitsky, *Chicago*
Cynthia Levy, *Gainesville*
Ming Li, *New Orleans*
Zhiping Li, *Baltimore*
Zhe-Xiong Lian, *Davis*
Lenard M Lichtenberger, *Houston*
Gary R Lichtenstein, *Philadelphia*
Otto Schiueh-Tzang Lin, *Seattle*
Martin Lipkin, *New York*
Chen Liu, *Gainesville*
Edward V Loftus, *Rocheste*
Robin G Lorenz, *Birmingham*
Michael R Lucey, *Madison*
James D Luketich, *Pittsburgh*
Guangbin Luo, *Cheveland*
Henry T Lynch, *Omaha*
Patrick M Lynch, *Houston*
John S Macdonald, *New York*
Bruce V MacFadyen, *Augusta*
Willis C Maddrey, *Dallas*
Ashok Malani, *Los Angeles*
Mercedes Susan Mandell, *Aurora*
Peter J Mannon, *Bethesda*
Charles M Mansbach, *Tennessee*
John F Di Mari, *Texas*
John M Mariadason, *Bronx*
Jorge A Marrero, *Ann Arbor*
Paul Martin, *New York*
Paulo Ney Aguiar Martins, *Boston*
Wendy M Mars, *Pittsburgh*
Laura E Matarese, *Pittsburgh*
Richard W McCallum, *Kansas*
Beth A McCormick, *Charlestown*
Lynne V McFarland, *Washington*
Kevin McGrath, *Pittsburgh*
Harihara Mehendale, *Monroe*
Ali Mencin, *New York*
Fanyin Meng, *Ohio*
Stephan Menne, *New York*
Didier Merlin, *Atlanta*
Howard Mertz, *Nashville*
George W Meyer, *Sacramento*
George Michalopoulos, *Pittsburgh*
James M Millis, *Chicago*
Fabrizio Michelassi, *New York*
Albert D Min, *New York*
Pramod K Mistry, *New Haven*
Emiko Mizoguchi, *Boston*
Smruti R Mohanty, *Chicago*
Satdarshan S Monga, *Pittsburgh*
Timothy H Moran, *Baltimore*
Peter L Moses, *Burlington*
Steven F Moss, *Providence*
Andrew J Muir, *Durham*
Milton G Mutchnick, *Detroit*
Masaki Nagaya, *Boston*
Victor Navarro, *Philadelphia*
Laura E Nagy, *Cleveland*
Hiroshi Nakagawa, *Philadelphia*
Douglas B Nelson, *Minneapolis*
Justin H Nguyen, *Florida*
Patrick G Northup, *Charlottesville*
Christopher O'Brien, *Miami*
Robert D Odze, *Boston*
Brant K Oelschlager, *Washington*
Curtis T Okamoto, *Los Angeles*
Stephen JD O'Keefe, *Pittsburgh*
Dimitry Oleynikov, *Omaha*
Stephen J Pandol, *Los Angeles*
Georgios Papachristou, *Pittsburgh*
Pankaj J Pasricha, *Galveston*
Zhiheng Pei, *New York*
Michael A Pezzone, *Pittsburgh*
CS Pitchumoni, *New Brunswiuc*
Paul J Pockros, *La Jolla*
Jay Pravda, *Gainesville*
Massimo Raimondo, *Jacksonville*
GS Raju, *Galveston*
Raymund R Razonable, *Minnesota*
Murray B Resnick, *Providence*
Adrian Reuben, *Charleston*
Douglas K Rex, *Indianapolis*
Victor E Reyes, *Galveston*
Basil Rigas, *New York*
Yehuda Ringel, *Chapel Hill*
Richard A Rippe, *Chapel Hill*
Maribel Rodriguez-Torres, *Santurce*
Marcos Rojkind, *Washington*
Philip Rosenthal, *San Francisco*
Barry Rosser, *Jacksonville Florida*
Hemant K Roy, *Evanston*
Sammy Saab, *Los Angeles*
Shawn D Safford, *Norfolk*
Dushyant V Sahani, *Boston*
Bruce E Sands, *Boston*
James M Scheiman, *Ann Arbor*
Eugene R Schiff, *Miami*
Nicholas J Shaheen, *Chapel Hill*
Vanessa M Shami, *Charlottesville*
Prateek Sharma, *Kansas City*
Harvey L Sharp, *Minneapolis*
Stuart Sherman, *Indianapolis*
Shivendra Shukla, *Columbia*
Alphonse E Sirica, *Virginia*
Shanthi V Sitaraman, *Atlanta*
Stuart J Spechler, *Dallas*
Shanthi Srinivasan, *Atlanta*
Michael Steer, *Boston*
Peter D Stevens, *New York*
Charmaine A Stewart, *Rochester*
Christian D Stone, *Saint Louis*
Gary D Stoner, *Columbus*
R Todd Stravitz, *Richmond*
Liping Su, *Chicago*
Christina Surawicz, *Seattle*
Robert W Summers, *Iowa City*
Wing-Kin Syn, *Durham*
Gyongyi Szabo, *Worcester*
Yvette Taché, *Los Angeles*
Seng-Lai Tan, *Seattle*
Andrzej S Tarnawski, *Orange*
K-M Tchou-Wong, *New York*
Jonathan P Terdiman, *San Francisco*
Neil D Theise, *New York*
Christopher C Thompson, *Boston*
Swan N Thung, *New York*
Michael Torbenson, *Baltimore*
Natalie J Torok, *Sacramento*
RA Travagli, *Baton Rouge*
George Triadafilopoulos, *Stanford*
Chung-Jyi Tsai, *Lexington*
Janet Elizabeth Tuttle-Newhall, *Durham*
Andrew Ukleja, *Florida*
Michael F Vaezi, *Nashville*
Hugo E Vargas, *Scottsdale*
Arnold Wald, *Wisconsin*
Scott A Waldman, *Philadelphia*
Jian-Ying Wang, *Baltimore*
Timothy C Wang, *New York*
Irving Waxman, *Chicago*
Steven A Weinman, *Galveston*
Steven D Wexner, *Weston*
Keith T Wilson, *Baltimore*
Jacqueline L Wolf, *Boston*
Jackie Wood, *Ohio*
George Y Wu, *Farmington*
Jian Wu, *Sacramento*
Samuel Wyllie, *Houston*
Wen Xie, *Pittsburgh*
Vijay Yajnik, *Boston*
Vincent W Yang, *Atlanta*
Francis Y Yao, *San Francisco*
Hal F Yee, *San Francisco*
Xiao-Ming Yin, *Pittsburgh*
Min You, *Tampa*
Zobair M Younossi, *Virginia*
Liqing Yu, *Winston-Salem*
David Yule, *Rochester*
Ruben Zamora, *Pittsburgh*
Michael E Zenilman, *New York*
Zhi Zhong, *Chapel Hill*
Michael A Zimmerman, *Colorado*
Stephen D Zucker, *Cincinnati*

Uruguay

Henry Cohen, *Montevideo*

[1]Passed away on October 20, 2007
[2]Passed away on June 11, 2007

National Journal Award 2005

World Journal of Gastroenterology®

Weekly Established in October 1995

Volume 14 Number 36
September 28, 2008

Contents

Contents

World Journal of Gastroenterology
Volume 14 **Number** 36 **September** 28, 2008

World Journal of Gastroenterology
Volume 14 **Number** 36 **September** 28, 2008

Contents

NAME OF JOURNAL
World Journal of Gastroenterology

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

EDITING
Editorial Board of *World Journal of Gastroenterology,* Room 903, Ocean International Center, Building D, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

PUBLISHING
The WJG Press and Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Ocean International Center, Building D, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

PRINTING
Beijing Kexin Printing House

OVERSEAS DISTRIBUTOR
Beijing Bureau for Distribution of Newspapers and Journals
(Code No. 82-261)
China International Book Trading Corporation PO Box 399, Beijing, China (Code No. M4481)

PUBLICATION DATE
September 28, 2008

EDITOR-IN-CHIEF
Lian-Sheng Ma, *Beijing*

SUBSCRIPTION
RMB 50 Yuan for each issue, RMB 2400 Yuan for one year

CSSN
ISSN 1007-9327
CN 14-1219/R

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5489

World J Gastroenterol 2008 September 28; 14(36): 5489-5490
World Journal of Gastroenterology ISSN 1007-9327

EDITORIAL

Crohnology: A tale of time and times and inflammatory bowel diseases

Fernando Gomollón, Javier P Gisbert, Miquel Ángel Gassull

Fernando Gomollón, Hospital Clínico Universitario, Zaragoza, Spain; Centro de Investigación Biomédica en Enfermedades Hepáticas y Digestivas, CIBEREHD, IACS, Spain
Javier P Gisbert, Hospital Universitario de la Princesa, CIBEREHD, Madrid, Spain
Miquel Ángel Gassull, Instituto de Investigación en Ciéncias de la Salud, Fundación Germans Trias i Pujol, CIBEREHD, Badalona, Spain
Author contributions: Gomollón F, Gisbert JP, and Gassull MÁ contributed equally to this work.
Correspondence to: Fernando Gomollón, Hospital Clínico Universitario "Lozano Blesa" Zaragoza, Avenida San Juan Bosco 15, Zaragoza 50009, Spain. fgomollon@gmail.com
Telephone: +34-87-6766000 Fax: +34-97-6768846
Received: July 29, 2008 Revised: August 6, 2008
Accepted: August 13, 2008
Published online: September 28, 2008

Abstract

Time, times and timing are key words in inflammatory bowel diseases (IBD). The leitmotif of this issue or *World Journal of Gastroenterology* is time. We have asked experts to review on the epidemiology of these diseases over time, the changes in innate immunity that could be present in the first time, and then the timing of key treatments. The correct time of using azathioprine, mercaptopurine, infliximab, cyclosporine and surgery are reviewed. We have chosen experts with not only great clinical expertise but also personal interest in clinical and basic investigation. Our goal in this monograph is to get an idea not only of the present but of the immediate future in some of the key management issues in IBD. To this end, we think that the authors are the most adequate.

Key words: Inflammatory bowel diseases; Epidemiology; Innate immunity; Treatment

Gomollón F, Gisbert JP, Gassull MÁ. Crohnology: A tale of time and times and inflammatory bowel diseases. *World J Gastroenterol* 2008; 14(36): 5489-5490 Available from: URL: http://www.wjgnet.com/1007-9327/14/5489.asp DOI: http://dx.doi.org/10.3748/wjg.14.5489

Life and time are concepts that can not be apart[1]. In fact, from a medical point of view, life, time and disease are inseparable concepts. The importance of time in life is being clarified by basic scientists and chronobiology is an area of knowledge steadily growing[2]. As we are writing on inflammatory bowel diseases (IBDs) we have misspelled on purpose the word *crohnology*, as new additional tribute to Burrill Bernard Crohn[3]. In fact, we borrowed the fundamental idea from another great expert, not only on Beethoven's piano sonatas, but on IBDs: David Sachar. He wrote some years ago an excellent editorial on this subject[4], and although he thinks that, at the end, no one reads his articles, he was wrong this time: we do. David Sachar wrote (sic): … *"time course* of disease progression should enter into our phenotyping schemes as elements in their own right" and also: "to understand the time course of Crohn's disease …, we have to figure out when and where to start the clock". Time is the *leitmotif* of this monographic issue of the *World Journal of Gastroenterology*.

First, IBDs are diseases of our times. Diseases affecting human beings have greatly changed over time[5]. In Spain, Crohn's disease could not to be found in most standard textbooks as late in the twentieth century as 1975. Now the Spanish working group on IBD (GETECCU, http://www.geteccu.org) has more than 300 gastroenterologists as active members. Of course, this can not be taken as an epidemiological figure, but it may be an idea about the quick changes in some of the health needs in our current world. Whatever the complex reasons for these dramatic changes, environmental factors must be a prominent influence, because the time taken to these changes is too short to blame genetics to explain them (at least in full). It is difficult to forget the similarities with cigarette smoking. "The IBDs' century "could be a very adequate title for a book on Crohn's disease (CD) and Ulcerative Colitis (UC)[6].

Second, we take a look to a very interesting hypothesis: "the first precursors of recognizable Crohn's disease"[3]. Professor Eduard Stange and co-workers, from Germany, suggests that IBDs are in fact the final consequence of a defective innate immunity against bacteria. Their data on defensins have been the matter of controversy in most recent meetings on IBDs. He has written a short review on the topic. The disturbances of adaptative immunology in advanced IBDs were first recognized; however, innate immunity errors are emerging as key clues to chronic inflammation[7]. The "defensins hyphothesis" is not unique, but it is very attractive and gives a refreshing point of view to the pathogenesis of IBD. In fact, human brain is more adapted to see what it is there, but has

serious difficulties to think of absent things[8]. Could it be possible that the lack of adequate defense mechanisms being the primary phenomenon, explain the final excess of inflammation? Certainly this seems an interesting and attractive way to explore.

Most of the chapters in this issue are directly related to the real life situations for a clinician in care of IBDs' patients. Clinicians have to take, or at least help to take, many decisions. Ideally, these decisions should be taken on time and always trying to avoid delays; that is, the time elapsed to take these decisions has to be the correct and needed time. Time is important for us, but much more for our patients. In certain occasions taking decisions on time it is not easy at all and we make errors[9]. These mistakes have sometimes very undesirable consequences to our patients. Timing of decisions is a key factor in our practice. In this issue the answer to some critical questions has been addressed.

In the very difficult clinical scenario of severe ulcerative colitis, there are two crucial questions: how long, if no response is obtained, should steroids be maintained to define *resistance*? and if steroids do not work: it is time for cyclosporine or for infliximab? These points are often discussed in meetings, but we do want the best answers. Therefore, we have asked these questions to real experts. Dr. María Esteve has been working for years both in basic and clinic research and at the same time she has great clinical experience; she is a real translational physician. She can approach steroid resistance from both points of view, and this was exactly our aim. The same can be said from Drs Gert van Asche and Severine Vermeire. Of course we know that currently there is not a formal clinical trial comparing cyclosporine and infliximab in severe UC, but patients are there and we have to help them just on time; today, not tomorrow. We expect that these two experts help us to understand the pros and cons for using these drugs at the proper time and for how long.

Azathioprine and mercaptopurine are the most important drugs in the maintenance treatment of IBDs[10]. Much has been written on these very useful old friends. However, do we start these treatments at the proper time or do delay theis use too much? This is a common and important clinical question, not very easy to answer. It is about prediction, a rather difficult science. Can we predict clinical course and therefore select the adequate timing for starting treatment? In any case, many patients are finally on immunomodulators, and many do stay quite well during years: so, is it a time to withdraw them?

Biologics are newer therapies[11], but are here to stay. There are, at least, two very different points of view. Many clinicians, as well as regulatory agencies[12], do think that these drugs should be used at later stages in therapy, after the failure of conventional treatments; high cost and high risks are the main arguments for this position. However, this way if thinking it seems rather intuitive (intuition is of course not sort of scientific proof) that administering treatment earlier could beneficially modify the natural history of disease. So many clinicians, as well as pharmaceutical companies, think that the earlier biological are administered, clearly the better. In fact there is some recent evidence supporting this argument[13]. The timing of this treatment seems critical. We think that giving both points of views would be interesting and useful for the clinician.

Finally, few decisions are as difficult as timing surgery in IBDs. Sometimes we decide too late (don't you have at least one patient telling you: Hey doc, why did we go through surgery so late?), perhaps sometimes the decision was taken too early (some refractory pouchitis could comment on this side). Few tips may help in those difficult clinical nodes. In addition, after surgery it is also time to act: you need to prevent recurrence. How is not easy to say, but we also need to know when.

Words are almost living beings. We have chosen times, time and timing, to think over IBDs. But, it is time to finish. It is time to continue working to help our IBDs' patients.

REFERENCES

1 **Mayr E**. What makes biology unique? London: Cambridge University Press, 2007: 1-232
2 **Fuller PM**, Lu J, Saper CB. Differential rescue of light- and food-entrainable circadian rhythms. *Science* 2008; **320**: 1074-1077
3 **Crohn BB**, Ginzburg L, Oppenheimer GD. Regional ileitis: a pathologic and clinical entity. 1932. *Mt Sinai J Med* 2000; **67**: 263-268
4 **Sachar DB**. Einstein, Darwin and conventional wisdom about Crohn's disease. *Dig Liver Dis* 2005; **37**: 227-229
5 **Diamond J**. Guns, Germs and Steel: The Fates of Human Society. New York: W.W. Norton, 1997: 1-480
6 **Brandt AM**. The cigarette century. New York: Basic Books, 2007: 1-672
7 **Kim EY**, Battaile JT, Patel AC, You Y, Agapov E, Grayson MH, Benoit LA, Byers DE, Alevy Y, Tucker J, Swanson S, Tidwell R, Tyner JW, Morton JD, Castro M, Polineni D, Patterson GA, Schwendener RA, Allard JD, Peltz G, Holtzman MJ. Persistent activation of an innate immune response translates respiratory viral infection into chronic lung disease. *Nat Med* 2008; **14**: 633-640
8 **Gilbert D**. Stumbling on happiness. 1st ed. New York: Vintage Books, 2007
9 **Gisbert JP**, Gomollon F. [Common errors in the management of the seriously ill patient with inflammatory bowel disease] *Gastroenterol Hepatol* 2007; **30**: 294-314
10 **Gisbert JP**, Gomollon F, Mate J, Pajares JM. [Questions and answers on the role of azathioprine and 6-mercaptopurine in the treatment of inflammatory bowel disease] *Gastroenterol Hepatol* 2002; **25**: 401-415
11 **Panes J**, Gomollon F, Taxonera C, Hinojosa J, Clofent J, Nos P. Crohn's disease: a review of current treatment with a focus on biologics. *Drugs* 2007; **67**: 2511-2537
12 **Chen YF**, Jobanputra P, Barton P, Jowett S, Bryan S, Clark W, Fry-Smith A, Burls A. A systematic review of the effectiveness of adalimumab, etanercept and infliximab for the treatment of rheumatoid arthritis in adults and an economic evaluation of their cost-effectiveness. *Health Technol Assess* 2006; **10**: iii-iv, xi-xiii, 1-229
13 **D'Haens G**, Baert F, van Assche G, Caenepeel P, Vergauwe P, Tuynman H, De Vos M, van Deventer S, Stitt L, Donner A, Vermeire S, Van de Mierop FJ, Coche JC, van der Woude J, Ochsenkuhn T, van Bodegraven AA, Van Hootegem PP, Lambrecht GL, Mana F, Rutgeerts P, Feagan BG, Hommes D. Early combined immunosuppression or conventional management in patients with newly diagnosed Crohn's disease: an open randomised trial. *Lancet* 2008; **371**: 660-667

S- Editor Xiao LL **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5491

World J Gastroenterol 2008 September 28; 14(36): 5491-5498
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Javier P Gisbert, Professor; Fernando Gomollón, Dr., MD, PhD, *Series Editors*

Inflammatory bowel diseases: A disease (s) of modern times? Is incidence still increasing?

Cristina Saro Gismera, Beatriz Sicilia Aladrén

Cristina Saro Gismera, Service of Digestive Diseases, Hospital de Cabueñes, Gijón, Spain
Beatriz Sicilia Aladrén, Service of Digestive Diseases, Alcañiz Hospital, Spain
Author contributions: Saro C and Sicilia B contributed equally to this work.
Correspondence to: Beatriz Sicilia Aladrén, Service of Digestive Diseases, Alcañiz Hospital, Dr. Repolles 2, 44600 Alcañiz, Teruel, Spain. bsicilia@salud.aragon.es
Telephone: +34-97-8830100 Fax: +34-97-8830977
Received: July 30, 2008 Revised: August 6, 2008
Accepted: August 13, 2008
Published online: September 28, 2008

Abstract

Inflammatory bowel diseases (IBD) are a heterogeneous group of diseases, not always easy to diagnose, even more difficult to classify, and diagnostic criteria are not always uniform. Well done population-based studies are not abundant, and so comparisons among different geographical areas or populations are not always very reliable. In this article, we have reviewed epidemiological studies available on the world's population while making a critical review of published data.

Key words: Inflammatory bowel diseases; Epidemiological; Incidence; Diagnostic criteria

Saro C, Sicilia B. Inflammatory bowel diseases: A disease (s) of modern times? Is incidence still increasing? *World J Gastroenterol* 2008; 14(36): 5491-5498 Available from: URL: http://www.wjgnet.com/1007-9327/14/5491.asp DOI: http://dx.doi.org/10.3748/wjg.14.5491

INTRODUCTION

There is no doubt about the key role of epidemiology in modern medicine. The burden of inflammatory bowel diseases (IBDs) is changing so quickly, and shows such geographical heterogeneity, that the challenge for epidemiologists is a formidable one. The main goal of this review is to furnish a *panorama* of these studies all over the world.

First, we have to underline some methodological difficulties. IBDs are a heterogeneous group of diseases, not always easy to diagnose, even more difficult to classify, and diagnostic criteria are not always uniform. And we can not forget that in many countries IBD were rare only 30 years ago. In these stages, hospital based studies are rather common, but of course with many bias. Well done population-based studies are not abundant, and so comparisons among different geographical areas or populations are not always very reliable. The heterogeneity of genotypes and phenotypes has been confirmed in the most recent reports. So, sound studies should be based on clear definitions and internationally accepted classification. Otherwise, some common misconceptions will be perpetuated.

For instance, it is common to find in the introduction of epidemiological reports these type of sentences: "One of the few aspects of the epidemiology of inflammatory bowel disease that seems indisputable is the emergence of both ulcerative colitis and Crohn's disease in the economically developed nations of Western Europe and North America during the past century"[1] or "It has been suggested that the incidence of inflammatory bowel disease (IBD), which includes Ulcerative colitis and Crohn's disease, is three or more times higher in Northern than in Southern Europe"[2]. It is the widespread idea that incidence of this group of diseases is low in Africa and Asia, as opposed to the more developed countries, particularly in Northern Europe and Central and North-America. However, other authors are critical with the geographical difference theory and try to clarify the concept: "The incidence figures for Ulcerative colitis and Crohn's disease have been difficult to interpret, and geographical variations may be due to differences in classification criteria and study design."[3]

We can see in Figure 1, how epidemiological studies published so far with high quality in methodology (clear definitions, prospective, population-based), are mostly grouped in the same areas[2,4-45] while in areas considered low incidence, coinciding with areas of low socioeconomic level, there are no relevant studies. In most undeveloped countries there are few epidemiological studies and the few published until now are limited to very specific areas for a centralized hospital and often retrospectively. The authors of these reports emphasize

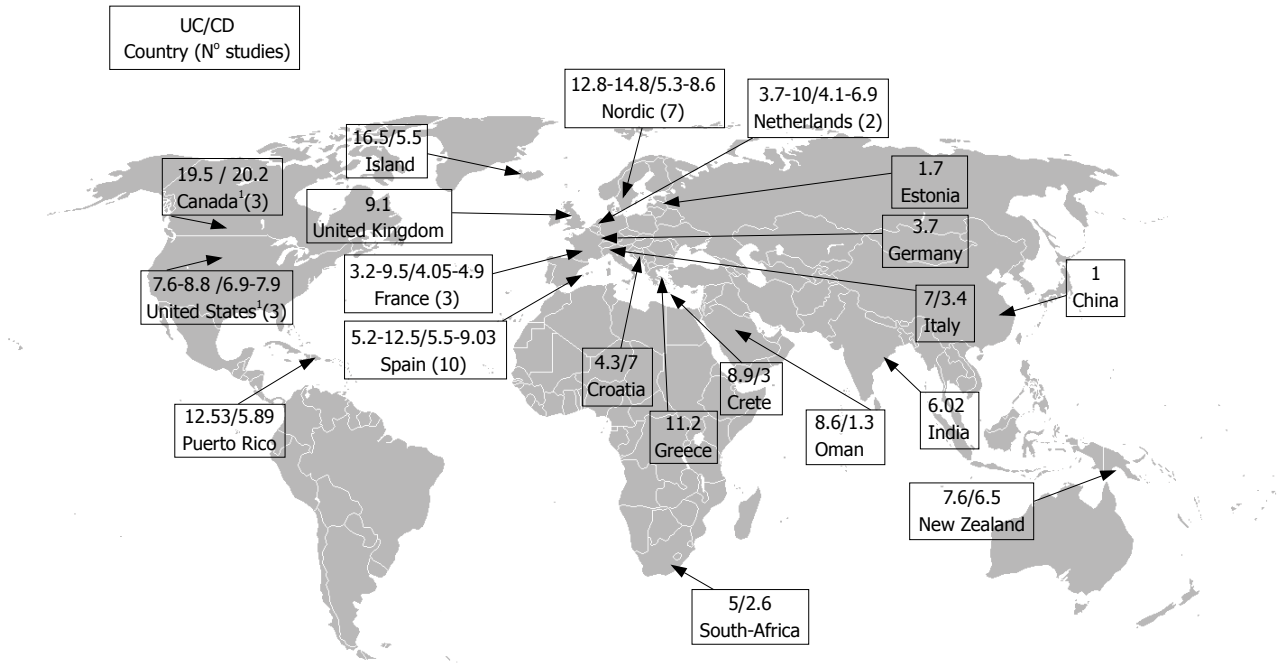


Figure 1 Prospective and population-based studies: UC and CD. [1]Retrospective but population-based data base of inflammatory bowel disease. Nº studies: Number of published studies.

the probable bias of their results[46].

In Asia, there is only 1 prospective study in Hong Kong[41] and 4 retrospective studies in Japan[47], Singapore[48] and Korea[46,49]. Just one study in India[42] and another one in the Arabian Peninsula, in Oman[43]. Studies of Israel are included in Europe. In Africa, data arrive from Cape Town[44]. Recently, a study conducted in New Zealand[45] describes incidence rates similar to those described in "developed" countries or "Northern Europe".

In the United States, where the health system is not ideal for conducting population studies because 45 million inhabitants are not covered by a health care system, the available studies, though retrospective, are based on the implementation of a "population-based data base of inflammatory bowel disease" which allows the identification of these diseases with a high sensitivity and specificity with the advantage of results over extensive periods of time[7-9]. In Canada it is been used the same methodology for collecting data[4-6].

After all, the general data are not very consistent, so it is difficult to make comparisons. In this article, we have reviewed epidemiological studies available on the world's population while making a critical review of published data.

INCIDENCE RATES IN DIFFERENT EPIDEMIOLOGICAL STUDIES

Retrospective studies

We show in Table 1 a summary of retrospective studies published until now[4-9,46-92] and the different rates of incidence: as you can see, many of the them are hospital-based[47,48,54,64,77,88,90], not surprisingly showing a worse clinical course with increased morbidity. Population-based studies demonstrate that at least 50% of patients do not require hospital admission, so hospital-based studies are clearly biased showing only the most severe group of patients. We would like to note also that incidence rates are highly variable even in the same geographical areas (for example in UK, incidence varies from 7.1 to 15 for UC)[52,53], a fact explained by methodological heterogeneity.

Prospective and population-based studies

The results of prospective studies (Figure 1) show much more homogeneity. However, there are high incidence areas such as Iceland[11] and Puerto Rico[10] and very low incidence areas such as China[41] and Oman[43]. The other studies do show intermediate rates and, in general the more recent the study the higher the incidence rates[12,20,27]. In most prospective studies, the incidence rates are higher for UC than for CD; only in the Croatian study[39] this ratio is reversed, as is found also in pediatric studies. In Canadian reports incidence rate of UC and CD tend to equalize[4-6], reflecting the high incidence of CD in this country.

In Europe we have multiple population-based and prospective studies, allowing us to comparing different areas (Figures 1, 2 and 3)[2,11-40]. In a multicenter, international, and well designed study led by Shivananda[2] from 1991 to 1993 and covering several areas of Europe comparing the incidence rates of inflammatory bowel disease (IBD) between North and South, the authors did confirm higher incidence rates for Northern (UC = 11.4 and CD = 6.3) than for Southern Europe (UC = 8 and CD = 3.6); however, when you take off extreme data studies (Iceland, Oslo, Lisbon and Ioannina), this difference loses its significance: for Northern Europe (UC = 9.5 and CD = 5.83) *vs* (UC = 8.79 and CD = 3.84) in Southern

Table 1 Incidence rates of ulcerative colitis and Crohn's disease (cases per 100 000 habitant per year) in retrospective studies

Authors	Study	Location	Year	Incidence UC	Incidence CD
Gilat *et al*[50]	RP	Tel Aviv-Jafo	1961-1970	3.7	-
Sinclair *et al*[51]	RP	Nor east Scotia	1975	15.8	-
Morris *et al*[52]	RP	Cardiff (UK)	1968-1977	7.2	-
Devlin *et al*[53]	RP	North Tees (UK)	1971-1977	15.1	5.3
Binder *et al*[54]	RH	Copenhagen	1962-1978	8.1	2.1
Jacobson *et al*[55]	RP	Jerusalem	1973-1978	6.3	-
Stonnington *et al*[56]	RP	Olmsted (EEUU)	1960-1979	15	-
Nyhlin *et al*[57]	RP	Sweden	1974-1981	-	4.9
Stewénius *et al*[58]	RP	Malmo (Sweden)	1958-1982	5.5	-
Grossman *et al*[59]	RP	Central Israel	1979	5.1	-
Ekbom *et al*[60]	RP	Sweden	1965-1983	10.4	-
Shivananda *et al*[61]	RP	Leiden (Holland)	1979-1983	7	-
Odes *et al*[62]	RP	South Israel	1961-1985	5.8	-
Odes *et al*[63]	RP	Beer Shiva	1981-1985	5.8	-
Stowe *et al*[64]	RH	Rochester (USA)	1973-1986	2.3	3.9
Niv *et al*[65]	RP	North Israel	1977-1986	3.8	-
Lindberg *et al*[66]	RP	Sweden	1963-1987	-	6.1
Langholz *et al*[67]	RP	Copenhagen	1962-1987	8.1	-
Munkholm *et al*[68]	RP	Copenhagen	1962-1987	-	4.1
Lapidus *et al*[69]	RP	Stockholm	1955-1989	-	4.6
Bjornsson *et al*[70]	RP	Island	1980-1989	11.7	3.1
Thomas *et al*[71]	RP	Cardiff (UK)	1986-1990	-	5.9
Tan *et al*[48]	RH	Singapore	1981-1991	8.6	1.3
Morita *et al*[47]	RH	Japan	1991	5.85	0.51
Tsianos *et al*[72]	RP	Nor East Greece	1982-1991	4	0.3
Odes *et al*[73]	RP	Israel	1987-1992	-	4.2
Fonager *et al*[74]	RP	Denmark	1981-1992	13.2	4.6
Tragnone *et al*[75]	RP	Italy	1981-1992	5.2	2.3
Trallori *et al*[76]	RP	Florence	1990-1992	9.6	3.4
Linares de la Cal *et al*[77]	RH	Panama Argentina	1987-1993	1.2	0
Loftus *et al*[7,8]	RP	Olmsted (USA)	1940-1993	7.6	6.9
Shapira *et al*[78]	RP	Kineret (Israel)	1965-1994	3.5	-
Bernstein *et al*[4]	RP	Canada	1989-1994	14.3	14.6
Blanchard *et al*[5]	RP	Canada	1987-1996	15.6	15.6
Yapp *et al*[79]	RP	Cardiff (UK)	1991-1995	-	5.6
Rubin *et al*[80]	RP	UK	1995	13.9	8.3
Niv *et al*[81]	RP	Israel	1987-1997	-	5
Yang *et al*[49]	RP	Seoul (Chorea)	1986-1997	0.68	-
García Rodríguez *et al*[82]	RP	UK	1995-1997	11	8
Tsianos *et al*[83]	RP	Greece	1982-1997	4	0.3
Molinie *et al*[84]	RP	France	1988-1999	4	6
Bernstein *et al*[6]	RP	Canada	1998-2000	11.8	13.4
Loftus *et al*[9]	RP	Olmsted (USA)	1940-2000	8.8	7.9
Lakatos *et al*[85]	RP	Hungary	1977-2001	11.01	4.68
Lapidus *et al*[86]	RP	Stockholm	1990-2001	-	8.3
Tsironi *et al*[87]	RP	UK	1997-2001	8.2	7.3
Al-Gamdi *et al*[88]	RH	Indian	1983-2002	-	0.94
Jacobsen BA *et al*[89]	RP	Denmark	1998-2002	16.8	9.6
Gheorghe *et al*[90]	RH	Rumania	2002-2003	0.97	0.5
Nerich *et al*[91]	RP	France	2000-2003	7.2	8.2
Yang *et al*[46]	RP	Seoul	1986-2005	0.53	1.51
Cachia *et al*[92]	RP	Malta	1993-2005	8.16	1.3

RP: Retrospective poblational; RH: Retrospective hospital.

Europe. It is worth to remember that in 1991 the socioeconomic status varied greatly in Europe, with Northern countries considerably more wealthy, a gap that is considerably less profound in 2008. With these results, we would like to highlight data from different areas: Exceeding 10 UC patients/100 000 habitants/year: Iceland[11], Norway[15-17], Copenhagen[12], Heraklion (Greece)[34] and more recent Spanish studies[27,28]; Between 5 and 10 UC patients/100 000 habitants/year: Leicester (UK)[21], Maastricht[33], France[20], Italy[38], and most Spanish studies[23-25]; Below 5 UC patients/100 000 habitants/year: Croatia[39], North of France[18,19], Brussels[32], Germany[37] and Estonia[40].

Spain is one of the European countries with more prospective and population-based published studies[22-31] (Figure 3). The results are fairly uniform in the studies made at the same time periods, with high incidence areas in the latest published studies as Navarra[28] and Asturias[27]. The results of Spanish studies are not different from those presented throughout Europe (data varying from 5 to 10 cases of UC/100 000 habitants/year and from 3.5 to 6.5 cases

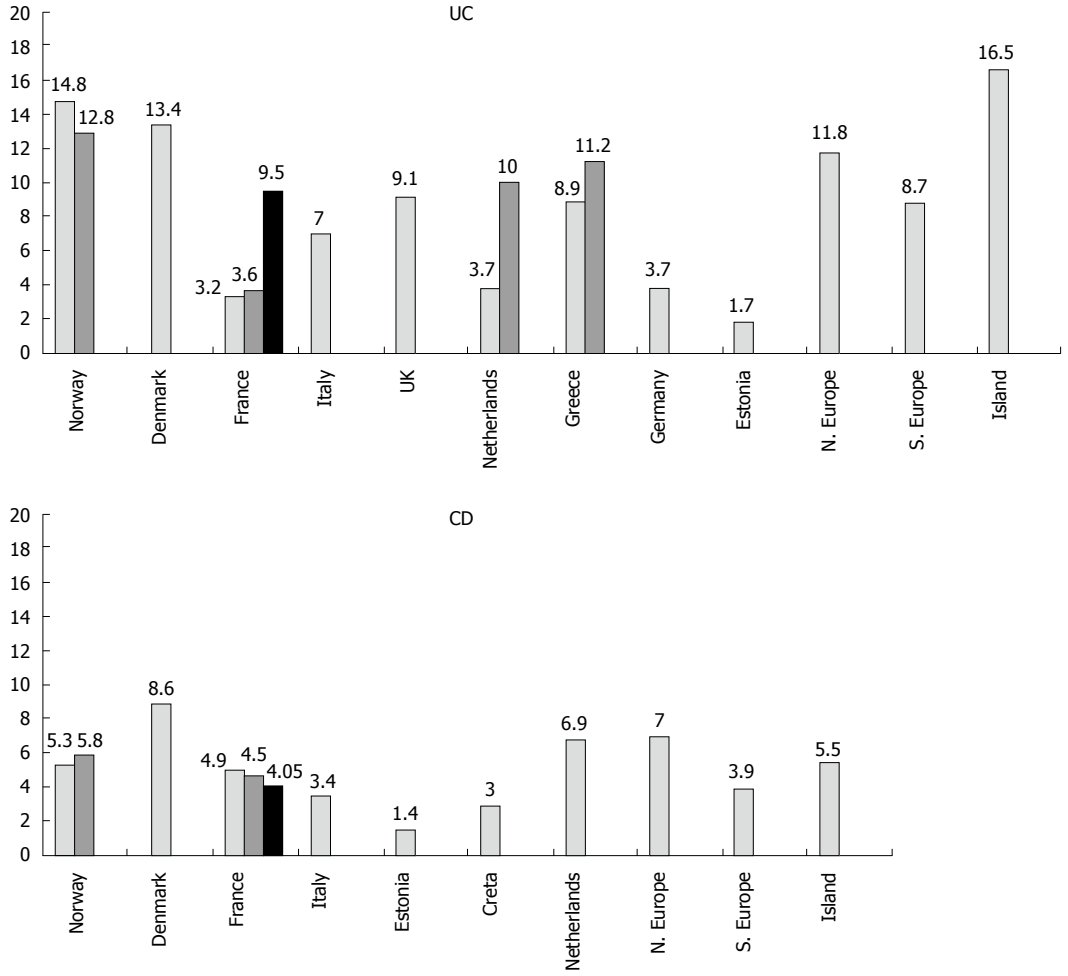


Figure 2 UC and CD incidence in prospective and poblational. European studies excepting Spain. Different color means there is more than one study in the same country and goes in chronological order from the first published study to the last.

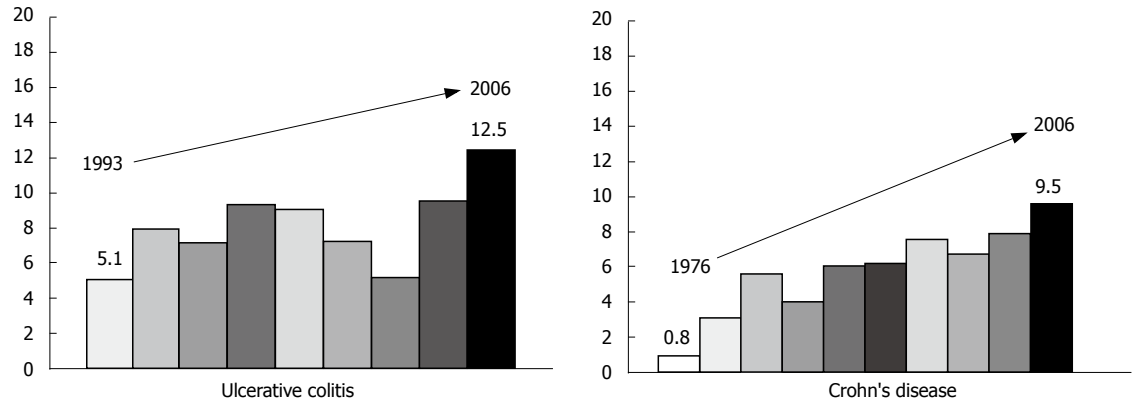


Figure 3 Prospective and population-based Spanish studies over time. UC: Incidence rates from 1993 to 2006; CD: Incidence rates from 1976 to 2006.

of CD/100 000 habitants per year). When comparing the results of the European study, data from Spanish studies are similar: North Europe (UC-CD = 9.5-5.83/100 000 habitants per year) *versus* (UC-CD = 8.79-3.84/100 000 habitants per year) in southern Europe versus (UC-CD = 7-9.3/4.8-6.08/100 000 habitants per year) in Spain.

VARIATIONS IN INCIDENCE OVER TIME

The general observations in northern Europe and America show that incidence of UC increased substantially in the early and middle third of 1900's, without changes afterwards, while the incidence of CD has been rising since the mid-1900s[5]. In Western world, it has been a clear increase in incidence in both CD and UC during the sixties and seventies after reaching a plateau between 7 and 12 new cases/100 000 habitants per year, respectively. Nowadays, rates tend to stabilize in areas of high incidence as in northern Europe and America, while in areas with low incidence as southern Europe, Asia and developing countries[93] these incidence

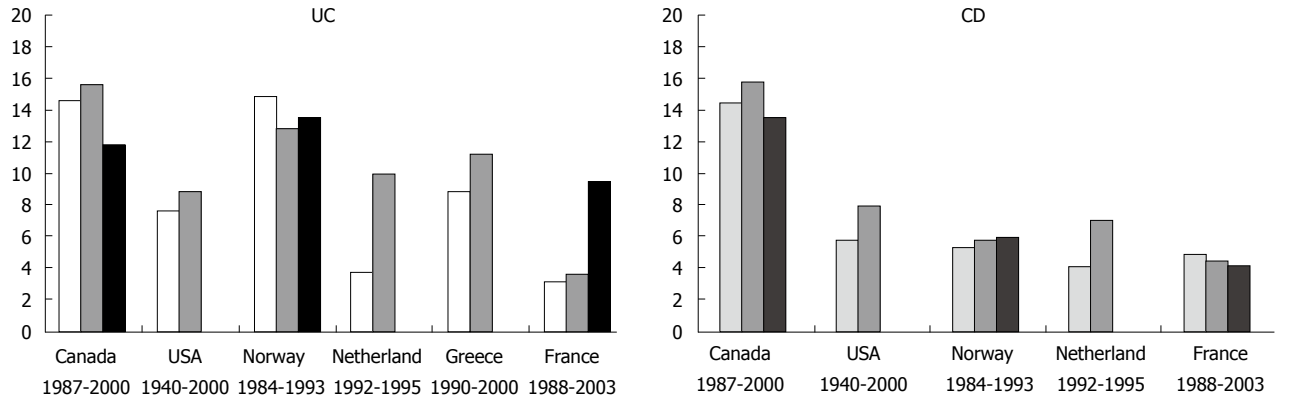


Figure 4 Incidence studies through time.

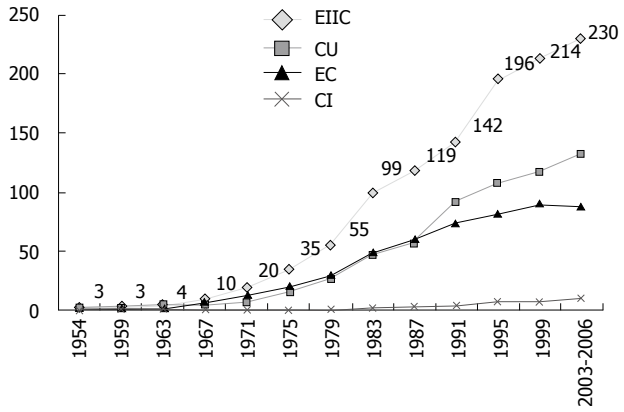


Figure 5 UC and CD incidence rates in Asturias from 1954 to 2006.

dada keep raising.

However, the truth is that we do have little reliable data to establish these comparisons. Few studies are repeated in the same areas and with the same diagnosed criteria or at different consecutive periods or long-term monitoring. In many cases, rates come indirectly from studies with records of hospital admissions and mortality studies. Moreover no less than 12% of patients diagnosed of UC have to be reclassified, as it has been described in some studies[3]. In Figures 3 and 4 we show the studies in the same areas at different periods of time describing differences in incidence rates over time.

In Canada and the United States, and although we have already commented that the methodology is retrospective, data collection of incident cases is comprehensive and based on a database created specifically to collect all incident cases of inflammatory bowel disease; the advantage of these studies is to be able to observe how incidence of these diseases varies over time because data are collected using the same methodology and results are easily comparable. The American Group has published 3 studies[7-9] analyzing the incidence of UC and CD between 1940 and 2000, describing a progressive increase since 1940 in both diseases, afterwards their data remain stable about 30 years, and later on, incidence starts increasing again mainly with CD (increase of 31%) since 1991 remaining UC incidence stable and even declining (7%). The Canadian group has also published 3 studies[4-6] showing the higher incidence of CD never published and described since 1987, remaining stable since then until now; the incidence rates of UC are also high, but similar to other European countries (Iceland, Denmark) and also remains stable.

Analyzing the evolution of incidence rates in European countries we can see that in most of them both rates of UC and CD have been increasing over the years. In relation to UC, we see how the population studies conducted in France[18-20], Greece[34-36] and Netherlands[32,33] show a gradual increase over time; in Norway remains stable and even "declining" but with very high levels (14.8-13.6/100 000 inhabitants per year). In relation to CD, we can see analogous evolution as UC except in France where this trend seems to be the opposite, but with little difference between the studies (4.9-4.05/100 000 habitants per year).

In Spain, we can see how incidence of UC increases gradually with the only exception of one study[29] and also, we can see how clearly CD incidence has increased progressively since 1978 in which the first prospective and population study of CD[22] was conducted in our country with rates of 0.5 patients/100 000 habit per year. The 2 studies conducted more recently in Spain, show really high incidence rates of both UC and CD: Arin *et al*[28] in Navarra observed an incidence between 2001 and 2003 from UC and EC of 9.57 and 585 patients/100 000 habit per year respectively; the Asturian study (Figure 5), with a long-term follow-up (historical cohort from 1954 until December 2006)[27] noted how since 1954 incident cases of IBD have been gradually increasing, with two points of inflection in the 80's and in 1991. The first coincides with the introduction of endoscopy in our hospitals as a diagnostic method and the second coincides with the beginning of the prospective study. You can see a clear increase of incidence in the second period of the study, but these results may be biased, since in the first period, the data are retrospective. Finally, in prospective study (1991-2006) there are no significant differences in incidence rates ($P = 0.08$).

CONCLUSION

To summarize, we can conclude that: Incidence rates have been increasing in developed countries and finally reaching a plateau in most studies. Data from underdeveloped countries are scarce and of low methodological quality, but most suggest lower incidence rates of IBDs in these areas.

The geographical variability is, in part, the consequence of differences in methods and diagnostic resources. However, when sequential and reliable data are available from the same area, incidence rates do really change.

The rather quick change of incidence does strongly suggest that environmental factors have a clear etiological role in IBDs. The factor (or factors) has something in common with economical development: those diseases seem to raise in parallel with per capita income[94].

New, methodologically sound studies are needed. We need transversal studies, but longitudinal ones with long-term follow-up will be probably more important to reveal new clues for etiological investigation.

REFERENCES

1 **Logan RF**. Inflammatory bowel disease incidence: up, down or unchanged? *Gut* 1998; **42**: 309-311
2 **Shivananda S**, Lennard-Jones J, Logan R, Fear N, Price A, Carpenter L, van Blankenstein M. Incidence of inflammatory bowel disease across Europe: is there a difference between north and south? Results of the European Collaborative Study on Inflammatory Bowel Disease (EC-IBD). *Gut* 1996; **39**: 690-697
3 **Moum B**, Ekbom A, Vatn MH, Aadland E, Sauar J, Lygren I, Schulz T, Stray N, Fausa O. Inflammatory bowel disease: re-evaluation of the diagnosis in a prospective population based study in south eastern Norway. *Gut* 1997; **40**: 328-332
4 **Bernstein CN**, Blanchard JF, Rawsthorne P, Wajda A. Epidemiology of Crohn's disease and ulcerative colitis in a central Canadian province: a population-based study. *Am J Epidemiol* 1999; **149**: 916-924
5 **Blanchard JF**, Bernstein CN, Wajda A, Rawsthorne P. Small-area variations and sociodemographic correlates for the incidence of Crohn's disease and ulcerative colitis. *Am J Epidemiol* 2001; **154**: 328-335
6 **Bernstein CN**, Wajda A, Svenson LW, MacKenzie A, Koehoorn M, Jackson M, Fedorak R, Israel D, Blanchard JF. The epidemiology of inflammatory bowel disease in Canada: a population-based study. *Am J Gastroenterol* 2006; **101**: 1559-1568
7 **Loftus EV Jr**, Silverstein MD, Sandborn WJ, Tremaine WJ, Harmsen WS, Zinsmeister AR. Crohn's disease in Olmsted County, Minnesota, 1940-1993: incidence, prevalence, and survival. *Gastroenterology* 1998; **114**: 1161-1168
8 **Loftus EV Jr**, Silverstein MD, Sandborn WJ, Tremaine WJ, Harmsen WS, Zinsmeister AR. Ulcerative colitis in Olmsted County, Minnesota, 1940-1993: incidence, prevalence, and survival. *Gut* 2000; **46**: 336-343
9 **Loftus CG**, Loftus EV Jr, Harmsen WS, Zinsmeister AR, Tremaine WJ, Melton LJ 3rd, Sandborn WJ. Update on the incidence and prevalence of Crohn's disease and ulcerative colitis in Olmsted County, Minnesota, 1940-2000. *Inflamm Bowel Dis* 2007; **13**: 254-261
10 **Appleyard CB**, Hernandez G, Rios-Bedoya CF. Basic epidemiology of inflammatory bowel disease in Puerto Rico. *Inflamm Bowel Dis* 2004; **10**: 106-111
11 **Bjyrnsson S**, Jyhannsson JH. Inflammatory bowel disease in Iceland, 1990-1994: a prospective, nationwide, epidemiological study. *Eur J Gastroenterol Hepatol* 2000; **12**: 31-38
12 **Vind I**, Riis L, Jess T, Knudsen E, Pedersen N, Elkjaer M, Bak Andersen I, Wewer V, Norregaard P, Moesgaard F, Bendtsen F, Munkholm P. Increasing incidences of inflammatory bowel disease and decreasing surgery rates in Copenhagen City and County, 2003-2005: a population-based study from the Danish Crohn colitis database. *Am J Gastroenterol* 2006; **101**: 1274-1282
13 **Haug K**, Schrumpf E, Halvorsen JF, Fluge G, Hamre E, Hamre T, Skjollingstad R. Epidemiology of Crohn's disease in western Norway. Study group of Inflammatory Bowel Disease in Western Norway. *Scand J Gastroenterol* 1989; **24**: 1271-1275
14 **Haug K**, Schrumpf E, Barstad S, Fluge G, Halvorsen JF. Epidemiology of ulcerative colitis in western Norway. *Scand J Gastroenterol* 1988; **23**: 517-522
15 **Kildebo S**, Nordgaard K, Aronsen O, Breckan R, Burhol PG, Jorde R. The incidence of ulcerative colitis in Northern Norway from 1983 to 1986. The Northern Norwegian Gastroenterology Society. *Scand J Gastroenterol* 1990; **25**: 890-896
16 **Moum B**, Vatn MH, Ekbom A, Aadland E, Fausa O, Lygren I, Stray N, Sauar J, Schulz T. Incidence of Crohn's disease in four counties in southeastern Norway, 1990-93. A prospective population-based study. The Inflammatory Bowel South-Eastern Norway (IBSEN) Study Group of Gastroenterologists. *Scand J Gastroenterol* 1996; **31**: 355-361
17 **Moum B**, Vatn MH, Ekbom A, Aadland E, Fausa O, Lygren I, Sauar J, Schulz T, Stray N. Incidence of ulcerative colitis and indeterminate colitis in four counties of southeastern Norway, 1990-93. A prospective population-based study. The Inflammatory Bowel South-Eastern Norway (IBSEN) Study Group of Gastroenterologists. *Scand J Gastroenterol* 1996; **31**: 362-366
18 **Gower-Rousseau C**, Salomez JL, Dupas JL, Marti R, Nuttens MC, Votte A, Lemahieu M, Lemaire B, Colombel JF, Cortot A. Incidence of inflammatory bowel disease in northern France (1988-1990). *Gut* 1994; **35**: 1433-1438
19 **Latour P**, Louis E, Belaiche J. Incidence of inflammatory bowel disease in the area of Liege: a 3 years prospective study (1993-1996). *Acta Gastroenterol Belg* 1998; **61**: 410-413
20 **Abakar-Mahamat A**, Filippi J, Pradier C, Dozol A, Hebuterne X. Incidence of inflammatory bowel disease in Corsica from 2002 to 2003. *Gastroenterol Clin Biol* 2007; **31**: 1098-1103
21 **Carr I**, Mayberry JF. The effects of migration on ulcerative colitis: a three-year prospective study among Europeans and first- and second- generation South Asians in Leicester (1991-1994). *Am J Gastroenterol* 1999; **94**: 2918-2922
22 **Ruiz Ochoa V**. [Epidemiologic study of Crohn's disease in Galicia from 1976 to 1983] *Rev Esp Enferm Apar Dig* 1984; **66**: 273-279
23 **Brullet E**, Bonfill X, Urrutia G, Ruiz Ochoa V, Cueto M Clofent J. Estudio epidemiológico sobre la incidencia de Enfermedad Inflamatoria Intestinal en cuatro áreas españolas. *Med Clin* 1998; **110**: 651-656
24 **Gomollón F**, López J, Sainz R. High incidence of inflammatory bowel disease in Aragón (Spain): a prospective population based study. *Gastroenterology* 1995; **108** Suppl: A865
25 **Lopez Miguel C**, Sicilia B, Sierra E, Lopez Zaborras J, Arribas F, Gomollon F. [Incidence of inflammatory bowel disease in Aragon: outcome of a prospective population-based study] *Gastroenterol Hepatol* 1999; **22**: 323-328
26 **Saro Gismera C**, Lacort Fernández M, Argüelles Fernández G, Antón Magarzo J, García López R, Navascues CA, Suárez González A, Díaz Alvarez G, González Bernal A, Palacios Galán A, Altadill Arregui A, Vizoso F. [Incidence and prevalence of inflammatory bowel disease in Gijon,

Asturias, Spain] *Gastroenterol Hepatol* 2000; **23**: 322-327
27 **Saro Gismera C**, de la Coba C, Lacort Fernández M, González Bernal A, Álvarez Álvarez A, Pérez-Pariente JM, Navascues CA. Changes in incidence of inflammatory bowel disease during the last 15 years: A epidemiological prospective Population-Based Study from Gijón (Spain) (1992-2006). *Gut* 2007; **39** (Suppl 1): A129
28 **Arin Letamendia A**, Borda Celaya F, Burusco Paternain MJ, Prieto Martínez C, Martínez Echeverría A, Elizalde Apestegui I, Laiglesia Izquierdo M, Macias Mendizábal E, Tamburri Moso P, Sánchez Valverde F. [High incidence rates of inflammatory bowel disease in Navarra (Spain). Results of a prospective, population-based study] *Gastroenterol Hepatol* 2008; **31**: 111-116
29 **Garrido A**, Martinez MJ, Ortega JA, Lobato A, Rodriguez MJ, Guerrero FJ. Epidemiology of chronic inflammatory bowel disease in the Northern area of Huelva. *Rev Esp Enferm Dig* 2004; **96**: 687-691; 691-694
30 **Rodrigo L**, Riestra S, Nino P, Cadahia V, Tojo R, Fuentes D, Moreno M, Gonzrlez Ballina E, Fernandez E. A population-based study on the incidence of inflammatory bowel disease in Oviedo (Northern Spain). *Rev Esp Enferm Dig* 2004; **96**: 296-305
31 **Rivera Irigoín R**, de Sola Earle C, Ubiña Aznar E, Perea-Milla E, Fernández Pérez F, Navarro Jarabo JM, García Fernández G. [Incidence and clinico-epidemiological aspects of ulcerative colitis in the area of the Hospital Costa del Sol] *Gastroenterol Hepatol* 2007; **30**: 7-10
32 **Van Gossum A**, Adler M, De Reuck M, Devis G, Fiasse R, Vanheurverzwijn R, Willocx R. Epidemiology of inflammatory bowel disease in Brussels' area (1992-1993). *Acta Gastroenterol Belg* 1996; **59**: 7-9
33 **Russel MG**, Dorant E, Volovics A, Brummer RJ, Pop P, Muris JW, Bos LP, Limonard CB, Stockbrugger RW. High incidence of inflammatory bowel disease in The Netherlands: results of a prospective study. The South Limburg IBD Study Group. *Dis Colon Rectum* 1998; **41**: 33-40
34 **Manousos ON**, Giannadaki E, Mouzas IA, Tzardi M, Koutroubakis I, Skordilis P, Vassilakis S, Kouroumalis E, Vlachonikolis IG. Ulcerative colitis is as common in Crete as in northern Europe: a 5-year prospective study. *Eur J Gastroenterol Hepatol* 1996; **8**: 893-898
35 **Manousos ON**, Koutroubakis I, Potamianos S, Roussomoustakaki M, Gourtsoyiannis N, Vlachonikolis IG. A prospective epidemiologic study of Crohn's disease in Heraklion, Crete. Incidence over a 5-year period. *Scand J Gastroenterol* 1996; **31**: 599-603
36 **Ladas SD**, Mallas E, Giorgiotis K, Karamanolis G, Trigonis D, Markadas A, Sipsa V, Raptis SA. Incidence of ulcerative colitis in Central Greece: a prospective study. *World J Gastroenterol* 2005; **11**: 1785-1787
37 **Timmer A**, Goebell H. Incidence of ulcerative colitis, 1980-1995--a prospective study in an urban population in Germany. *Z Gastroenterol* 1999; **37**: 1079-1084
38 **Ranzi T**, Bodini P, Zambelli A, Politi P, Lupinacci G, Campanini MC, Dal Lago AL, Lisciandrano D, Bianchi PA. Epidemiological aspects of inflammatory bowel disease in a north Italian population: a 4-year prospective study. *Eur J Gastroenterol Hepatol* 1996; **8**: 657-661
39 **Sincic BM**, Vucelic B, Persic M, Brncic N, Erzen DJ, Radakovic B, Mićović V, Stimac D. Incidence of inflammatory bowel disease in Primorsko-goranska County, Croatia, 2000-2004: A prospective population-based study. *Scand J Gastroenterol* 2006; **41**: 437-444
40 **Salupere R**. Inflammatory bowel disease in Estonia: a prospective epidemiologic study 1993-1998. *World J Gastroenterol* 2001; **7**: 387-388
41 **Leong RW**, Lau JY, Sung JJ. The epidemiology and phenotype of Crohn's disease in the Chinese population. *Inflamm Bowel Dis* 2004; **10**: 646-651
42 **Sood A**, Midha V, Sood N, Bhatia AS, Avasthi G. Incidence and prevalence of ulcerative colitis in Punjab, North India. *Gut* 2003; **52**: 1587-1590
43 **Radhakrishnan S**, Zubaidi G, Daniel M, Sachdev GK, Mohan AN. Ulcerative colitis in Oman. A prospective study of the incidence and disease pattern from 1987 to 1994. *Digestion* 1997; **58**: 266-270
44 **Wright JP**, Froggatt J, O'Keefe EA, Ackerman S, Watermeyer S, Louw J, Adams G, Girdwood AH, Burns DG, Marks IN. The epidemiology of inflammatory bowel disease in Cape Town 1980-1984. *S Afr Med J* 1986; **70**: 10-15
45 **Gearry RB**, Richardson A, Frampton CM, Collett JA, Burt MJ, Chapman BA, Barclay ML. High incidence of Crohn's disease in Canterbury, New Zealand: results of an epidemiologic study. *Inflamm Bowel Dis* 2006; **12**: 936-943
46 **Yang SK**, Yun S, Kim JH, Park JY, Kim HY, Kim YH, Chang DK, Kim JS, Song IS, Park JB, Park ER, Kim KJ, Moon G, Yang SH. Epidemiology of inflammatory bowel disease in the Songpa-Kangdong district, Seoul, Korea, 1986-2005: a KASID study. *Inflamm Bowel Dis* 2008; **14**: 542-549
47 **Morita N**, Toki S, Hirohashi T, Minoda T, Ogawa K, Kono S, Tamakoshi A, Ohno Y, Sawada T, Muto T. Incidence and prevalence of inflammatory bowel disease in Japan: nationwide epidemiological survey during the year 1991. *J Gastroenterol* 1995; **30** Suppl 8: 1-4
48 **Tan CC**, Kang JY, Guan R, Yap I, Tay HH. Inflammatory bowel disease: an uncommon problem in Singapore. *J Gastroenterol Hepatol* 1992; **7**: 360-362
49 **Yang SK**, Hong WS, Min YI, Kim HY, Yoo JY, Rhee PL, Rhee JC, Chang DK, Song IS, Jung SA, Park EB, Yoo HM, Lee DK, Kim YK. Incidence and prevalence of ulcerative colitis in the Songpa-Kangdong District, Seoul, Korea, 1986-1997. *J Gastroenterol Hepatol* 2000; **15**: 1037-1042
50 **Gilat T**, Ribak J, Benaroya Y, Zemishlany Z, Weissman I. Ulcerative colitis in the Jewish population of Tel-Aviv Jafo. I. Epidemiology. *Gastroenterology* 1974; **66**: 335-342
51 **Sinclair TS**, Brunt PW, Mowat NA. Nonspecific proctocolitis in northeastern Scotland: a community study. *Gastroenterology* 1983; **85**: 1-11
52 **Morris T**, Rhodes J. Incidence of ulcerative colitis in the Cardiff region 1968-1977. *Gut* 1984; **25**: 846-848
53 **Devlin HB**, Datta D, Dellipiani AW. The incidence and prevalence of inflammatory bowel disease in North Tees Health District. *World J Surg* 1980; **4**: 183-193
54 **Binder V**, Both H, Hansen PK, Hendriksen C, Kreiner S, Torp-Pedersen K. Incidence and prevalence of ulcerative colitis and Crohn's disease in the County of Copenhagen, 1962 to 1978. *Gastroenterology* 1982; **83**: 563-568
55 **Jacobsohn WZ**, Levine Y. Incidence and prevalence of ulcerative colitis in the Jewish population of Jerusalem. *Isr J Med Sci* 1986; **22**: 559-563
56 **Stonnington CM**, Phillips SF, Melton LJ 3rd, Zinsmeister AR. Chronic ulcerative colitis: incidence and prevalence in a community. *Gut* 1987; **28**: 402-409
57 **Nyhlin H**, Danielsson A. Incidence of Crohn's disease in a defined population in northern Sweden, 1974-1981. *Scand J Gastroenterol* 1986; **21**: 1185-1192
58 **Stewénius J**, Adnerhill I, Ekelund G, Florén CH, Fork FT, Janzon L, Lindström C, Mars I, Nyman M, Rosengren JE. Ulcerative colitis and indeterminate colitis in the city of Malmo, Sweden. A 25-year incidence study. *Scand J Gastroenterol* 1995; **30**: 38-43
59 **Grossman A**, Fireman Z, Lilos P, Novis B, Rozen P, Gilat T. Epidemiology of ulcerative colitis in the Jewish population of central Israel 1970-1980. *Hepatogastroenterology* 1989; **36**: 193-197
60 **Ekbom A**, Helmick C, Zack M, Adami HO. The epidemiology of inflammatory bowel disease: a large, population-based study in Sweden. *Gastroenterology* 1991; **100**: 350-358
61 **Shivananda S**, Pena AS, Mayberry JF, Ruitenberg EJ, Hoedemaeker PJ. Epidemiology of proctocolitis in the region of Leiden, The Netherlands. A population study from 1979 to 1983. *Scand J Gastroenterol* 1987; **22**: 993-1002

62 **Odes HS**, Fraser D, Krawiec J. Ulcerative colitis in the Jewish population of southern Israel 1961-1985: epidemiological and clinical study. *Gut* 1987; **28**: 1630-1636
63 **Odes HS**, Fraser D, Krawiec J. Incidence of idiopathic ulcerative colitis in Jewish population subgroups in the Beer Sheva region of Israel. *Am J Gastroenterol* 1987; **82**: 854-858
64 **Stowe SP**, Redmond SR, Stormont JM, Shah AN, Chessin LN, Segal HL, Chey WY. An epidemiologic study of inflammatory bowel disease in Rochester, New York. Hospital incidence. *Gastroenterology* 1990; **98**: 104-110
65 **Niv Y**, Torten D, Tamir A, Epstein L. Incidence and prevalence of ulcerative colitis in the upper Galilee, Northern Israel, 1967-1986. *Am J Gastroenterol* 1990; **85**: 1580-1583
66 **Lindberg E**, Jarnerot G. The incidence of Crohn's disease is not decreasing in Sweden. *Scand J Gastroenterol* 1991; **26**: 495-500
67 **Langholz E**, Munkholm P, Nielsen OH, Kreiner S, Binder V. Incidence and prevalence of ulcerative colitis in Copenhagen county from 1962 to 1987. *Scand J Gastroenterol* 1991; **26**: 1247-1256
68 **Munkholm P**, Langholz E, Nielsen OH, Kreiner S, Binder V. Incidence and prevalence of Crohn's disease in the county of Copenhagen, 1962-87: a sixfold increase in incidence. *Scand J Gastroenterol* 1992; **27**: 609-614
69 **Lapidus A**, Bernell O, Hellers G, Persson PG, Lofberg R. Incidence of Crohn's disease in Stockholm County 1955-1989. *Gut* 1997; **41**: 480-486
70 **Bjornsson S**, Johannsson JH, Oddsson E. Inflammatory bowel disease in Iceland, 1980-89. A retrospective nationwide epidemiologic study. *Scand J Gastroenterol* 1998; **33**: 71-77
71 **Thomas GA**, Millar-Jones D, Rhodes J, Roberts GM, Williams GT, Mayberry JF. Incidence of Crohn's disease in Cardiff over 60 years: 1986-1990 an update. *Eur J Gastroenterol Hepatol* 1995; **7**: 401-405
72 **Tsianos EV**, Masalas CN, Merkouropoulos M, Dalekos GN, Logan RF. Incidence of inflammatory bowel disease in north west Greece: rarity of Crohn's disease in an area where ulcerative colitis is common. *Gut* 1994; **35**: 369-372
73 **Odes HS**, Locker C, Neumann L, Zirkin HJ, Weizman Z, Sperber AD, Fraser GM, Krugliak P, Gaspar N, Eidelman L. Epidemiology of Crohn's disease in southern Israel. *Am J Gastroenterol* 1994; **89**: 1859-1862
74 **Fonager K**, Sorensen HT, Olsen J. Change in incidence of Crohn's disease and ulcerative colitis in Denmark. A study based on the National Registry of Patients, 1981-1992. *Int J Epidemiol* 1997; **26**: 1003-1008
75 **Tragnone A**, Corrao G, Miglio F, Caprilli R, Lanfranchi GA. Incidence of inflammatory bowel disease in Italy: a nationwide population-based study. Gruppo Italiano per lo Studio del Colon e del Retto (GISC). *Int J Epidemiol* 1996; **25**: 1044-1052
76 **Trallori G**, Palli D, Saieva C, Bardazzi G, Bonanomi AG, d'Albasio G, Galli M, Vannozzi G, Milla M, Tarantino O, Renai F, Messori A, Amorosi A, Pacini F, Morettini A. A population-based study of inflammatory bowel disease in Florence over 15 years (1978-92). *Scand J Gastroenterol* 1996; **31**: 892-899
77 **Linares de la Cal JA**, Canton C, Hermida C, Perez-Miranda M, Mate-Jimenez J. Estimated incidence of inflammatory bowel disease in Argentina and Panama (1987-1993). *Rev Esp Enferm Dig* 1999; **91**: 277-286
78 **Shapira M**, Tamir A. Ulcerative colitis in the Kinneret sub district, Israel 1965-1994: incidence and prevalence in different subgroups. *J Clin Gastroenterol* 1998; **27**: 134-137
79 **Yapp TR**, Stenson R, Thomas GA, Lawrie BW, Williams GT, Hawthorne AB. Crohn's disease incidence in Cardiff from 1930: an update for 1991-1995. *Eur J Gastroenterol Hepatol* 2000; **12**: 907-911
80 **Rubin GP**, Hungin AP, Kelly PJ, Ling J. Inflammatory bowel disease: epidemiology and management in an English general practice population. *Aliment Pharmacol Ther* 2000; **14**: 1553-1559
81 **Niv Y**, Abuksis G, Fraser GM. Epidemiology of Crohn's disease in Israel: a survey of Israeli kibbutz settlements. *Am J Gastroenterol* 1999; **94**: 2961-2965
82 **García Rodríguez LA**, Gonzalez-Perez A, Johansson S, Wallander MA. Risk factors for inflammatory bowel disease in the general population. *Aliment Pharmacol Ther* 2005; **22**: 309-315
83 **Tsianos EV**, Katsanos KH, Christodoulou D, Dimoliatis I, Kogevinas A, Logan RF. Continuing low incidence of Crohn's disease in Northwest Greece. *Dig Liver Dis* 2003; **35**: 99-103
84 **Molinie F**, Gower-Rousseau C, Yzet T, Merle V, Grandbastien B, Marti R, Lerebours E, Dupas JL, Colombel JF, Salomez JL, Cortot A. Opposite evolution in incidence of Crohn's disease and ulcerative colitis in Northern France (1988-1999). *Gut* 2004; **53**: 843-848
85 **Lakatos L**, Mester G, Erdelyi Z, Balogh M, Szipocs I, Kamaras G, Lakatos PL. Striking elevation in incidence and prevalence of inflammatory bowel disease in a province of western Hungary between 1977-2001. *World J Gastroenterol* 2004; **10**: 404-409
86 **Lapidus A**. Crohn's disease in Stockholm County during 1990-2001: an epidemiological update. *World J Gastroenterol* 2006; **12**: 75-81
87 **Tsironi E**, Feakins RM, Probert CS, Rampton DS, Phil D. Incidence of inflammatory bowel disease is rising and abdominal tuberculosis is falling in Bangladeshis in East London, United Kingdom. *Am J Gastroenterol* 2004; **99**: 1749-1755
88 **Al-Ghamdi AS**, Al-Mofleh IA, Al-Rashed RS, Al-Amri SM, Aljebreen AM, Isnani AC, El-Badawi R. Epidemiology and outcome of Crohn's disease in a teaching hospital in Riyadh. *World J Gastroenterol* 2004; **10**: 1341-1344
89 **Jacobsen BA**, Fallingborg J, Rasmussen HH, Nielsen KR, Drewes AM, Puho E, Nielsen GL, Sorensen HT. Increase in incidence and prevalence of inflammatory bowel disease in northern Denmark: a population-based study, 1978-2002. *Eur J Gastroenterol Hepatol* 2006; **18**: 601-606
90 **Gheorghe C**, Pascu O, Gheorghe L, Iacob R, Dumitru E, Tantau M, Vadan R, Goldis A, Balan G, Iacob S, Dobru D, Saftoiu A. Epidemiology of inflammatory bowel disease in adults who refer to gastroenterology care in Romania: a multicentre study. *Eur J Gastroenterol Hepatol* 2004; **16**: 1153-1159
91 **Nerich V**, Monnet E, Etienne A, Louafi S, Ramee C, Rican S, Weill A, Vallier N, Vanbockstael V, Auleley GR, Allemand H, Carbonnel F. Geographical variations of inflammatory bowel disease in France: a study based on national health insurance data. *Inflamm Bowel Dis* 2006; **12**: 218-226
92 **Cachia E**, Calleja N, Aakeroy R, Degaetano J, Vassallo M. Incidence of inflammatory bowel disease in Malta between 1993 and 2005: a retrospective study. *Inflamm Bowel Dis* 2008; **14**: 550-553
93 **Loftus EV Jr**. Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. *Gastroenterology* 2004; **126**: 1504-1517
94 **Bach JF**. The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 2002; **347**: 911-920

S- Editor Xiao LL **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5499

World J Gastroenterol 2008 September 28; 14(36): 5499-5503
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Javier P Gisbert, Professor; Fernando Gomollón, Dr., MD, PhD, *Series Editors*

Crohn's disease-Defect in innate defence

Michael Gersemann, Jan Wehkamp, Klaus Fellermann, Eduard Friedrich Stange

Michael Gersemann, Jan Wehkamp, Klaus Fellermann, Eduard Friedrich Stange, Robert Bosch Hospital and Dr. Margarete Fischer Bosch Institute of Clinical Pharmacology and University of Tübingen, Stuttgart, Germany
Author contributions: Gersemann M, Wehkamp J, Fellermann K, and Stange EF are working on the pathogenesis of both intestinal bowel diseases, Crohn's disease and ulcerative colitis since many years resulting in a couple of original works. This review article was a joint work.
Supported by The Robert Bosch Foundation, Stuttgart, Germany and the Emmy Noether program (J.W.) of the Deutsche Forschungsgemeinschaft (DFG)
Correspondence to: Eduard Friedrich Stange, MD, Robert Bosch Hospital, Internal Medicine I Auerbachstr. 110, D-70376 Stuttgart, Germany. eduard.stange@rbk.de
Telephone: +49-711-81013406 Fax: +49-711-81013793
Received: July 30, 2008 Revised: August 5, 2008
Accepted: August 12, 2008
Published online: September 28, 2008

Abstract

Crohn's disease may prinicipally involve the whole gastrointestinal tract. Most commonly, the inflammation occurs in the small intestine and/or in the colon with stable disease location over the years. The pathogenesis of both disease phenotypes is complex, the likely primary defect lies in the innate rather than adaptive immunity, particularly in the chemical antimicrobial barrier of the mucosa. Crohn's ileitis is associated with a reduced expression of the Wnt signalling pathway transcription factor T-cell factor 4 (TCF4), which is regulating Paneth cell differentiation. As a result, the alpha-defensins and principal Paneth cell products HD5 and HD6 are deficiently expressed in ileal disease, independent of current inflammation. In contrast, Crohn's colitis is typically associated with an impaired induction of the beta-defensins HBD2 and HBD3 caused by fewer gene copy numbers in the gene locus of the beta-defensins on chromosome 8. This ileal and colonic defect in innate defence mediated by a deficiency of the protective alpha- and beta-defensins may enable the luminal microbes to invade the mucosa and trigger the inflammation. A better understanding of the exact molecular mechanisms behind ileal and colonic Crohn's disease may give rise to new therapeutic strategies based on a stimulation of the protective innate immune system.

Key words: Crohn's disease; Intestinal stem cell; Differentiation; Defensins; Transcription factor T-cell factor 4

Gersemann M, Wehkamp J, Fellermann K, Stange EF. Crohn's disease-Defect in innate defence. *World J Gastroenterol* 2008; 14(36): 5499-5503 Available from: URL: http://www.wjgnet.com/1007-9327/14/5499.asp DOI: http://dx.doi.org/10.3748/wjg.14.5499

INTRODUCTION

Crohn's disease is characterized by a chronic inflammation of the whole intestine most commonly occurring in the distal ileum and/or in the colon[1]. Since both disease locations contain high concentrations of intestinal bacteria (10^7-10^8 organisms/gram luminal content in the distal ileum and 10^{11}-10^{12} in the colon as compared to 0-10^3 organisms/gram luminal content in the stomach, duodenum, jejunum and proximal ileum)[2] it is conceivable that luminal microbes are suggested to play an important role in the pathogenesis of Crohn's disease[3]. For example, it was demonstrated that surgical diversion of the fecal stream effectively resolves Crohn's disease inflammation distal to the surgical site[4]. Moreover, an abnormal microbial composition was found in intestinal bowel disease[5,6], especially with an enhanced *E.coli* virulence in Crohn's disease[7]. The most convincing evidence for the involvement of enteric microbiota in the pathogenesis of Crohn´s disease was demonstrated by Swidsinsky *et al* by fluorescent in situ hybridization studies (FISH) showing a drastic increase of mucosa-associated and invasive bacteria in active Crohn's disease, whereas these bacteria are absent from the normal small and large bowel epithelium[8].

In the past few years, several investigations implicated a central role for the adaptive immunity mediated by defensins and other antimicrobial peptides in Crohn's disease[9]. A relative deficiency of secreted defensins in Crohn's disease was shown in several human and animal studies shifting the balance between antimicrobial peptides and luminal bacteria towards the microbial flora[9]. As a result, an abnormal microbial composition in Crohn's disease allows the intestinal microbes to adhere and invade into the mucosa triggering the inflammation[10].

Since Crohn's disease is characterized by different

disease locations, such as small intestinal *versus* colonic disease, we discuss in this review not only the concept of a defective antimicrobial shield in both phenotypes of Crohn's disease, but also focus on the mechanisms behind ileal and colonic defensin deficiency beginning with the intestinal stem cell.

INSTESTINAL STEM CELLS

The whole intestinal tract undergoes a rapid epithelial cell turnover maintained by a population of intestinal stem cells. These enigmatic cells are characterized by a relatively undifferentiated, immature phenotype, which makes them difficult to be clearly identified even if recent studies found leucine-rich-repeat-containing G-protein-coupled receptor 5 (Lgr5) as a marker for these cells[11,12]. The stem cell compartment is located at the base of the crypts, in the small intestine intermingled with the Paneth cells[12]. They are surrounded by epithelial and mesenchymal cells, which regulate stem cell behaviour by paracrine secretion of extra-cellular substrates, such as growth factors and cytokines[13].

In this optimal microenvironment, the intestinal stem cells can give rise to four epithelial cell types[13]: The most abundant cells in the epithelium are the columnar cells, which are specialised for absorption by the presence of apical microvilli. Goblet cells are mucin producing and secreting cells forming the protective luminal mucosal layer. Neuroendocrine cells release hormones in an endo- and paracrine fashion, and finally the Paneth cells, which are found in the crypt base of the small intestine and ascending colon. Their main function is to secrete defensins and other antimicrobial molecules to keep the crypts sterile[14].

Stem cell behaviour is regulated by several cell signalling pathways, such as Wnt, Notch, Hedgehog and BMP[15,16]. Derangement of these pathways plays a crucial role in the development of malignancy within the intestinal tract[13,16,17]. The role of these pathways in inflammatory diseases, such as inflammatory bowel disease, is a new finding. In our recent work we focused on the differentiation from intestinal stem cells towards Paneth cells in ileal Crohn's disease as a potential pathogenetic mechanism of this disease[18].

PANETH CELLS

Paneth cells are located at the crypt base adjacent to the stem cells. Their main function is to protect the intestinal mucosa against microbes by the secretion of several antimicrobial molecules into the lumen of the crypt. The principal Paneth cell products are the alpha-defensins HD5 and HD6, but also other antimicrobials like lysozyme and phospholipase A2[19]. These secretory molecules enable the Paneth cells to defend the small intestine against a broad spectrum of agents, including gram-positive and gram-negative bacteria, fungi and even some viruses[20].

The differentiation from the intestinal stem cell to the Paneth cell is mainly controlled by the Wnt signaling pathway[21]. As shown by embryonic mouse intestine studies, the Wnt transcription factor TCF4 seems to be essential for the Paneth cell gene program[21]. The activation of Wnt signaling leads to the formation of an intracellular β-catenin-TCF4-complex. This complex translocates into the nucleus, where TCF4 acts as a transcription factor to control the expression of several downstream target genes, such as Paneth cell alpha-defensins[21]. In TCF4 null mice, a direct link exists between TCF4 and cryptidins (the mouse homolog to human alpha-defensins)[21], whereas in humans, especially in Crohn´s disease evidence was missing. Therefore, we looked at the expression of Paneth cell defensins and the Wnt signaling transcription factor TCF4 in ileal Crohn's disease.

THE ALPHA-DEFENSIN DEFICIENCY IN ILEAL CROHN'S DISEASE IS LINKED TO A REDUCED EXPRESSION OF THE TRANSCRIPTION FACTOR TCF4

In ileal Crohn's disease, we demonstrated in two independent cohorts a decreased expression of the alpha-defensins HD5 and HD6, whereas the other Paneth cell products were unchanged or even increased when compared with controls[19,22]. Remarkably, this observation was independent of the degree of inflammation and not detected in colonic biopsies from patients with Crohn's disease and ulcerative colitis, as well as in pouchitis samples, which were used as inflammatory controls[19,22]. Since the antibacterial activity in intestinal mucosal extracts from patients with ileal Crohn's disease was also reduced, the decreased expression of HD5 and HD6 results in a defective antimicrobial shield in humans[19]. Transgenic mice with human HD5 expression levels (reflecting the antibacterial conditions in Crohn's disease) had an abnormal composition of the luminal microbial flora, which also supports the relevance of alpha-defensins in regulating the bacterial flora[19]. Mutations in the NOD2 (nucleotide-binding oligomerization domain containing 2) gene, an intracellular receptor for bacterial muramyl dipeptide, are clearly associated with ileal Crohn's disease[23,24]. It is noteworthy that Paneth cells, which are responsible for a good effective antibacterial shield in the small intestine, also express NOD2[25]. Interestingly, we found an even more pronounced decrease of alpha-defensin transcripts in patients with a NOD2 mutation as compared to the wild types[19,22]. The low defensin synthesis in those patients has been confirmed by Elphick *et al*[26]. Another study confirmed the low defensin formation in Crohn's disease but linked it to inflammation and loss of Paneth cells[27]. However, this study was flawed by using different tissue samples such as biopsies *versus*

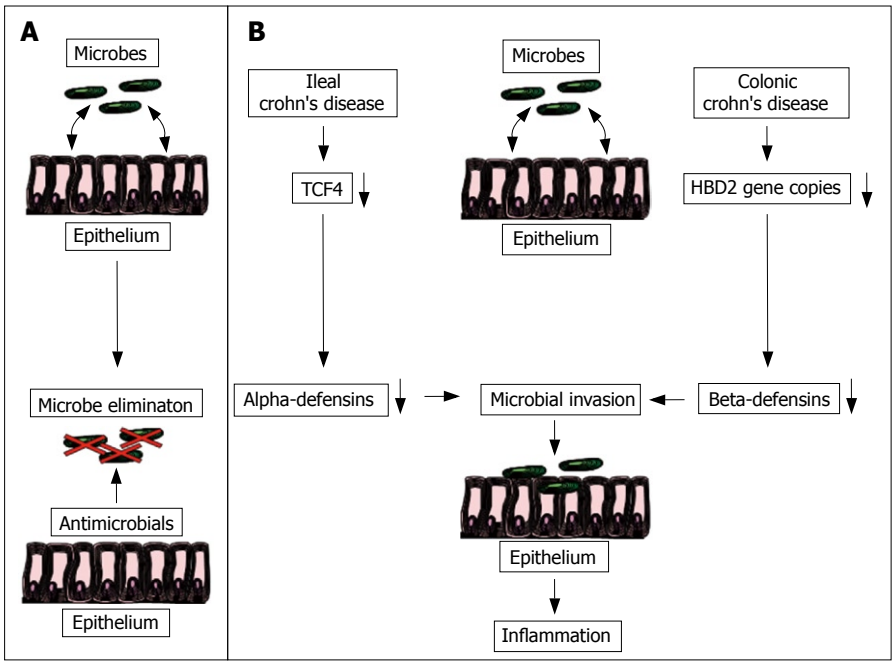


Figure 1 Proposed model for the pathogenesis of ileal and colonic Crohn's disease. In the healthy ileal and colonic mucosa, luminal microbes are sufficiently eliminated by an adequate secretion of antimicrobials peptides (**A**). In ileal Crohn's disease, a reduction of the Wnt transcription factor TCF4 leads to a decreased alpha-defensin secretion. In colonic Crohn´s disease a reduced HBD2 copy number causes a weakened antimicrobial barrier mediated by the beta-defensins. Both changes may allow the luminal microbes to attach and invade in the mucosa triggering the inflammation (**B**).

segments from controls *versus* diseased patients as well as other methodological problems in normalizing their data.

In summary, these studies above implicate that the specific deficiency of Paneth cell defensins in ileal Crohn's disease, which is even more pronounced in case of NOD2 mutations, results in a dysfunction of the mucosal barrier. The defect in innate immune defense of the ileal mucosa appears to be primary and could cause and/or maintain this inflammatory disease.

Wnt signaling is one of the crucial pathways in the differentiation from the intestinal stem cell towards the Paneth cell. In particular the Wnt transcription factor TCF4 is a known regulator of Paneth cell differentiation and also alpha-defensin expression[21]. We were able to show a reduced expression of TCF4 in patients with ileal Crohn's disease as compared to colonic Crohn´s disease and ulcerative colitis[18]. Remarkably, this decrease of TCF4 in ileal Crohn's disease was independent of the degree of inflammation and independent of the NOD2 genotype[18]. In accordance to prior animal models[21], TCF4 expression correlated also in human samples significantly with HD5 and HD6[18]. Moreover, we were able to demonstrate the functional relevance of this TCF4 decrease in heterozygous TCF4-knockout mice. These mice were characterized by a reduced TCF4 expression leading to a significant decrease of Paneth cell alpha-defensins and also bacterial killing[18]. We were also able to detect a high-affinity binding site in the HD5 and HD6 promotor for TCF4, which showed a significantly reduced activity in ileal Crohn's disease[18]. Overall, a defect in intestinal stem cell differentiation towards Paneth cells mediated *via* the Wnt signaling transcription factor TCF4 could results in a defective antibacterial shield in ileal Crohn's diseases by the absence of a sufficient expression of the protective Paneth cell defensins HD5 and HD6 (Figure 1).

THE BETA-DEFENSIN DEFICIENCY IN COLONIC CROHN'S DISEASE IS RELATED TO FEWER HBD2 GENE COPY NUMBERS

Since Paneth cells are rare in the colon, the contribution of the alpha-defensins to the antimicrobial defence in the large intestine is only limited. Therefore, it is not surprising that colonic HD5 and HD6 expression was weak and almost equal in Crohn´s disease and ulcerative colitis[22,28]. In contrast, the beta-defensins HBD1-3 are preferentially expressed in the colon. HBD1 is constitutively expressed in Crohn's disease independent of the grade of inflammation[29,30], whereas HBD2 and HBD3 are inducible in intestinal bowel disease [30-32]. As shown in two separate cohorts, the induction of both beta-defensins, HBD2 and HBD3, was less pronounced in colonic mucosa of Crohn's disease patients as compared to ulcerative colitis[31,32]. This deficiency of HBD2 expression in Crohn's colitis was also confirmed on the protein level by immunohistochemistry[30]. HBD2 and HBD3 are strongly correlated to each other, especially in case of inflammation[31,32]. Similar to these two beta-defensins, other antimicrobial peptides like elafin, SLPI and LL37 also followed the pattern of diminished induction in Crohn's disease compared to ulcerative colitis[33,34]. This observed colonic defect of the antimicrobial barrier caused by a diminished expression of beta-defensins and other antimicrobials was supported by a recent study, where we found a significantly lower bacterial killing using biopsy extracts from Crohn's disease patients compared to ulcerative colitis and healthy controls[35].

In humans, the beta-defensins HBD2 and HBD3 are neighbouring genes on chromosome 8p23.1. The DNA copy number of this beta-defensin gene cluster is highly polymorphic within the healthy population[36].

A genome wide copy number profiling using a DNA microarray showed that the defective induction of HBD2 and HBD3 in colonic CD may be due to a lower gene copy number in this gene area[37]: The median number of HBD2 gene copies in the control group and in patients with ulcerative colitis was 4. In contrast, two different cohorts (an exploratory surgical cohort with ileal or colonic CD and a large confirmatory cohort with inflammatory bowel diseases those with ileal resections/ disease) showed a median of only 3 copies per genome in colonic CD. Taken together, the number of HBD2 gene copies was shifted to lower numbers in colonic CD as compared to controls (3 *vs* 4)[37]. Since patients with 3 or less HBD2 gene copies were shown to have a significantly higher risk of developing colonic CD as compared to individuals with four or more copies (OR: 3.06, 95% CI: 1.46-6.45), the number of HBD2 gene copies seems to be functionally relevant. It is not surprising that HBD2 gene copy numbers of < 4 are associated with a diminished mucosal HBD2 mRNA expression[37]. In conclusion, a lower HBD2 gene copy number in the beta-defensin locus predisposes to colonic CD, which is most likely mediated through a diminished beta-defensin expression (Figure 1).

WHAT ABOUT ULCERATIVE COLITIS?

In active ulcerative colitis the epithelial expression of the inducible beta-defensins HBD2 (about 1000-fold), HBD3 (about 300-fold) and other antimicrobials is enhanced[9,34]. Accordingly, we were able to demonstrate an increase of the antibacterial activity toward various bacteria of the normal gut flora in cationic biopsy extracts of patients with ulcerative colitis suggesting an intact antibacterial shield in ulcerative colitis[35]. Interestingly, psoriasis, which is a non-bacterial inflammatory disease of the skin, is linked to an increased gene copy number in the beta-defensin locus[38]. It could be possible that the tremendous induction of defensins and other antimicrobials in ulcerative colitis may lead to a better antibacterial protection at the expense of enhancing the inflammatory process, since many antibacterials have proinflammatory and sometimes chemokine like effects.

CONCLUSION

The exact mechanisms behind both phenotypes of Crohn's disease are certainly complex but the presented concept has already opened new avenues of thinking. The long nurtured alterations in adaptive immunity may be secondary to this defect in innate mucosal immunity allowing mucosal invasion of bacteria. In addition to the defect in Paneth cell differentiation in ileal disease, and a lower HBD2 gene copy number in colonic disease other mechanisms are likely to exist which may require a more systematic study of the antibacterial mucosal peptide system. Nevertheless, in the future new therapeutic strategies in the treatment of Crohn's disease should focus on a stimulation of the protective innate immune system rather than broadly suppressing the adequate immunological response at the expense of opportunistic infections.

REFERENCES

1 **Podolsky DK**. Inflammatory bowel disease. *N Engl J Med* 2002; **347**: 417-429
2 **Eckburg PB**, Relman DA. The role of microbes in Crohn's disease. *Clin Infect Dis* 2007; **44**: 256-262
3 **Sartor RB**. Microbial influences in inflammatory bowel diseases. *Gastroenterology* 2008; **134**: 577-594
4 **Rutgeerts P**, Goboes K, Peeters M, Hiele M, Penninckx F, Aerts R, Kerremans R, Vantrappen G. Effect of faecal stream diversion on recurrence of Crohn's disease in the neoterminal ileum. *Lancet* 1991; **338**: 771-774
5 **Bibiloni R**, Mangold M, Madsen KL, Fedorak RN, Tannock GW. The bacteriology of biopsies differs between newly diagnosed, untreated, Crohn's disease and ulcerative colitis patients. *J Med Microbiol* 2006; **55**: 1141-1149
6 **Frank DN**, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci USA* 2007; **104**: 13780-13785
7 **Darfeuille-Michaud A**, Neut C, Barnich N, Lederman E, Di Martino P, Desreumaux P, Gambiez L, Joly B, Cortot A, Colombel JF. Presence of adherent Escherichia coli strains in ileal mucosa of patients with Crohn's disease. *Gastroenterology* 1998; **115**: 1405-1413
8 **Swidsinski A**, Ladhoff A, Pernthaler A, Swidsinski S, Loening-Baucke V, Ortner M, Weber J, Hoffmann U, Schreiber S, Dietel M, Lochs H. Mucosal flora in inflammatory bowel disease. *Gastroenterology* 2002; **122**: 44-54
9 **Wehkamp J**, Schmid M, Stange EF. Defensins and other antimicrobial peptides in inflammatory bowel disease. *Curr Opin Gastroenterol* 2007; **23**: 370-378
10 **Wehkamp J**, Schmid M, Fellermann K, Stange EF. Defensin deficiency, intestinal microbes, and the clinical phenotypes of Crohn's disease. *J Leukoc Biol* 2005; **77**: 460-465
11 **Barker N**, Clevers H. Tracking down the stem cells of the intestine: strategies to identify adult stem cells. *Gastroenterology* 2007; **133**: 1755-1760
12 **Barker N**, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ, Clevers H. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 2007; **449**: 1003-1007
13 **Leedham SJ**, Brittan M, McDonald SA, Wright NA. Intestinal stem cells. *J Cell Mol Med* 2005; **9**: 11-24
14 **Ouellette AJ**. Defensin-mediated innate immunity in the small intestine. *Best Pract Res Clin Gastroenterol* 2004; **18**: 405-419
15 **Crosnier C**, Stamataki D, Lewis J. Organizing cell renewal in the intestine: stem cells, signals and combinatorial control. *Nat Rev Genet* 2006; **7**: 349-359
16 **Katoh M**, Katoh M. Notch signaling in gastrointestinal tract (review). *Int J Oncol* 2007; **30**: 247-251
17 **Bach SP**, Renehan AG, Potten CS. Stem cells: the intestinal stem cell as a paradigm. *Carcinogenesis* 2000; **21**: 469-476
18 **Wehkamp J**, Wang G, Kubler I, Nuding S, Gregorieff A, Schnabel A, Kays RJ, Fellermann K, Burk O, Schwab M, Clevers H, Bevins CL, Stange EF. The Paneth cell alpha-defensin deficiency of ileal Crohn's disease is linked to Wnt/Tcf-4. *J Immunol* 2007; **179**: 3109-3118
19 **Wehkamp J**, Salzman NH, Porter E, Nuding S, Weichenthal M, Petras RE, Shen B, Schaeffeler E, Schwab M, Linzmeier R, Feathers RW, Chu H, Lima H Jr, Fellermann K, Ganz T, Stange EF, Bevins CL. Reduced Paneth cell alpha-defensins in ileal Crohn's disease. *Proc Natl Acad Sci USA* 2005; **102**: 18129-18134

20 **Ganz T**. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol* 2003; **3**: 710-720

21 **van Es JH**, Jay P, Gregorieff A, van Gijn ME, Jonkheer S, Hatzis P, Thiele A, van den Born M, Begthel H, Brabletz T, Taketo MM, Clevers H. Wnt signalling induces maturation of Paneth cells in intestinal crypts. *Nat Cell Biol* 2005; **7**: 381-386

22 **Wehkamp J**, Harder J, Weichenthal M, Schwab M, Schaffeler E, Schlee M, Herrlinger KR, Stallmach A, Noack F, Fritz P, Schroder JM, Bevins CL, Fellermann K, Stange EF. NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. *Gut* 2004; **53**: 1658-1664

23 **Hugot JP**, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, Almer S, Tysk C, O'Morain CA, Gassull M, Binder V, Finkel Y, Cortot A, Modigliani R, Laurent-Puig P, Gower-Rousseau C, Macry J, Colombel JF, Sahbatou M, Thomas G. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 2001; **411**: 599-603

24 **Ogura Y**, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, Britton H, Moran T, Karaliuskas R, Duerr RH, Achkar JP, Brant SR, Bayless TM, Kirschner BS, Hanauer SB, Nunez G, Cho JH. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 2001; **411**: 603-606

25 **Lala S**, Ogura Y, Osborne C, Hor SY, Bromfield A, Davies S, Ogunbiyi O, Nunez G, Keshav S. Crohn's disease and the NOD2 gene: a role for paneth cells. *Gastroenterology* 2003; **125**: 47-57

26 **Elphick D**, Liddell S, Mahida YR. Impaired luminal processing of human defensin-5 in Crohn's disease: persistence in a complex with chymotrypsinogen and trypsin. *Am J Pathol* 2008; **172**: 702-713

27 **Simms LA**, Doecke JD, Walsh MD, Huang N, Fowler EV, Radford-Smith GL. Reduced alpha-defensin expression is associated with inflammation and not NOD2 mutation status in ileal Crohn's disease. *Gut* 2008; **57**: 903-910

28 **Wehkamp J**, Schwind B, Herrlinger KR, Baxmann S, Schmidt K, Duchrow M, Wohlschlager C, Feller AC, Stange EF, Fellermann K. Innate immunity and colonic inflammation: enhanced expression of epithelial alpha-defensins. *Dig Dis Sci* 2002; **47**: 1349-1355

29 **O'Neil DA**, Porter EM, Elewaut D, Anderson GM, Eckmann L, Ganz T, Kagnoff MF. Expression and regulation of the human beta-defensins hBD-1 and hBD-2 in intestinal epithelium. *J Immunol* 1999; **163**: 6718-6724

30 **Wehkamp J**, Fellermann K, Herrlinger KR, Baxmann S, Schmidt K, Schwind B, Duchrow M, Wohlschlager C, Feller AC, Stange EF. Human beta-defensin 2 but not beta-defensin 1 is expressed preferentially in colonic mucosa of inflammatory bowel disease. *Eur J Gastroenterol Hepatol* 2002; **14**: 745-752

31 **Fahlgren A**, Hammarstrom S, Danielsson A, Hammarstrom ML. Increased expression of antimicrobial peptides and lysozyme in colonic epithelial cells of patients with ulcerative colitis. *Clin Exp Immunol* 2003; **131**: 90-101

32 **Wehkamp J**, Harder J, Weichenthal M, Mueller O, Herrlinger KR, Fellermann K, Schroeder JM, Stange EF. Inducible and constitutive beta-defensins are differentially expressed in Crohn's disease and ulcerative colitis. *Inflamm Bowel Dis* 2003; **9**: 215-223

33 **Schauber J**, Rieger D, Weiler F, Wehkamp J, Eck M, Fellermann K, Scheppach W, Gallo RL, Stange EF. Heterogeneous expression of human cathelicidin hCAP18/LL-37 in inflammatory bowel diseases. *Eur J Gastroenterol Hepatol* 2006; **18**: 615-621

34 **Schmid M**, Fellermann K, Fritz P, Wiedow O, Stange EF, Wehkamp J. Attenuated induction of epithelial and leukocyte serine antiproteases elafin and secretory leukocyte protease inhibitor in Crohn's disease. *J Leukoc Biol* 2007; **81**: 907-915

35 **Nuding S**, Fellermann K, Wehkamp J, Stange EF. Reduced mucosal antimicrobial activity in Crohn's disease of the colon. *Gut* 2007; **56**: 1240-1247

36 **Hollox EJ**, Armour JA, Barber JC. Extensive normal copy number variation of a beta-defensin antimicrobial-gene cluster. *Am J Hum Genet* 2003; **73**: 591-600

37 **Fellermann K**, Stange DE, Schaeffeler E, Schmalzl H, Wehkamp J, Bevins CL, Reinisch W, Teml A, Schwab M, Lichter P, Radlwimmer B, Stange EF. A chromosome 8 gene-cluster polymorphism with low human beta-defensin 2 gene copy number predisposes to Crohn disease of the colon. *Am J Hum Genet* 2006; **79**: 439-448

38 **Hollox EJ**, Huffmeier U, Zeeuwen PL, Palla R, Lascorz J, Rodijk-Olthuis D, van de Kerkhof PC, Traupe H, de Jongh G, den Heijer M, Reis A, Armour JA, Schalkwijk J. Psoriasis is associated with increased beta-defensin genomic copy number. *Nat Genet* 2008; **40**: 23-25

S- Editor Xiao LL **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5504

World J Gastroenterol 2008 September 28; 14(36): 5504-5507
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Javier P Gisbert, Professor; Fernando Gomollón, Dr., MD, PhD, *Series Editors*

Severe ulcerative colitis: At what point should we define resistance to steroids?

Maria Esteve, Javier P Gisbert

Maria Esteve, Department of Gastroenterology, Hospital Universitari Mútua de Terrassa, Fundació per la Recerca Mútua de Terrassa, University of Barcelona, Terrassa, Catalonia, Spain
Javier P Gisbert, Department of Gastroenterology, Hospital Universitario de la Princesa, Madrid, and Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas, Spain
Author contributions: Esteve M is responsible for the manuscript writing and intellectual content; Gisbert JP is responsible of the critical revision and intellectual content.
Correspondence to: Maria Esteve, MD, Department of Gastroenterology, Hospital Universitari Mútua de Terrassa, University of Barcelona Plaça Dr Robert nº 508221 Terrassa, Barcelona, Catalonia, Spain. mestevecomas@telefonica.net
Telephone: +34-93-7365050 Fax: +34-93-7365043
Received: August 18, 2008 Revised: August 25, 2008
Accepted: September 1, 2008
Published online: September 28, 2008

Abstract

Corticosteroids are still the first-line treatment for active ulcerative colitis more than 50 years after the publication of trials assessing their beneficial effect, with about a 50% remission rate in cases of severe disease. The mortality related to severe attacks of ulcerative colitis has decreased dramatically, to less than 1%, in experienced centers, due to the appropriate use of intensive therapeutic measures (intravenous steroids, fluids and electrolytes, artificial nutritional support, antibiotics, *etc*), along with timely decision-making about second-line medical therapy and early identification of patients requiring colectomy. One of the most difficult decisions in the management of severe ulcerative colitis is knowing for how long corticosteroids should be administered before deciding that a patient is a non-responder. Studies assessing the outcome of acute attacks after steroid initiation have demonstrated that, in steroid-sensitive patients, the response generally occurs early on, in the first days of treatment. Different indexes to predict treatment failure, when applied on the third day of treatment, have demonstrated a high positive predictive value for colectomy. In contrast to this resolute approach, which is the most widely accepted, other authors have suggested that in some patients a complete and prolonged response to steroids may take longer. Either way, physicians taking care of these patients need to recognize that severe ulcerative colitis may be life-threatening, and they need to be careful with excessively prolonged medical treatment and delayed surgery.

Key words: Ulcerative colitis; Steroids; Severe; Resistance

Esteve M, Gisbert JP. Severe ulcerative colitis: At what point should we define resistance to steroids? *World J Gastroenterol* 2008; 14(36): 5504-5507 Available from: URL: http://www.wjgnet.com/1007-9327/14/5504.asp DOI: http://dx.doi.org/10.3748/wjg.14.5504

RESISTANCE TO CORTICOSTEROIDS RELATED TO DOSE OR ROUTE OF ADMINISTRATION

The wide use of glucocorticosteroids in the management of active ulcerative colitis (UC) in the second half of the twentieth century had a major role in dramatically reducing the high mortality rate of this condition, from 75%[1] to less than 1%[2]. In 1955 Truelove and Witts published a summary of controlled trials using cortisone in 109 UC patients, most of them with moderate disease[3]. In 1978, Truelove *et al*[4] reported, in an uncontrolled study, a 60% remission rate in 87 patients with severe UC by using 60 mg intravenous prednisolone. More than 50 years after the publication of the initial trial, Corticosteroids are still the first-line treatment for moderate to severely active UC. Similar percentages of response and remission rates were reproduced in a number of subsequent studies[2]. However, little evidence-based data are available indicating the optimal dose and route of administration in severe disease. Only one study performed in outpatients with moderate disease compared doses of 20, 40 and 60 mg of oral prednisone, and demonstrated a similar effect for 40 and 60 mg, and significantly higher than that for

20 mg/d[5]. No trials assessing a dose-response rate have been performed in severe UC, and the majority of studies have used fixed doses, not adjusted to body weight, ranging from 40-80 mg of methylprednisolone and 100-400 mg of hydrocortisone. A recently published systematic review of cohort studies and controlled clinical trials assessing the response to Corticosteroids in severe UC addressed the issue of dose-related efficacy of steroids[2]. The dose administered was reported in 24 studies, and no relationship was found between the dose administered-standardized as methylprednisolone equivalent while using a mean adult weight of 70 kg and colectomy rate. However, the mean daily dose was 68 ± 13 mg (range 40-100 mg); only 3 studies have used doses lower than 50 mg/d. Therefore, doses equivalent to 1 mg of prednisone per kg body weight seem to be the most effective, with higher doses apparently not adding more benefit. However, supraphysiological doses (equivalent to 1000 mg methylprednisolone), as used in other immunomediated inflammatory disorders, were evaluated in only one pilot study that used 100 mg dexamethasone pulse therapy for 3 d with good short term outcome[6].

Previous to establish the criteria of steroid resistance, it is accepted that corticosteroids should be administered intravenously when a lack of response to oral treatment is observed. This recommendation is based on classical pharmacokinetic studies, performed with limited numbers of patients, which demonstrated delayed prednisolone absorption after an oral dose in patients with acute colitis as compared to healthy controls, and better plasma levels after intravenous administration[7,8]. In addition, intravenous administration in the hospital setting ensures good treatment compliance.

Hospital admission for intensive intravenous treatment is consequently mandatory for those patients with moderate disease who have failed to respond to oral prednisone or those having clinical criteria of severe disease as defined by the classical clinical index[3,9]. In addition to the life-saving benefit of corticosteroid administration, the decreased morbidity and mortality in severe UC was thought to be due to the appropriate use of intensive supportive measures (fluid and electrolyte replacement, artificial nutritional support, antibiotics, *etc*) and early identification of patients requiring colectomy[10,11]. In fact, unacceptably high mortality rates have recently been reported in non-specialized centers, probably due to delayed decisions in perioperative management[12,13]. Physicians treating UC patients should be able to recognize the severity of the condition and related complications and to be aware of the limits of all the therapeutic armamentarium, thereby avoiding unnecessary exposure to ineffective treatment. In this sense, the availability of several therapeutic alternatives such as cyclosporine, infliximab and granulocyteapheresis stimulated the search for predictive factors of treatment failure at an early stage. Thus, one of the most difficult questions in the management of severe UC is not deciding when corticosteroids should be administered, but rather for how long they should be administered, and with what limits for their efficacy.

AT WHAT POINT SHOULD WE DEFINE RESISTANCE TO STEROIDS: 3, 7 OR 21 DAYS AFTER INITIATION?

The colectomy rate in severe UC (30%-35%), as well as percentages of remission and response, have remained unchanged since the introduction of corticosteroids in UC treatment, and similar results have been reported in both clinical trials[2] and in the clinical setting[14]. The classic limit of 7-10 d for establishing the criteria of steroid resistance was based on the results of historical series showing that the median time of remission was 7.5 d and that prolonged administration beyond 10 d did not increase the percentage of remission[15].

However, some authors have argued in favor of a more conservative approach since a group of "slow responders" have been identified[16,17]. These patients showed a partial response within 10 d of admission, defined as a decrease in stool frequency, with little or no blood. In the largest retrospective series of a single experienced hospital (149 episodes in 115 patients), 19% fulfilling this criteria entered into remission within the first 21 d of treatment[16]. More importantly, the long-term follow-up of these "slow-responders" showed that none of them required colectomy within a median follow-up period of 49 mo. These data are in contrast with the high colectomy rate of corticoesteroid refractory patients one year after ciclosporin-induced remission when azathioprine is not administered to maintain it[18].

In contrast with this point of view, a more resolute approach is based on the results of several studies which have identified factors predictive of treatment failure soon after corticosteroid initiation[19-22]. The study of Travis *et al*[19] was a pioneer in this area, demonstrating a prospective day-by-day evolution, during the first days after admission, of several inflammatory parameters (C reactive protein (CRP), erythrocyte sedimentation rate (ESR), platelets, *etc*) and clinical symptoms (bowel movements, blood in stools, pulse rate, *etc*) in 51 consecutive episodes of severe UC. This study identified a turning point in the disease outcome on the third day of treatment, establishing a critical limit for deciding whether patients are responders or non-responders. Two simple parameters (stool frequency of > 8 per day or 3-8 bowel movements per day and CRP > 45 mg/dL on the third day of therapy) have a positive predictive value (PPV) of 85% for colectomy. Similar results (decreased CRP and Montreal classification[23] of UC activity as independent predictive factors for response on the 3rd day of treatment) have been obtained in a recent validated prospective study published as an abstract form[20]. Two additional studies retrospectively analyzed clinical and biochemical data at 1, 3 and 7 d[21], or within the first 3 d[22] of medical therapy, to obtain

predictive models of the likelihood of colectomy. In one of these, a value higher than 8 obtained by the formula *stool frequency/d + 0.14 × CRP (mg/dL)* calculated on the 3rd day after initiation of treatment had a PPV for colectomy of 72%[21]. This study confirmed that a regression formula including the same simple parameters used in the Travis *et al* study allows prediction of treatment failure and colectomy in a high percentage of severe UC patients. These results were prospectively validated in the only randomized, placebo-controlled study assessing infliximab as rescue therapy in severe UC, showing that patients fulfilling an index criteria of fulminant colitis (value ⩾ 8) in the placebo arm had a PPV for colectomy of 69%[24]. In the other retrospective study, a risk score was proposed to identify patients who are at low, intermediate or high likelihood of not responding to intensive medical treatment, aiding in the early selection of patients for second-line medical therapy or colectomy[22]. Multiple logistic regression analysis identified mean stool frequency (graded from 0 to 4 points) and colonic dilatation (4 points) within the first three days and hypoalbuminemia (1 point) on day 1 of treatment, as significantly predictive of the need for surgery within the hospitalization period. The risk score allowed the stratification of patients into those with low (11%; score 0-1), intermediate (45%; score 2-3) and high risk (85%; score ⩾ 4) of not responding to medical therapy. With a cut-off of ⩾ 4 points, the sensitivity and specificity in predicting non-response to medical therapy were 85% and 75% respectively.

The results of these studies are in agreement with previous observations showing that complete remission, which takes longer than response, is achieved, in the majority of steroid-sensitive cases, within 7 to 8 d of beginning corticosteroid treatment[15].

In conclusion, to evaluate steroid resistance, steroids must be intravenously administered, in the hospital setting, at a dose equivalent to 1 mg/kg per body weight of prednisone. The lack of response at day three of steroid treatment, as defined by well-established clinical parameters, suggests a high probability of colectomy and should be used in general as a limit point to define steroid resistance. Whether 3, 7 or 21 d are used as a limit marker for steroid resistance, judicious decisions by physicians, skilled in Inflammatory Bowel Disease management, should prevail. The risks and benefits of any decision have to be carefully weighed, taking into account that the priority in the management of severe UC is to save the patient and, if possible, the colon.

ACKNOWLEDGMENTS

CIBEREHD is funded by the Instituto de Salud Carlos Ⅲ.

REFERENCES

1 **Hardy TL**, Bulmer E. Ulcerative colitis. *Br Med J* 1933; **2**: 812-815
2 **Turner D**, Walsh CM, Steinhart AH, Griffiths AM. Response to corticosteroids in severe ulcerative colitis: a systematic review of the literature and a meta-regression. *Clin Gastroenterol Hepatol* 2007; **5**: 103-110
3 **Truelove SC**, Witts LJ. Cortisone in ulcerative colitis; preliminary report on a therapeutic trial. *Br Med J* 1954; **2**: 375-378
4 **Truelove SC**, Willoughby CP, Lee EG, Kettlewell MG. Further experience in the treatment of severe attacks of ulcerative colitis. *Lancet* 1978; **2**: 1086-1088
5 **Baron JH**, Connell AM, Kanaghinis TG, Lenneard-Jones JE, Jones AF. Out-patient treatment of ulcerative colitis. Comparison between three doses of oral prednisone. *Br Med J* 1962; **2**: 441-443
6 **Sood A**, Midha V, Sood N, Awasthi G. A prospective, open-label trial assessing dexamethasone pulse therapy in moderate to severe ulcerative colitis. *J Clin Gastroenterol* 2002; **35**: 328-331
7 **Elliott PR**, Powell-Tuck J, Gillespie PE, Laidlow JM, Lennard-Jones JE, English J, Chakraborty J, Marks V. Prednisolone absorption in acute colitis. *Gut* 1980; **21**: 49-51
8 **Berghouse LM**, Elliott PR, Lennard-Jones JE, English J, Marks V. Plasma prednisolone levels during intravenous therapy in acute colitis. *Gut* 1982; **23**: 980-983
9 **Seo M**, Okada M, Yao T, Ueki M, Arima S, Okumura M. An index of disease activity in patients with ulcerative colitis. *Am J Gastroenterol* 1992; **87**: 971-976
10 **Travis SP**. Predicting outcome in severe ulcerative colitis. *Dig Liver Dis* 2004; **36**: 448-449
11 **Travis S**. Review article: saving the colon in severe colitis - the case for medical therapy. *Aliment Pharmacol Ther* 2006; **24** Suppl 3: 68-73
12 **Stenner JMC**, White P, Gould SR, Lim AG. Audit of the management of severe ulcerative colitis in a DGH. *Gut* 2001; **48** Suppl 1: A87
13 **Dolwani S**, Hawthorne AB. Mortality caused by inflammatory bowel disease in the UK: a two year survey. *Gut* 2003; **52** Suppl 1: A12
14 **Hawthorne AB**, Travis SP. Outcome of inpatient management of severe ulcerative colitis: a BSG IBD clinical trials network survey. *Gut* 2002; **50**: A16
15 **Meyers S**, Lerer PK, Feuer EJ, Johnson JW, Janowitz HD. Predicting the outcome of corticoid therapy for acute ulcerative colitis. Results of a prospective, randomized, double-blind clinical trial. *J Clin Gastroenterol* 1987; **9**: 50-54
16 **Daperno M**, Sostegni R, Scaglione N, Ercole E, Rigazio C, Rocca R, Pera A. Outcome of a conservative approach in severe ulcerative colitis. *Dig Liver Dis* 2004; **36**: 21-28
17 **Gold DM**, Levine JJ, Weinstein TA, Kessler B, Pettei MJ. Prolonged medical therapy for severe pediatric ulcerative colitis. *Am J Gastroenterol* 1995; **90**: 732-735
18 **Carbonnel F**, Boruchowicz A, Duclos B, Soule JC, Lerebours E, Lemann M, Belaiche J, Colombel JF, Cosnes J, Gendre JP. Intravenous cyclosporine in attacks of ulcerative colitis: short-term and long-term responses. *Dig Dis Sci* 1996; **41**: 2471-2476
19 **Travis SP**, Farrant JM, Ricketts C, Nolan DJ, Mortensen NM, Kettlewell MG, Jewell DP. Predicting outcome in severe ulcerative colitis. *Gut* 1996; **38**: 905-910
20 **Mañosa M**, Doménech E, Gordillo J, Esteve M, García-Planella E, Cabré E, Bernal I, Zabana Y, Gassull MA. Prospective study of early predictors of response to corticosteroids (CS) in moderate to severe acute flares of ulcerative colitis (UC) and validation in an independent cohort. *Gut* 2007; **56** suppl: A155
21 **Lindgren SC**, Flood LM, Kilander AF, Lofberg R, Persson TB, Sjodahl RI. Early predictors of glucocorticosteroid treatment failure in severe and moderately severe attacks of ulcerative colitis. *Eur J Gastroenterol Hepatol* 1998; **10**: 831-835
22 **Ho GT**, Mowat C, Goddard CJ, Fennell JM, Shah NB, Prescott RJ, Satsangi J. Predicting the outcome of severe

ulcerative colitis: development of a novel risk score to aid early selection of patients for second-line medical therapy or surgery. *Aliment Pharmacol Ther* 2004; **19**: 1079-1087

23 **Silverberg MS**, Satsangi J, Ahmad T, Arnott ID, Bernstein CN, Brant SR, Caprilli R, Colombel JF, Gasche C, Geboes K, Jewell DP, Karban A, Loftus Jr EV, Pena AS, Riddell RH, Sachar DB, Schreiber S, Steinhart AH, Targan SR, Vermeire S, Warren BF. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Can J Gastroenterol* 2005; **19** Suppl A: 5-36

24 **Järnerot G**, Hertervig E, Friis-Liby I, Blomquist L, Karlén P, Grännö C, Vilien M, Ström M, Danielsson A, Verbaan H, Hellström PM, Magnuson A, Curman B. Infliximab as rescue therapy in severe to moderately severe ulcerative colitis: a randomized, placebo-controlled study. *Gastroenterology* 2005; **128**: 1805-1811

S- Editor Xiao LL **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5508

World J Gastroenterol 2008 September 28; 14(36): 5508-5511
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Javier P Gisbert, Professor; Fernando Gomollón, Dr., MD, PhD, *Series Editors*

Treatment of severe steroid refractory ulcerative colitis

Gert Van Assche, Séverine Vermeire, Paul Rutgeerts

Gert Van Assche, Séverine Vermeire, Division of Gastroenterology, University of Leuven Hospitals, Herestraat 49, B-3000 Leuven, Belgium
Paul Rutgeerts, Division of Gastroenterology, University of Leuven Hospitals, Herestraat 49, B-3000 Leuven, Belgium
Author contributions: Van Assche G, Vermeire S and Rutgeerts P contributed equally to this work.
Correspondence to: Dr. Gert Van Assche, Professor, Division of Gastroenterology, University of Leuven Hospitals, Herestraat 49, B-3000 Leuven,
Belgium. gert.vanassche@uz.kuleuven.be
Telephone: +32-16344225 Fax: +32-16344419
Received: August 18, 2008 Revised: August 26, 2008
Accepted: September 2, 2008
Published online: September 28, 2008

Abstract

Although systemic steroids are highly efficacious in ulcerative colitis (UC), failure to respond to steroids still poses an important challenge to the surgeon and physician alike. Even if the life time risk of a fulminant UC flare is only 20%, this condition is potentially life threatening and should be managed in hospital. If patients fail 3 to 5 d of intravenous corticosteroids and optimal supportive care, they should be considered for any of three options: intravenous cyclosporine (2 mg/kg for 7 d, and serum level controlled), infliximab (5 mg/kg IV, 0-2-6 wk) or total colectomy. The choice between these three options is a medical-surgical decision based on clinical signs, radiological and endoscopic findings and blood analysis (CRP, serum albumin). Between 65 and 85% of patients will initially respond to cyclosporine and avoid colectomy on the short term. Over 5 years only 50% of initial responders avoid colectomy and outcomes are better in patients naive to azathioprine (bridging strategy). The data on infliximab as a medical rescue in fulminant colitis are more limited although the efficacy of this anti tumor necrosis factor (TNF) monoclonal antibody has been demonstrated in a controlled trial. Controlled data on the comparative efficacy of cyclosporine and infliximab are not available at this moment. Both drugs are immunosuppressants and are used in combination with steroids and azathioprine, which infers a risk of serious, even fatal, opportunistic infections. Therefore, patients not responding to these agents within 5-7 d should be considered for colectomy and responders should be closely monitored for infections.

Key words: Ulcerative colitis; Steroids; Corticosteroids; Azathioprine; Cyclosporine; Infliximab; Immunosuppressants

Van Assche G, Vermeire S, Rutgeerts P. Treatment of severe steroid refractory ulcerative colitis. *World J Gastroenterol* 2008; 14(36): 5508-5511 Available from: URL: http://www.wjgnet.com/1007-9327/14/5508.asp DOI: http://dx.doi.org/10.3748/wjg.14.5508

INTRODUCTION

Some patients with ulcerative colitis (UC) remain symptomatic despite optimal doses of oral 5-aminosalicylic acid (5-ASA) drugs, topical therapy with either 5-ASA or steroids, and systemic corticosteroids. This can occur regardless of the extent of colonic involvement. However, steroids are highly efficacious in UC. Failure to induce remission will occur in only 40% of patients after a first course of oral systemic steroids and only 20% will have no improvement of symptoms whatsoever[1,2]. In patients with early relapse after an initial successful course of systemic steroids, subsequent courses of steroids are probably less efficacious, but this has never been studied in a controlled trial. The term "steroid refractory" has been used to define patients whose symptoms never responded to corticosteroids and those who respond initially but developed recurrence while continuing treatment. The distinction can be important since treatment options in the two settings may differ. In patients who have initially responded, the dose of oral steroids can be temporarily increased to the dose level that controlled symptoms before, whereas patients never responding need alternative options on the short term.

GENERAL TREATMENT RECOMMENDATIONS

The approach to treatment should be based on the severity of the disease flare. Moderately ill patients can usually be managed in an outpatient setting with the approaches summarized below. Disease flares are considered to be moderate or moderate-to-severe when

symptoms impair with activities of daily life, but are not necessitating immediate hospitalization. Fulminant UC necessitates IV therapy in hospital as described below and regardless of previous oral steroid use[3].

APPROACH TO SEVERE ATTACKS OF ULCERATIVE COLITIS

Only 15 to 20 percent of patients with UC will ever experience an attack of fulminant colitis[4]. Patients with pancolitis appear to be predisposed to severe flares (Figure 1). Severe UC is a serious, potentially life threatening condition and hospitalization should be considered in all patients who have more than 6-10 bloody stools per day, associated with fever, dehydration, tachycardia malaise and/or increased C-reactive Proteine (CRP)[3,4,5]. Patients entering hospital with fulminant colitis should be evaluated for other causes of severe colitis. We always perform an unprepped sigmoidoscopy to assess disease severity, to obtain mucosal biopsies, and to provide a baseline assessment. Total colonoscopy and ileoscopy should not be attempted as it carries the risk of inducing toxic megacolon. As a rule sigmoidoscopy in fulminant colitis should be performed by an experienced endoscopist with minimal inflation and at the first sign of discomfort from the patient no further proximal progression should be attempted. Infectious colitis should be excluded with stool culture for bacterial pathogens, C. difficile toxin test in stools and ova/parasites in a fresh stool sample and careful assessment of the mucosal biopsies. The likelihood of infectious colitis is geographically determined but should be considered in all patients. Although the precise role of active cyto-megalo virus (CMV) replication in fulminant colitis is still debated, the presence of CMV inclusions in a colonic biopsy should be ruled out. In the recent patient history foreign travel and non steroidal anti inflammatory drugs (NSAID) use should be recorded. Clinically disease severity should be assessed using the criteria in the Lichtiger score including stool frequency, nocturnal diarrhea and fecal incontinence, rectal bleeding, abdominal cramping and tenderness and general well being. Others signs of fulminant colitis include fever, lethargy and dehydration. Blood analysis should include: serum albumine, electrolytes, hemoglobin, white blood cell count (WBD) and differential, CRP, blood urea nitrogen (creatinine) (BUN) and liver tests[2]. A plain abdominal X-ray should be obtained initially and at regular intervals during the hospitalization. We generally treat those patients with IV prednisolone (60 mg daily) or equivalent as a continuous infusion regardless of prior oral corticosteroid therapy. Pioneering studies by Truelove *et al*[1] have shown that applying this strategy 64 percent of patients will enter clinical remission and only 23 percent require rescue total procto-colectomy. Doses higher than 60 mg or 1 mg/kg prednisolone equivalent are not recommended. Although recent controlled evidence failed to show superiority of a continuous infusion of methyl-

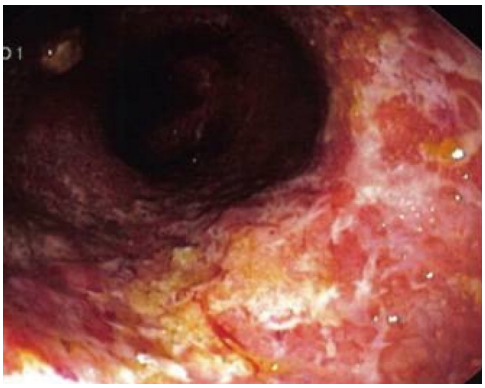


Figure 1 Endoscopic lesions compatible with severe colitis.

prednisolone 1 mg/kg IV daily over and IV bolus therapy at the same dose, most centers still prefer continuous infusion. Supportive therapy for this condition includes relative bowel rest and parenteral nutrition if needed. The value of antibiotics as a prophylactic or adjuvant therapy in the setting of fulminant colitis has not been established. However, patients with high fever, signs of peritonitis or high CRP levels should receive broad spectrum antibiotics (e.g. ciprofloxacin + metronidazole). Even so, initiation of antibiotics should never postpone the decision for procto-colectomy.

If patients fail to respond to three to five days of IV steroids, they should be considered for intensified medical therapy (described below) or colectomy[3,4]. Daily clinical follow up of these patients by both an expert surgeon and physician is required from that stage on and colectomy should be considered if the clinical condition of a patient worsens. Addition of rectal hydrocortisone drips or mesalamine enemas can be considered at this stage particularly in patients with symptoms secondary to left sided colitis.

THERAPY OPTIONS FOR PATIENTS FAILING IV CORTICOSTEROIDS

When patients fail three to five days of IV corticosteroids at adequate doses and continue to report frequent bloody diarrhea with fever or high CRP levels, they should be considered for surgical colectomy or rescue medical treatment[3,4]. Complications such as toxic megacolon or uncontrolled bleeding should favor the decision towards surgical intervention.

Intravenous cyclosporine has been shown to be an effective rescue therapy for severe UC attacks in two controlled trials[6,7]. In the trial by Lichtiger *et al*, 9 out of 11 IV 4 mg/kg cyclosporine treated patients avoided colectomy versus none of the 9 placebo treated patients. Data from one mono center controlled trial in 73 patients indicate that 2 mg/kg per day IV cyclosporine initial treatment may prove as effective for severe attacks of UC, although not all of these patients were failing intravenous corticosteroids[8]. When results from controlled and non-controlled trials are pooled 76 to 85 percent of patients will respond to IV

cyclosporine and avoid colectomy short term. Before the initiation of Ⅳ cyclosporine hypomagnesemia and hypocholesterolemia should be corrected to decrease the risk neurologic toxicity. After an initial dose of 2 mg/kg, daily cyclosporine doses should be adjusted to achieve therapeutic blood levels from day two onwards. Therapeutic ranges for cyclosporine blood levels may vary based on the assay used. The median time to response for Ⅳ cyclosporine is four to five days and in patients responding, initiation of oral cyclosporine therapy at 5-8 mg/kg divided in two doses should be considered along with gradual steroid tapering and initiation of azathioprine or 6-mercaptopurine. While patients are on a triple immunosuppressive regimen prophylaxis against *Pneumocystis carinii* pneumonia should be given and alertness for opportunistic infections in general should be high. Cyclosporine use in UC has been associated with mortality and most of the fatalities were due to opportunistic infections[9]. Other complications of cyclosporine therapy include nephrotoxicity, tremor and convulsions, hypertension, gingival hyperplasia and hypertrichosis. Rare cases of anaphylaxis are contributed to the solvent in Sandimmun, the commercially available formulation of cyclosporine, and occurrence of anaphylaxis allows treatment with oral cyclosporine.

Following initial response to cyclosporine for fulminant UC about 50 percent of patients avoid colectomy at three years[9-12]. Lower colectomy free rates have been recently reported with follow up extending to seven years[11]. The patient population already failing adequate courses of azathioprine or 6-MP is most prone to colectomy following initial response to cyclosporine[10,11].

Tacrolimus an oral cyclosporine can be considered to treat severe attacks of ulcerative colitis but only retrospective uncontrolled data are available[13-15].

INFLIXIMAB

The efficacy of infliximab in the setting of severe UC not responding to therapy with intravenous steroids has been demonstrated recently in a small placebo controlled trial. Significantly more patients treated with placebo (14/21) required surgical colectomy by three months as compared to those treated with a single dose of infliximab 5 mg/kg Ⅳ (7/24)[16]. Open label experience in patients with severe UC attacks has been inconsistent[17,18]. In a recent publication from the colorectal surgery group at the Mayo Clinic, Rochester, MN, an increased risk of infectious postoperative complications was found in a group of infliximab treated patients as compared to controls[19]. It should be noted however, that disease severity, use of immunosuppressives and Ⅳ steroids was higher in the infliximab group. Other retrospective cohorts and the controlled Scandinavian trial have not confirmed this increased complication risk. However, preliminary results from a cohort of patients treated at the Mount Sinai hospital in New York suggest that patients receiving infliximab followed by cyclosporine or vice versa have a substantial risk of serious adverse events including mortality[20]. Data on long term avoidance of colectomy with infliximab are as of yet not available, but there is no indication that infliximab would increase surgical complications.

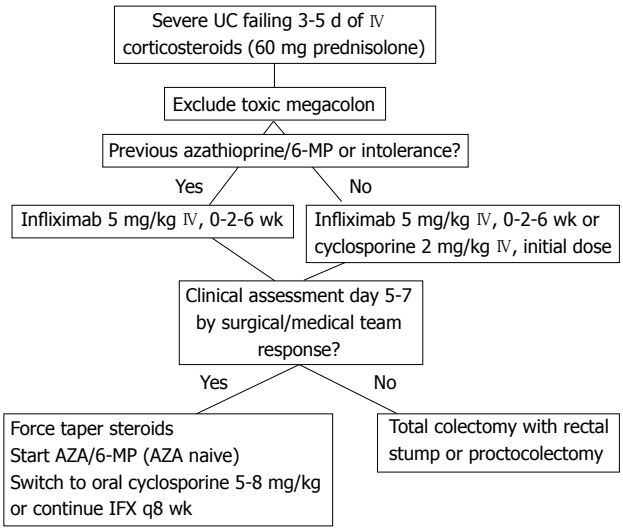


Figure 2 Proposed treatment algorithm for the management of severe steroid refractory ulcerative colitis.

SURGICAL COLECTOMY

Surgical proctocolectomy with ileo-anal pouch anastomosis is a valid option for patients with moderate to severe UC failing medical therapy. Patients should be counseled about the option of surgery, short term complication and long term outcomes of pouch surgery, early in the course of a severe flare of UC. Also from the first day of hospitalization the surgical team should be involved in the management of the patient with fulminant UC.

EXPERT OPINION

Patients with severe attacks of UC should be hospitalized and closely monitored. After failing three to five days of intravenous corticosteroids, patients should be considered for intravenous cyclosporine (2 mg/kg per day), for infliximab (5 mg/kg Ⅳ) or for surgical colectomy. Cyclosporine is most useful as a bridge to the effect of azathioprine or 6-MP and should be considered particularly in this setting. Long term colectomy free survival rates after initial response to cyclosporine are far from optimal and we have no long term data with infliximab yet. However, in patients with a dramatic response to any of the two immune therapies a delayed elective colectomy later in the disease course may be a noble goal *per se*. In patients failing Ⅳ steroids, he risk of, even fatal, serious infections with cyclosporine is clearly increased and also patients responding to infliximab should be closely monitored for opportunistic infections. Finally, only a head to head comparison of cyclosporine and infliximab in a prospective trial will be able to conclusively guide us in immediate decision making (Figure 2).

REFERENCES

1 **Truelove SC**, Witts LJ. Cortisone in ulcerative colitis. Final report on a therapeutic trial. *Br Med J* 1955; **2**: 104-108
2 **Baron JH**, Connell AM, Kanaghinis TG, Lennard-Jones JE, Jones AF. Out-patient treatment of ulcerative colitis. Comparison between three doses of oral prednisone. *Br Med J* 1962; **2**: 441-443
3 **Truelove SC**, Willoughby CP, Lee EG, Kettlewell MG. Further experience in the treatment of severe attacks of ulcerative colitis. *Lancet* 1978; **2**: 1086-1088
4 **Travis SP**, Farrant JM, Ricketts C, Nolan DJ, Mortensen NM, Kettlewell MG, Jewell DP. Predicting outcome in severe ulcerative colitis. *Gut* 1996; **38**: 905-910
5 **Edwards FC**, Truelove SC. The course and prognosis of ulcerative colitis. *Gut* 1963; **4**: 299-315
6 **Lichtiger S**, Present DH, Kornbluth A, Gelernt I, Bauer J, Galler G, Michelassi F, Hanauer S. Cyclosporine in severe ulcerative colitis refractory to steroid therapy. *N Engl J Med* 1994; **330**: 1841-1845
7 **D'Haens G**, Lemmens L, Geboes K, Vandeputte L, Van Acker F, Mortelmans L, Peeters M, Vermeire S, Penninckx F, Nevens F, Hiele M, Rutgeerts P. Intravenous cyclosporine versus intravenous corticosteroids as single therapy for severe attacks of ulcerative colitis. *Gastroenterology* 2001; **120**: 1323-1329
8 **Van Assche G**, D'Haens G, Noman M, Vermeire S, Hiele M, Asnong K, Arts J, D'Hoore A, Penninckx F, Rutgeerts P. Randomized, double-blind comparison of 4 mg/kg versus 2 mg/kg intravenous cyclosporine in severe ulcerative colitis. *Gastroenterology* 2003; **125**: 1025-1031
9 **Arts J**, D'Haens G, Zeegers M, Van Assche G, Hiele M, D'Hoore A, Penninckx F, Vermeire S, Rutgeerts P. Long-term outcome of treatment with intravenous cyclosporin in patients with severe ulcerative colitis. *Inflamm Bowel Dis* 2004; **10**: 73-78
10 **Cohen RD**, Stein R, Hanauer SB. Intravenous cyclosporin in ulcerative colitis: a five-year experience. *Am J Gastroenterol* 1999; **94**: 1587-1592
11 **Moskovitz DN**, Van Assche G, Maenhout B, Arts J, Ferrante M, Vermeire S, Rutgeerts P. Incidence of colectomy during long-term follow-up after cyclosporine-induced remission of severe ulcerative colitis. *Clin Gastroenterol Hepatol* 2006; **4**: 760-765
12 **Campbell S**, Travis S, Jewell D. Ciclosporin use in acute ulcerative colitis: a long-term experience. *Eur J Gastroenterol Hepatol* 2005; **17**: 79-84
13 **de Saussure P**, Soravia C, Morel P, Hadengue A. Low-dose oral microemulsion ciclosporin for severe, refractory ulcerative colitis. *Aliment Pharmacol Ther* 2005; **22**: 203-208
14 **Fellermann K**, Tanko Z, Herrlinger KR, Witthoeft T, Homann N, Bruening A, Ludwig D, Stange EF. Response of refractory colitis to intravenous or oral tacrolimus (FK506). *Inflamm Bowel Dis* 2002; **8**: 317-324
15 **Baumgart DC**, Pintoffl JP, Sturm A, Wiedenmann B, Dignass AU. Tacrolimus is safe and effective in patients with severe steroid-refractory or steroid-dependent inflammatory bowel disease--a long-term follow-up. *Am J Gastroenterol* 2006; **101**: 1048-1056
16 **Jarnerot G**, Hertervig E, Friis-Liby I, Blomquist L, Karlén P, Grännö C, Vilien M, Ström M, Danielsson A, Verbaan H, Hellström PM, Magnuson A, Curman B. Infliximab as rescue therapy in severe to moderately severe ulcerative colitis: a randomized, placebo-controlled study. *Gastroenterology* 2005; **128**: 1805-1811
17 **Medlinechey WY**. Infliximab for patients with refractory ulcerative colitis. *Inflamm Bowel Dis* 2001; **7** Suppl 1: S30-S33
18 **Regueiro M**, Curtis J, Plevy S. Infliximab for hospitalized patients with severe ulcerative colitis. *J Clin Gastroenterol* 2006; **40**: 476-481
19 **Maser E**, Deconda D, Lichtiger S, Present D, Kornbluth A. Cyclosporine (CsA) and infliximab (Infl) as acute salvage therapies for each other in severe steroid refractory ulcerative colitis (UC). *Gastroenterology* 2007; **132**: A180
20 **Selvasekar CR**, Cima RR, Larson DW, Dozois EJ, Harrington JR, Harmsen WS, Loftus EV Jr, Sandborn WJ, Wolff BG, Pemberton JH. Effect of infliximab on short-term complications in patients undergoing operation for chronic ulcerative colitis. *J Am Coll Surg* 2007; **204**: 956-962; discussion 962-963

S- Editor Xiao LL **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5512

World J Gastroenterol 2008 September 28; 14(36): 5512-5518
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Javier P Gisbert, Professor; Fernando Gomollón, Dr., MD, PhD, *Series Editors*

Are we giving azathioprine too late? The case for early immunomodulation in inflammatory bowel disease

María Josefina Etchevers, Montserrat Aceituno, Miquel Sans

María Josefina Etchevers, Department of Gastroenterology, Hospital Posadas, Buenos Aires, Argentina; Department of Gastroenterology, Hospital Clínic i Provincial/IDIBAPS, Barcelona, CIBER Enfermedades Hepáticas y Digestivas, Spain
Montserrat Aceituno, Miquel Sans, Department of Gastroenterology, Hospital Clínic i Provincial/IDIBAPS, Barcelona, CIBER Enfermedades Hepáticas y Digestivas, Spain
Author contributions: All authors contributed equally to this work.
Supported by Grants from Ministerio de Educación y Ciencia (SAF2005-00280) to MS
Correspondence to: Miquel Sans, MD, PhD, Department of Gastroenterology, Hospital Clínic/IDIBAPS, 170 Villarroel St., 08036 Barcelona, Spain. msans@clinic.ub.es
Telephone: +34-93-2275418 Fax: +34-93-2279387
Received: August 18, 2008 Revised: August 26, 2008
Accepted: September 2, 2008
Published online: September 28, 2008

Abstract

Inflammatory bowel disease (IBD) includes two entities, Crohn's disease and ulcerative colitis. Both are chronic conditions with frequent complications and surgical procedures and a great impact on patient's quality of life. The thiopurine antimetabolites azathioprine and 6-mercaptopurine are widely used in IBD patients. Current indications include maintenance therapy, steroid-dependant disease, fistula closure, prevention of infliximab immunogenicity and prevention of Crohn's disease recurrence. Surprisingly, the wide use of immunosuppressants in the last decades has not decreased the need of surgery, probably because these treatments are introduced at too late stages in disease course. An earlier use of immunossupressants is now advocated by some authors. The rational includes: (1) failure to modify IBD natural history of present therapeutic approach, (2) demonstration that azathioprine can induce mucosal healing, a relevant prognostic factor for Crohn's disease and ulcerative colitis, and (3) demonstration that early immunossupression has a very positive impact on pediatric, recently diagnosed Crohn's disease patients. We are now awaiting the results of new studies, to clarify the contribution of azathioprine, as compared to infliximab (SONIC Study), and to demonstrate the usefulness of azathioprine in recently diagnosed adult Crohn's disease patients (AZTEC study).

Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Immunosuppressants; Azathioprine

Etchevers MJ, Aceituno M, Sans M. Are we giving azathioprine too late? The case for early immunomodulation in inflammatory bowel disease. *World J Gastroenterol* 2008; 14(36): 5512-5518 Available from: URL: http://www.wjgnet.com/1007-9327/14/5512.asp DOI: http://dx.doi.org/10.3748/wjg.14.5512

INTRODUCTION

IBD and azathioprine

Inflammatory bowel disease (IBD) includes two main entities, ulcerative colitis (UC) and Crohn's disease (CD). Both are chronic, inflammatory disorders of the gastrointestinal tract, with an increasing prevalence in developed countries. IBD affects patients early in life, resulting in an enormous personal, social and economic burden. Although the etiology of IBD remains unknown, major progress has been done in our understanding of IBD pathophysiology in recent years. We now believe that IBD develops in genetically predisposed individuals, due to an abnormal recognition of microbiota antigens by certain elements and cells of the innate immunity, leading to a deregulated immune reaction and, ultimately, resulting in bowel inflammation and injury [1].

Treatment of IBD has greatly evolved in the last two decades. A better understanding of IBD pathophysiology has progressively resulted in a more frequent use of inmunossupressants, such as azathioprine (AZA), mercaptopurine and methotrexate and the arrival of the so called "biological therapy", represented by the anti-TNF-α antibodies. Whether this "more aggressive" approach has really had any impact on IBD patient's outcome is still a matter of controversy. As an example, an interesting work by Cosnes and colleagues revealed that, in spite of the striking increase in the azathioprine use in CD patients, the natural history of CD, as judged by the percentage of CD patients requiring surgical resection, had remain unchanged over the last 40 years

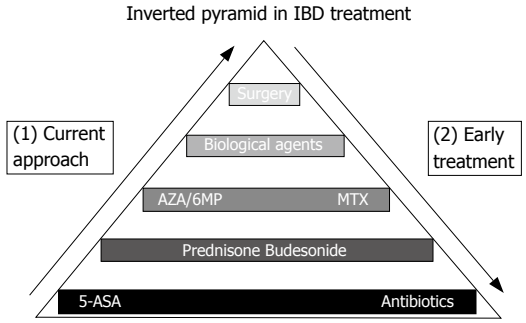


Figure 1 Inverted pyramid. Represents two different treatments approaches in IBD: (1) Current practice: Initially treatment with 5-ASA and antibiotics and then depends on the responsiveness scale to steroids, immunomodulators and biological agents. (2) Top-Down strategy: New tendencies. Initially aggressive treatment with early immunosuppresion and biological therapy.

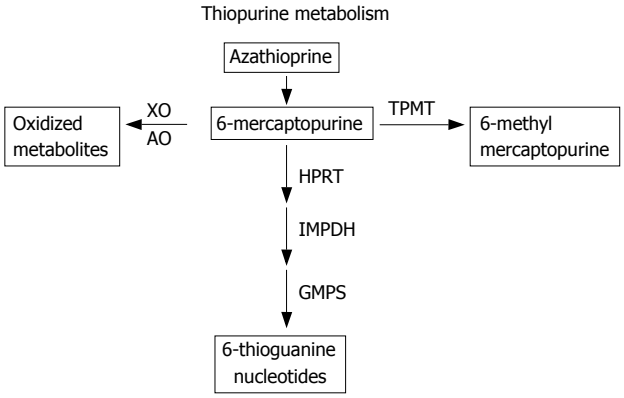


Figure 2 Metabolism of azathioprine (AZA): AZA is converted to 6-mercaptopurine (6-MP). 6-MP by thiopurine methyltransferase (TPMT) and xantino oxidase (XO) in inactive metabolites, but phosphoriboxyltransferase (HPRT), inosine monophosphate dehydrogenase (IMPDH) and guanosine monophosphate synthetase (GMPS) catalize the synthesis of active 6-thioguanine nucleotides (6-TGNs).

and until our days[2]. Although the results of this study might indicate indeed that azathioprine is unable to modify CD natural history, a closer view of this study reveals that in most CD patients azathioprine had been introduced really late in the patient's course, often after the development of a penetrating phenotype or following a surgical procedure. This fact sets the notion that an early use of immunosuppressants might result in a significant impact on IBD patient's natural course.

The aim of this article is to make a critical analysis of the current use of thiopurines, but also attempts to analyze the new tendencies in terms of optimal time to initiate immunosuppression with antimetabolites in IBD (Figure 1).

Pharmacology of azathioprine

6-Mercaptopurine (6-MP) and its prodrug azathioprine are thiopurine analogues and are immuno-modulatory agents. Of the AZA compound, 88% is converted *via* nonenzymatic process to 6-MP. Then 6-MP undergoes several enzymatic pathways and is transformed to active and inactive metabolites (Figure 2). The first step in the 6-MP metabolism is a catabolic process by xanthine-oxidase, which is present in the intestinal mucosa and liver, resulting in inactive oxidized metabolites, such as 6-thiouric acid. 6-MP also serves as a substrate for the thiopurine methyltransferase (TPMT) which, by methylation, converts 6-MP into inactive metabolites, such as 6-methyl mercaptopurine. Anabolic processes led to the synthesis of active metabolites. First hypoxanthine phosphoribosyltransferase initiates the transformation of 6-MP into active metabolites, or 6-thioguanine nucleotide[3-4]. These nucleotides act as purine antagonists interfering DNA and RNA synthesis which has been demonstrated to result in a significant inhibition of lymphocyte proliferation and a decreased immunity response.

Not all individuals methylate thiopurines equally, a series of processes that depend on the genetic variability in the TPMT activity. The TPMT gene is inherited as an autosomal co-dominant trait. Most of the Caucasian population (about 89%), have normal to high enzyme activity (known as homozygous wild-type TPMT), 11% have intermediate enzyme activity (heterozygous TPMT) and 0.3% have low or absent enzyme activity (homozygous mutated TPMT). The two last groups are associated with major elevations of 6-TGN levels and an increased risk of adverse effects[3,5]. This genetic variation can be studied at different levels. First, the genetic study will ascertain the patient's genotype. Second, it is also possible to measure the enzyme activity in a blood sample. Third, also the blood levels of active and inactive, methylated metabolites can be quantified. A widely accepted consensus on the best way to address this issue has not been achieved to date.

Safety of azathioprine

There is little doubt that patients treated with azathioprine have a higher rate of adverse events than placebo treated patients. A Cochrane meta-analysis, by Sandborn and colleagues, analyzed the usefulness and safety of AZA and 6-MP when used to induce remission in CD patients[6]. In this meta-analysis, adverse events occurred in 9.3% of patients taking AZA or 6-MP *versus* 2.3% of those taking placebo. The number needed to harm (number of patients that should be treated to develop a single adverse event) was 14. Pearson and colleagues also addressed the issue of azathioprine safety in another Cochrane meta-analysis, but specifically devoted to patients receiving this drug as maintenance therapy for CD. Drug withdrawal due to adverse effects were found in 5.8% of patients receiving thiopurines, as compared to 1.3% of patients without treatment. In this case, the number needed to harm was 19[7].

Classically, AZA-related adverse events have been categorized in two types: allergic, idiosyncratic or none dose dependent, and dose-dependent. Allergic reactions include, among others, malaise, rush, fever, pancreatitis and hepatitis. All of them are infrequent, occurring in a 5%-10% of AZA-treated patients. These adverse events are not related to the dose of AZA used or the variations in drug metabolism. In general, dose dependant adverse effects are much more frequent than the previous.

Table 1 Vienna classification of Crohn's disease[12]

Parameter	
Age at diagnosis	A1 < 40 yr A2 ⩾ 40 yr
Maximal location of disease prior to first surgery	L1 Terminal ileal L2 Colonic L3 Ileocolonic L4 Any upper GI, regardless of disease elsewhere in bowel
Disease behavior	B1 Nonstricturing, nonpenetrating (inflammatory) B2 Stricturing B3 Penetrating disease Behavior could be defined at any time after diagnosis

Table 2 Montreal classification of Crohn's disease[13]

Parameter	
Age at diagnosis	A1 ⩽ 16 yr A2 17-40 yr A3 ⩾ 40 yr
Maximal location of disease prior to first surgery	L1 Terminal ileal L2 Colonic L3 Ileocolonic L4 Upper GI only +L4 Additional designation to be added if patient has upper GI and distal disease
Disease behavior	B1 Nonstricturing, nonpenetrating (inflammatory) B2 Stricturing B3 Penetrating disease *P* = Perianal penetrating disease Recommended behavior assessed > 5 yr after diagnosis

Bone marrow suppression is the most common dose-dependent adverse effect. Leukopenia appears in 2% to 15% of AZA-treated patients, depending on the cut-off used for its definition, is influenced by the degree of TPMT activity and can modified by other concomitant drugs if they impact the enzyme activity. Such myelosuppression is reversible upon AZA dose reduction or transient suspension of the drug.

Another potential source of AZA-related adverse reactions steam from the immune suppression caused by the drug. AZA and 6-MP therapy is associated with an increased risk of infections ranging from 0.3% to 7.4%[8]. The most common are viral infections, such as cytomegalovirus, Epstein-Barr virus, varicella zoster virus and herpes simplex virus. Noteworthy, infections can occur in the absence of leukopenia. Thiopurine-induced liver toxicity is also a relevant issue. It's incidence varies between 3% and 10% of AZA-exposed patients and it can be classified into different entities: hypersensitivity, idiosyncratic cholestatic reaction, and endothelial cell injury (the later resulting in raised portal pressures, veno-occlusive disease or peliosis hepatis)[9]. The majority of these syndromes respond to drug withdrawal.

Finally the relationship between thiopurines and development of cancer, and specially hematologic malignancies such as lymphomas, remains a controversial topic. A meta-analysis of risk of malignancy associated to the use of immunosuppressive drugs in inflammatory bowel disease identified 9 studies reporting colorectal cancer, malignant melanoma, leukemia and lymphoma cases. The weighted mean difference of malignancy incidence in IBD patients who received immunosuppressive agents, as compared to IBD patients not exposed to immunosuppressants, was -0.3×10^{-3}/person per year. There was no significant difference when the authors analyzed the length of exposure to immunosuppressants or whether the patients had CD or UC[10]. The issue of relationship between lymphoma and IBD is complex, because the effect caused by the disease "*per se*", by disease activity, and by different IBD therapies clearly overlap. In a Meta-analysis of Kandiel and colleagues[11] they identify 6 cohort studies with AZA or 6-MP exposure that have been specifically designed to evaluate cancer as adverse outcome. The total number of observed cases was 11 with a pooled relative risk of 4.18. Because these data were obtained from observational studies it is not possible to fully exclude the possibility of severity of the disease as confounding factor. As a global conclusion, the consensus about the relationship between immunosuppressants and lymphoma is that, if any association exists, it would be of small magnitude and, in any case, the beneficial effects exerted by these drugs on IBD patient's outcome would clearly overweight the risk caused by the drug itself.

PRESENT USE OF AZATHIOPRINE IN IBD

Use in Crohn's disease

Crohn's disease is a heterogeneous entity that requires an individual approach. Several attempts have been done to homogenize CD evaluation. The classification obtained as result of the consensus effort held in Vienna, in 1998[12], categorize CD in subgroups according age at diagnosis, disease location and disease behavior (inflammatory, fistulating or stenosing) (Table 1).

In spite of its initial usefulness, significant advances have been made in recent years. It has become clear that children have different presentation, disease location can be simultaneous in different segments of the bowel and disease behavior is dynamic. Because of these in 2005 the Montreal classification[13] added a separate category for children with onset at ⩽ 16 years, acknowledged the coexistence of CD in the upper GI tract with distal disease and differentiated between internal and perianal penetrating disease (Table 2).

AZA to induce CD remission

Because of the delayed onset of action of thiopurines analogues, even with intravenous administration[14], this drugs have been most frequently used, with concomitant use of steroids or more recently with infliximab[15], in patients with active disease in the context of corticosteroid-dependent CD. A Cochrane database meta-analysis[6] found eight randomized,

placebo-controlled trials in adults that evaluated the use of azathioprine or 6-mercaptopurine in active Crohn's disease. The outcome measure was the proportion of patients with clinical improvement or remission (as defined by the CDAI score, the Harvey-Bradshaw Index, subjective evaluation or steroid sparing effect). The pooled response rate was 54% for the group with thiopurine analogues *versus* 34% for the placebo treated patients, with a peak response odds ratio reached at 17 wk of therapy. The number needed to treat is 5 in order to benefit 1 patient.

In clinical practice the use of azathioprine as monotherapy to induce remission in active CD is very rarely indicated, due to its delayed action. In mild-to-moderate, chronically active CD, AZA can be an option in selected patients.

AZA to obtain a steroid–sparing effect in CD

The efficacy of AZA and 6-MP, as compared with placebo, in the subgroup of corticosteroid-dependent CD is well established. This effect is observed in patients with active CD and in patients with quiescent but corticosteroid-dependent disease. Both scenarios were analyzed in two Cochrane Database Meta-analyses.

In active disease, five studies showed that the use of antimetabolites results in a pooled reduction from 65% to 36% in the percentage of patients receiving steroids, as compared to the placebo group. In this case, the number needed to treat to obtain a steroid-sparing effect was 3 patients[6].

In patients with clinical remission but corticosteroid-dependent behavior, only two small studies (overall 30 patients) were randomized, double-blind, placebo-controlled. The global effect in reduce steroid consumption was 87% (13/15) for the AZA group *versus* 53% (8/15) for the placebo group with a number needed to treat for one reduction in steroid consumption of 3[7].

AZA to maintain remission in CD

Once CD is quiescent, the thiopurine analogues are a very effective therapeutic option to maintain disease remission. This situation was analyzed by Pearson and colleagues in a Cochrane Meta-analysis. They found five trials that satisfied the inclusion criteria as randomized, double-blind, and placebo-controlled. In spite the variability in terms of doses and duration of therapy, the overall remission maintenance was 67% with AZA compared with 52% of the placebo group. It's worth to mention that the effect is dose-dependent with optimal benefit at dose of 2.5 mg/kg per day[7]. All these data support the use of antimetabolites as maintenance therapy.

In the pediatric study by Markowitz and colleagues[16], only 4% of patients (1 children) of the 6-MP group required another course of steroids within 540 d, after being weaned off of prednisone, clearly in contrast to the 57% of pediatric CD patients receiving placebo which need to restart prednisone within 360 d ($P < 0.0001$).

AZA to prevent disease recurrence after surgery in CD

The incidence of surgery in the course of CD is very high, reaching a cumulative risk of 78%-91% in some classic studies[17-18]. Postoperative recurrence approaches the 100% of patients, at 3 years, when assessed endoscopically[19]. Fortunately, clinical recurrence and the need for repeated surgery are lower (15%-45% after 3 years). AZA and 6-MP are effective as a therapy to prevent disease recurrence. AZA or 6-MP are probably more efficacious than 5-ASA compounds, but the design of the studies aimed at clarifying this issue does not allow a strong statement in that respect[20-21].

AZA to induce remission in fistulizing CD

The risk of developing internal or perianal fistulas is really high in CD patients and close to 50% of them will develop any form of penetrating behavior in a lifetime. The rate of complete healing with the available medical treatments is 50% and multiple relapses are frequents. In the Cochrane Database Meta-analyses perfomed by Sanborn and colleagues, 55% of the patients with AZA compared with 29% of the placebo treated patients achieved a response. The number needed to treat to observe one patient with fistula healing was 4, which confirms that AZA is a potent therapeutic strategy for this type of CD-related complications[6].

Use in ulcerative colitis

There are less robust data of the use of thipurine analogues in UC compared to CD. In active disease most of the studies evaluated the remission of the disease in the context of corticosteroid-dependant or resistant disease with co-medication with aminosalicylates, steroids or biological agents, not as monotherapy treatment. In a prospective trial of Ardizzone *et al*[22], 72 patients with steroid dependant UC were randomized to receive azathioprine 2 mg/kg per day or oral 5-aminosalicylic acid (5-ASA) 3.2 g/day for a 6 months follow up. The AZA group achieved a clinical and endoscopic remission of 53% compared with 19% of the 5-ASA group ($P = 0.006$). There is no doubt of the efficacy of AZA and 6-MP as steroid-sparing agents in subjects with corticoid- dependence and for maintenance in patients with remission induced by cyclosporine or in whom have failed or cannot tolerate standard maintenance therapy with aminosalicylates. A recent Cochrane Database Meta-analyses by Timmer *et al*[23], found 6 studies which examined the efficacy of purine antimetabolites compared to placebo or standard maintenance therapy in ulcerative colitis. In the pooled analysis, azathioprine was superior to placebo for maintenance of remission. 56% of patients treated with AZA were on disease free after one year of treatment compared to 35% of patients who received placebo. The number needed to treat is 5 in order to benefit 1 patient. In summary, azathioprine is effective in maintenance therapy for patients who have failed or cannot tolerate aminosalicylates and patients who require repeated courses of steroids.

Length of AZA treatment

The question how long thiopurines should be continued and until how long there is a really benefit of this therapy in patients that achieved remission is not

complete resolved. The study's results are contradictory. The first who evaluated this issue was O'Donoghue in 1978 in 51 CD patients who were receiving AZA for at least 6 mo. The cumulative probability of relapse at 1 year was 5% for the AZA treated group *versus* 41% in the placebo group ($P < 0.01$)[24]. After this first report, several studies tried to further clarify this issue. A multicenter, randomized, double-blind, noninferiority withdrawal trial in CD patients who were in remission on AZA treatment for $\geqslant$ 3.5 years showed that the mean relapse rate at 18 mo in patients who stopped the drug was nearly 3 times that observed in those who were maintained on treatment (21.3% *vs* 7.9%). Therefore the authors recommended continuing with AZA maintenance therapy beyond 3.5 years[25]. Another retrospectively study in 1176 patients with IBD (CD and UC) from 16 European centers showed that within the first 4 years of treatment, AZA diminished the incidence of flares and steroid consumption in both diseases and continuation beyond 4 years improved clinical activity and steroid requirements[26]. Conversely to the mentioned study, Bouhnik Y and colleagues found that in CD patients who were in clinical remission taking AZA or 6-MP after 4 years of remission on these drugs, the risk of relapse is similar whether the therapy was maintained or stopped[27]. Recently, Mantzaris *et al* published a prospective, investigator-blind study in patients with steroid-dependant Crohn's disease in remission on AZA. They stratified patients in two groups: Group A, consisted of patients receiving continuously azathioprine for between 2 and 4 years and Group B; which consisted of patients receiving azathioprine for between 4 and 8 years. The annual relapse rate in Group A was 19.6% *versus* 11.9% in the Group B without significant difference ($P = 0.67$)[28]. For the clinician's point of view, and taking into account the published evidences, an individual decision is often required.

THE FUTURE: TOWARDS AN EARLIER USE OF AZATHIOPRINE IN IBD

Why early azathioprine? Natural history of CD

CD is a progressive condition, characterized by a frequent development of CD-related complications, such as internal fistulas and abscesses, perianal fistulas and bowel strictures. This results is a high surgical requirement, with a significant proportion of CD patients receiving bowel resection at some point. One of the key factors determining the natural history of CD is the disease duration, since an increasing number of complications have been described over time. Louis *et al* assessed retrospectively the evolution of the disease after 1 to 25 years since diagnosis. At diagnosis, 73.7% of patients had an inflammatory phenotype while at 20 years only 12% had this phenotype and 32% and 48.8% had structuring and penetrating behavior respectively. The proportion of patients who had surgery over a 10 year period was 30.4% being higher in the subgroups B2 and B3[29].

In the study of Cosnes *et al*, they evaluated retrospectively 2002 patients with CD. The rate of complication was 80% at 20 years. At 5 and 20 years after diagnosis, the actuarial risks for stricturing disease alone were 12% and 18% respectively, whereas they were 40% and 70% respectively for penetrating disease[30].

Two main conclusions can be obtained from these results, both supporting an early use of AZA/Immunossupressants in CD. First, overall, CD patients have a poor outcome, regardless of the present medical therapy offered to them, and development of complications occur in three out of four patients in their lifetime. Second, CD offers a great opportunity for aggressive, earlier treatment, because almost all patients are complication-free at diagnosis. The mentioned penetrating and stenosing complications will slowly develop in the years following diagnosis which offers physicians a wide timeframe to introduce more efficacious drugs, early in CD course.

Mucosal healing

Mucosal healing (MH) is defined as a normal or mildly altered endoscopic appearance of the mucosa. The clinical relevance of MH has been recently underlined by different authors. In a Norweigan population cohort they prospective analyzed 740 incident patients diagnosed with UC and CD and evaluated MH at 1 and 5 years. At 5 years UC patients with MH had significant low risk of future colectomy ($P = 0.02$) and for patients with CD, MH was significantly associated with less inflammation ($P = 0.02$) and decreased future steroid treatment[31].

Not all IBD therapies impact equally MH. Glucocorticosteroids are not very effective in achieving MH in CD patients and a poor correlation between clinical and endoscopical parameters has been described. Moreover, endoscopic remission in colonic CD is of 29% and in ileal disease almost null[32-33]. Conversely to steroids, immunosuppressants and biological agents are associated with a high rate of MH. In a study of D'Haens *et al* of 19 patients with recurrent Crohn's ileitis treated with azathioprine, 15 could be re-evaluated at 6 mo, of them 6 patients had complete MH and 5 near complete healing[34]. Another study from the same group analyzed 20 patients with Crhon's colitis or ileocolitis who achieved symptoms relief with corticosteroids and in clinical remission with at least 9 mo of treatment with AZA. The ileocolonoscopy at 24.4 mo show 70% of complete healing and 10% of near-complete healing[35]. In respect to infliximab therapy, several studies have demonstrated efficacy for these drug in MH[36-38]. Two other studies demonstrated recently that combined immunosuppression with azathioprine and infliximab are more effective in terms of bowel MH respect to each one separately[15,39], but both were done at relative short term (1 and 2 years respectively).

If we believe that MH is indeed a relevant clinical outcome, as a growing body of evidence seem to suggest, then we have a strong reason to recommend an earlier and wider use of both immunosuppressants and biological agents, which have clearly demonstrated their

ability to induce MH. Although not formally proven yet, it seems very reasonable to admit that maintaining an endoscopically normal mucosa over time should result in a higher proportion of patients maintaining disease remission and also in a lower risk of developing CD-related complications, such as fistulas and strictures.

First results as proof of concept

The most solid, evidence-based prove to recommend an earlier use of immunosuppressants come from the pediatric study by Markowitz and colleagues[16]. They conducted a prospective, double-blind, placebo-controlled, 18 mo clinical trial in children newly diagnosed of CD. 55 subjects were included and were randomized to receive 6-mercaptopurine 1.5 mg/kg body weight daily in the treatment group, or placebo. Both groups received corticosteroids to achieve the control of the first flare of their CD. In the 6-MP group, the duration of steroid use was shorter (observed-to-expected ratio of days with prednisone of 0.73 *versus* 1.34 in the control group, $P < 0.001$). This results supports the use of 6-MP as induction therapy in combination with corticosteroids in active Crohn's disease.

The "step-up *vs* top-down" study, by D'Haens and colleagues[39] is also very relevant to support the notion that introducing the most efficacious IBD drugs early in disease course has a significant impact on CD patients outcome. This randomized study compared the conventional therapeutic approach ("step-up") with a newer, more aggressive strategy ("top-down"). In the "step-up group" patients were treated first with steroids and, in case of steroid dependency or resistance, immunosuppressants and infliximab were used. In the "top-down group" patients received upfront a combined treatment of immunossupressants and infliximab. This study clearly demonstrate the superiority of the "top-down" over the "step-up" approach, as demonstrated by a significantly higher proportion of patients in remission and showing MH in the "top-down", as compared to the "step-up" group. Interestingly, this higher efficacy did not carry a higher proportion of side effects in the "more aggressively", "top-down" treated patients. One of the drawbacks of this study is the lack of long-term follow up, especially in respect to the potential impact of prolonged, intense immune suppression on the risk of developing severe infections or cancer.

Several studies, ongoing at present, will help to clarify this issue and, maybe, will provide further evidence to support an early use of azatioprine in IBD. One is the SONIC study, aimed at comparing the use of AZA alone *versus* infliximab alone *versus* combined therapy. The other, the AZTEC study, a Spanish multicenter randomized study aimed at reproducing the efficacy of AZA in recently diagnosed CD, but in adults.

REFERENCES

1 **Scaldaferri F**, Fiocchi C. Inflammatory bowel disease: progress and current concepts of etiopathogenesis. *J Dig Dis* 2007; 8: 171-178

2 **Cosnes J**, Nion-Larmurier I, Beaugerie L, Afchain P, Tiret E, Gendre JP. Impact of the increasing use of immunosuppressants in Crohn's disease on the need for intestinal surgery. *Gut* 2005; **54**: 237-241

3 **Dubinsky MC**. Azathioprine, 6-mercaptopurine in inflammatory bowel disease: pharmacology, efficacy, and safety. *Clin Gastroenterol Hepatol* 2004; **2**: 731-743

4 **Siegel CA**, Sands BE. Review article: practical management of inflammatory bowel disease patients taking immunomodulators. *Aliment Pharmacol Ther* 2005; **22**: 1-16

5 **Su C**, Lichtenstein GR. Treatment of inflammatory bowel disease with azathioprine and 6-mercaptopurine. *Gastroenterol Clin North Am* 2004; **33**: 209-234, viii

6 **Sandborn W**, Sutherland L, Pearson D, May G, Modigliani R, Prantera C. Azathioprine or 6-mercaptopurine for inducing remission of Crohn's disease. *Cochrane Database Syst Rev* 2000; CD000545

7 **Pearson DC**, May GR, Fick G, Sutherland LR. Azathioprine for maintaining remission of Crohn's disease. *Cochrane Database Syst Rev* 2000; CD000067

8 **Present DH**, Meltzer SJ, Krumholz MP, Wolke A, Korelitz BI. 6-Mercaptopurine in the management of inflammatory bowel disease: short- and long-term toxicity. *Ann Intern Med* 1989; **111**: 641-649

9 **Gisbert JP**, Gonzelez-Lama Y, Mate J. Thiopurine-induced liver injury in patients with inflammatory bowel disease: a systematic review. *Am J Gastroenterol* 2007; **102**: 1518-1527

10 **Masunaga Y**, Ohno K, Ogawa R, Hashiguchi M, Echizen H, Ogata H. Meta-analysis of risk of malignancy with immunosuppressive drugs in inflammatory bowel disease. *Ann Pharmacother* 2007; **41**: 21-28

11 **Kandiel A**, Fraser AG, Korelitz BI, Brensinger C, Lewis JD. Increased risk of lymphoma among inflammatory bowel disease patients treated with azathioprine and 6-mercaptopurine. *Gut* 2005; **54**: 1121-1125

12 **Gasche C**, Scholmerich J, Brynskov J, D'Haens G, Hanauer SB, Irvine EJ, Jewell DP, Rachmilewitz D, Sachar DB, Sandborn WJ, Sutherland LR. A simple classification of Crohn's disease: report of the Working Party for the World Congresses of Gastroenterology, Vienna 1998. *Inflamm Bowel Dis* 2000; **6**: 8-15

13 **Silverberg MS**, Satsangi J, Ahmad T, Arnott ID, Bernstein CN, Brant SR, Caprilli R, Colombel JF, Gasche C, Geboes K, Jewell DP, Karban A, Loftus Jr EV, Pena AS, Riddell RH, Sachar DB, Schreiber S, Steinhart AH, Targan SR, Vermeire S, Warren BF. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Can J Gastroenterol* 2005; **19** Suppl A: 5-36

14 **Sandborn WJ**, Tremaine WJ, Wolf DC, Targan SR, Sninsky CA, Sutherland LR, Hanauer SB, McDonald JW, Feagan BG, Fedorak RN, Isaacs KL, Pike MG, Mays DC, Lipsky JJ, Gordon S, Kleoudis CS, Murdock RH Jr. Lack of effect of intravenous administration on time to respond to azathioprine for steroid-treated Crohn's disease. North American Azathioprine Study Group. *Gastroenterology* 1999; **117**: 527-535

15 **Lemann M**, Mary JY, Duclos B, Veyrac M, Dupas JL, Delchier JC, Laharie D, Moreau J, Cadiot G, Picon L, Bourreille A, Sobahni I, Colombel JF. Infliximab plus azathioprine for steroid-dependent Crohn's disease patients: a randomized placebo-controlled trial. *Gastroenterology* 2006; **130**: 1054-1061

16 **Markowitz J**, Grancher K, Kohn N, Lesser M, Daum F. A multicenter trial of 6-mercaptopurine and prednisone in children with newly diagnosed Crohn's disease. *Gastroenterology* 2000; **119**: 895-902

17 **Mekhjian HS**, Switz DM, Watts HD, Deren JJ, Katon RM, Beman FM. National Cooperative Crohn's Disease Study: factors determining recurrence of Crohn's disease after surgery. *Gastroenterology* 1979; **77**: 907-913

18 **Farmer RG**, Whelan G, Fazio VW. Long-term follow-up

of patients with Crohn's disease. Relationship between the clinical pattern and prognosis. *Gastroenterology* 1985; **88**: 1818-1825

19 **Olaison G**, Smedh K, Sjodahl R. Natural course of Crohn's disease after ileocolic resection: endoscopically visualised ileal ulcers preceding symptoms. *Gut* 1992; **33**: 331-335

20 **Ardizzone S**, Maconi G, Sampietro GM, Russo A, Radice E, Colombo E, Imbesi V, Molteni M, Danelli PG, Taschieri AM, Bianchi Porro G. Azathioprine and mesalamine for prevention of relapse after conservative surgery for Crohn's disease. *Gastroenterology* 2004; **127**: 730-740

21 **Hanauer SB**, Korelitz BI, Rutgeerts P, Peppercorn MA, Thisted RA, Cohen RD, Present DH. Postoperative maintenance of Crohn's disease remission with 6-mercaptopurine, mesalamine, or placebo: a 2-year trial. *Gastroenterology* 2004; **127**: 723-729

22 **Ardizzone S**, Maconi G, Russo A, Imbesi V, Colombo E, Bianchi Porro G. Randomised controlled trial of azathioprine and 5-aminosalicylic acid for treatment of steroid dependent ulcerative colitis. *Gut* 2006; **55**: 47-53

23 **Timmer A**, McDonald JW, Macdonald JK. Azathioprine and 6-mercaptopurine for maintenance of remission in ulcerative colitis. *Cochrane Database Syst Rev* 2007; CD000478

24 **O'Donoghue DP**, Dawson AM, Powell-Tuck J, Bown RL, Lennard-Jones JE. Double-blind withdrawal trial of azathioprine as maintenance treatment for Crohn's disease. *Lancet* 1978; **2**: 955-957

25 **Lemann M**, Mary JY, Colombel JF, Duclos B, Soule JC, Lerebours E, Modigliani R, Bouhnik Y. A randomized, double-blind, controlled withdrawal trial in Crohn's disease patients in long-term remission on azathioprine. *Gastroenterology* 2005; **128**: 1812-1818

26 **Holtmann MH**, Krummenauer F, Claas C, Kremeyer K, Lorenz D, Rainer O, Vogel I, Böcker U, Böhm S, Büning C, Duchmann R, Gerken G, Herfarth H, Lügering N, Kruis W, Reinshagen M, Schmidt J, Stallmach A, Stein J, Sturm A, Galle PR, Hommes DW, D'Haens G, Rutgeerts P, Neurath MF. Long-term effectiveness of azathioprine in IBD beyond 4 years: a European multicenter study in 1176 patients. *Dig Dis Sci* 2006; **51**: 1516-1524

27 **Bouhnik Y**, Lemann M, Mary JY, Scemama G, Tai R, Matuchansky C, Modigliani R, Rambaud JC. Long-term follow-up of patients with Crohn's disease treated with azathioprine or 6-mercaptopurine. *Lancet* 1996; **347**: 215-219

28 **Mantzarisa GJ**, Roussosa A, Christidoua A, Koilakoua S, Kalantzisb CN, Petrakia K, Sfakianakisc M, Karagiannidisa A, Polyzou P. The long-term efficacy of azathioprine does not wane after four years of continuous treatment in patients with steroid-dependent luminal Crohn's disease. *J Crohn's Colitis* 2007; **1**: 28-34

29 **Louis E**, Collard A, Oger AF, Degroote E, Aboul Nasr El Yafi FA, Belaiche J. Behaviour of Crohn's disease according to the Vienna classification: changing pattern over the course of the disease. *Gut* 2001; **49**: 777-782

30 **Cosnes J**, Cattan S, Blain A, Beaugerie L, Carbonnel F, Parc R, Gendre JP. Long-term evolution of disease behavior of Crohn's disease. *Inflamm Bowel Dis* 2002; **8**: 244-250

31 **Froslie KF**, Jahnsen J, Moum BA, Vatn MH. Mucosal healing in inflammatory bowel disease: results from a Norwegian population-based cohort. *Gastroenterology* 2007; **133**: 412-422

32 **Modigliani R**, Mary JY, Simon JF, Cortot A, Soule JC, Gendre JP, Rene E. Clinical, biological, and endoscopic picture of attacks of Crohn's disease. Evolution on prednisolone. Groupe d'Etude Therapeutique des Affections Inflammatoires Digestives. *Gastroenterology* 1990; **98**: 811-818

33 **Olaison G**, Sjodahl R, Tagesson C. Glucocorticoid treatment in ileal Crohn's disease: relief of symptoms but not of endoscopically viewed inflammation. *Gut* 1990; **31**: 325-328

34 **D'Haens G**, Geboes K, Ponette E, Penninckx F, Rutgeerts P. Healing of severe recurrent ileitis with azathioprine therapy in patients with Crohn's disease. *Gastroenterology* 1997; **112**: 1475-1481

35 **D'Haens G**, Geboes K, Rutgeerts P. Endoscopic and histologic healing of Crohn's (ileo-) colitis with azathioprine. *Gastrointest Endosc* 1999; **50**: 667-671

36 **D'haens G**, Van Deventer S, Van Hogezand R, Chalmers D, Kothe C, Baert F, Braakman T, Schaible T, Geboes K, Rutgeerts P. Endoscopic and histological healing with infliximab anti-tumor necrosis factor antibodies in Crohn's disease: A European multicenter trial. *Gastroenterology* 1999; **116**: 1029-1034

37 **Rutgeerts P**, Diamond RH, Bala M, Olson A, Lichtenstein GR, Bao W, Patel K, Wolf DC, Safdi M, Colombel JF, Lashner B, Hanauer SB. Scheduled maintenance treatment with infliximab is superior to episodic treatment for the healing of mucosal ulceration associated with Crohn's disease. *Gastrointest Endosc* 2006; **63**: 433-442; quiz 464

38 **Hanauer SB**, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, Rachmilewitz D, Wolf DC, Olson A, Bao W, Rutgeerts P. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet* 2002; **359**: 1541-1549

39 **D'Haens G**, Baert F, van Assche G, Caenepeel P, Vergauwe P, Tuynman H, De Vos M, van Deventer S, Stitt L, Donner A, Vermeire S, Van de Mierop FJ, Coche JC, van der Woude J, Ochsenkuhn T, van Bodegraven AA, Van Hootegem PP, Lambrecht GL, Mana F, Rutgeerts P, Feagan BG, Hommes D. Early combined immunosuppression or conventional management in patients with newly diagnosed Crohn's disease: an open randomised trial. *Lancet* 2008; **371**: 660-667

S- Editor Xiao LL **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5519

World J Gastroenterol 2008 September 28; 14(36): 5519-5522
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Javier P Gisbert, Professor; Fernando Gomollón, Dr., MD, PhD, *Series Editors*

Are we giving azathioprine too much time?

Fernando Gomollón, Santiago García López

Fernando Gomollón, Department of Digestive Diseases, Clinic University Hospital "Lozano Blesa", CIBEREHD, IACS, Zaragoza, Spain
Santiago García López, Department of Digestive Diseases, University Hospital "Miguel Servet", Zaragoza, Spain
Author contributions: Gomollón F and García López S contributed equally to this work.
Correspondence to: Fernando Gomollón, Hospital Clínico Universitario "Lozano Blesa" Zaragoza, Avenida San Juan Bosco 15, Zaragoza 50009, Spain. fgomollon@gmail.com
Telephone: +34-87-6766000 Fax: +34-97-6768846
Received: August 18, 2008 Revised: August 25, 2008
Accepted: September 1, 2008
Published online: September 28, 2008

Abstract

Azathioprine is currently the key drug in the maintenance treatment of inflammatory bowel diseases. However, there are still some practical issues to be resolved: one is how long we must maintain the drug. Given that inflammatory bowel diseases are to date chronic, non-curable conditions, treatment should be indefinite and only the loss of efficacy or the appearance of serious side effects may cause withdrawal. As regards to efficacy and their maintenance over time, evidence supports the continuous usefulness of the drug in the long term: in fact its withdrawal very substantially increases the risk of relapse. About side effects, azathioprine is a relatively well tolerated drug and even indefinite use seems safe. The main theoretical risks of prolonged use would be the myelotoxicity, hepatotoxicity, and the development of cancer. In fact, serious bone marrow suppression or serious liver damage are uncommon, and can be minimized with proper use of the drug. Recent metanalysis suggests that the risk of lymphoma is real, but the individual risk is rather low, and decision analysis suggests a favorable benefit/risk ratio in the long term. Therefore, in patients with inflammatory bowel diseases in whom azathioprine is effective and well tolerated, the drug should not be stopped. This recommendation concerns the use of azathioprine as a single maintenance drug, and is not necessarily applicable to patients receiving concomitant biological therapy.

Key words: Azathioprine; Inflammatory bowel diseases; Maintenance treatment

Gomollón F, García López S. Are we giving azathioprine too much time? *World J Gastroenterol* 2008; 14(36): 5519-5522 Available from: URL: http://www.wjgnet.com/1007-9327/14/5519.asp DOI: http://dx.doi.org/10.3748/wjg.14.5519

INTRODUCTION

Azathioprine (AZA) is an effective drug in the treatment of inflammatory bowel disease (IBD), particularly in the maintenance of remission of patients with a complex clinical course, and there is little doubt that once indicated should be maintained for an extended period of time. However, when it takes some time without symptoms or signs of disease activity, both clinicians and patients often arise: should I withdraw the drug? After this question lies another: if I should withdraw it, when it's time? Given the chronic nature of these diseases, only two reasons could actually lead to the withdrawal: a loss of efficacy over time or appearance of adverse events that exceed the benefits of the drug. Our goal is to review the available evidence for answering the main question posed by the title of the article. Note that although we refer in the article usually only to AZA, all the above aspects are also valid for its active metabolite, mercaptopurine.

DOES AZATHIOPRINE MAINTAIN ITS EFFECTIVENESS IN THE LONG-TERM?

The long-term effectiveness of AZA in the maintenance of Crohn's disease (CD) is demonstrated in several clinical trials (5 randomized control trials[1-5]), a meta-analysis[6] and a Cochrane review[7]. In this last review, the rate of remission with and without AZA was 67% and 52% respectively, with an odds ratio of 2.16 (95% CI 1.35-3.47) and an NNT of 7 to prevent a relapse. These figures greatly improve if you use the adequate dose of the drug (using 2.5 mg/kg odds ratio increases to 4.13). This same study reflects its value in saving steroids with an NNT of approximately 3[7]. In addition, observational studies corroborate these findings. Thus, in one of the most important of these studies (many patients included and longer follow up), which include CD and ulcerative colitis patients, the life table analyses show that maintenance with AZA is clearly useful for up to 5 years

in both entities[8]. In another multicenter european study, aimed to evaluate the long-term effectiveness of the immunomodulators in IBD, results are quite similar[9]. It included 818 CD and 358 CU IBD patients treated with thiopurinic immunomodulators. The prolonged treatment with these drugs was associated with a lower relapse rate and steroid use. In the case of ulcerative colitis (UC), the data are somewhat more limited but also demonstrate long-term effectiveness of AZA as a maintenance therapy. Several randomized studies have been published although the number of included patients is usually low. A recent Cochrane meta-analysis examines 6 randomized controlled studies (286 patients) of at least 12 mo of duration, which compare AZA (or mercaptopurine) against placebo or mesalazine for UC[10]. The meta-analysis concludes that AZA is more effective than placebo (4 trials) while it is not possible to analyse properly the comparison with the salicylates, due to the significant heterogeneity of studies included (2 trials). The effect of saving steroids, although not evaluated in this meta-analysis, is evident in other 3 controlled trials. In the first of them, a recent study not included in the previous meta-analysis and may be the best trial of AZA in UC to date, effectiveness of AZA in active steroid dependent UC is demonstrated[11]. In this study 72 patients with steroid dependent active UC, are randomized to receive mesalamine or AZA plus prednisolone. At 6 mo, clinical and endoscopic remission free of steroids was achieved in 53% of patients on AZA compared to 21% on mesalamina (OR 4.78, 95% CI 1.57-14.5). In the second trial, 30 patients with steroid dependent UC were treated with AZA or placebo for 6 mo[12]. While in the placebo group was not possible to reduce the doses of steroids, in the AZA group did it. In the third study, with very similar design, the results were similar but even more marked: in the placebo group steroid dose was decreased to 13 mg/day and up to 2.3 mg/day in the AZA group[13]. The observational data provided similar results in support of AZA for UC maintenance. The best evidence of these studies comes from the 30-years cohort from Oxford[8]. In this study the proportion of patients on strict remission at 5 years were 62%, and the median time to relapse 1.5 years. Another important data is that derived form a recent Spanish study[14]. In this prospective study, AZA therapy results in a clear steroid sparing effect and reduction in the number of hospitalizations and surgery in 394 IBD patients (CD and UC, with similar effectiveness). In addition we must emphasize that both in CD and UC, we have over 40 years of clinical experience which confirms the results of the studies previously mentioned.

Once established the effectiveness of AZA in the long-term maintenance, we will analyze what happens if the drug is withdrawn. Virtually all papers reveal a marked increase in the rate of relapse following the discontinuation of AZA. In the case of Crohn's disease, three randomized controlled trials specifically analyze the impact of withdrawing AZA, in CD patients in remission with this drug over a period of time more or less prolonged[4,15,16]. The three trials clearly show the damage that causes the withdrawal of AZA. First study included 51 patients with CD in remission for more than 6 months on AZA, which are randomized to continue on or discontinue AZA[4]. One year of follow up, the cumulative risk of relapse was 5% against 41% in groups with and without AZA ($P < 0.01$). In the second study, open one-year trial, CD patients treated with AZA in remission for 2 or more years, were randomized to continue or not with the drug[15]. At one year, 11/13 patients on AZA and 7/15 without AZA maintained remission ($P = 0043$), being the differences more pronounced when higher doses of AZA were used. In the third trial results are similar, but even when the patients maintained remission on AZA for longer period of time. In this study, 83 CD patients in remission on AZA at least 3.5 years ($\geq$ 42 mo), were randomized to continue or not the drug. One year and a half later, relapse rate was 8% in patients continuing AZA and 21% in patients that stopped the drug[16]. Observational studies commented previously, show similar data, although some with longer surveillance. This is the case study of Fraser, in which the proportion of patients (both CD and UC) still in remission after 12, 24, 36, 48 and 60 mo was 0.63, 0.44, 0.34, 0.28 and 0.25 respectively[8]. Duration of remission on prior AZA did not affect the relapse rate after stopping the drug. However, some authors initially suggested the withdrawal of AZA if the patient maintains remission for a prolonged period (about 4 years). This idea comes essentially from a study published in 1996[17], which showed that while the effectiveness of AZA is maintained over time, extended therapy more than 4 years could have no additional clinical benefit. Although this study had a surprising impact, it is retrospective and has important limitations (does not take into account factors such as smoking or causes of withdrawal of the drug, number of patients followed over 5 years was very small). Only appear somewhat similar results in another study, also commented previously[9]. These data contrast sharply with those offered by controlled studies and other observational studies, which show clearly beneficial to continue indefinitely AZA in the CD (see previously). In the case of ulcerative colitis, available data may be considered weaker again, although there are one controlled trial and some observational studies. The controlled study showed that withdrawal of AZA was associated with a higher relapse rate than when the drug was maintained[18]. It was a randomized trial although did not use a double blind design. Seventy nine UC patients on AZA for more than 6 mo were randomized to continue or not with the drug. The one year relapse rate was 36% and 59% in group with and without AZA respectively. Observational data clearly suggest that AZA treatment is useful, maintain this efficacy over time and that discontinuation of AZA is followed by higher rate of relapse, even in patients being in prolonged remission with the drug[19,20,8,9].

In short, solid evidence sustains that AZA (or mercaptopurine) is effective long-term maintenance

treatment of IBD and that their withdrawal is followed by a clear increase of relapse rate. No "safe" period of time being in remission on AZA (or mercaptopurine) has been established after which these drugs could be stopped with no risk of relapse.

WHICH IS THE SAFETY OF USING AZATHIOPRINE INDEFINITELY?

The safety profile of AZA is well known and we have very extensive follow up and long term data with this drug. Side effects are relatively common and can lead the withdrawal of treatment between 10% and 20% (for example in the Cochrane review for CD, adverse effects that conditioned the withdrawal of AZA were at 9.3%)[7]. However, most adverse events are seen at the beginning of treatment and once after the first few weeks, tolerance to the drug is generally very good. The main risks of indefinite use of AZA are myelotoxicity, hepatotoxicity and perhaps development of neoplasms. Myelotoxicity has an incidence of 3 cases per patient and year, is serious in only a small proportion (less than 10%), and can be prevented partly with proper analytical monitoring[21]. Serious hepatotoxicity is also rare, and usually less relevant from a clinical point of view[22]. Regarding to a theoretical increase of cancer, lymphomas especially, it is an issue still unresolved after years of use of the drug, but has made questioned long-term use of AZA. The results of individual studies are inconclusive. A recent meta-analysis assesses the results of the 6 cohort studies designed to analyze cancer as a side effect of treatment[23]. In this study, the pooled relative risk in IBD patients treated with AZA versus general population was 4.18 (95% confidence interval 2.07-7.51; 11 cases observed, 2.63 expected). As the studies are all observational, the increased risk of lymphoma could be a result of the medications, the severity of the underlying disease, or a combination of the two previous factors. The only real way to evaluate this risk at this moment is through a decision analysis, which suggests that the benefits clearly outweigh the risk[24]. This decision analysis was planned to evaluate the impact of AZA therapy on survival and quality-adjusted life expectancy, taking into account both benefits of therapy and potential risk of lymphoma. In the base-case analysis, CD treatment of patients with a steroid-induced remission with AZA resulted in an increase in average life expectancy of 0.04 years and 0.05 quality-adjusted years. The incremental gain in life expectancy decreased with increasing patient age and increasing risk of lymphoma. These results show that AZA in CD patients results in increased quality-adjusted life expectancy. This benefit was greatest in young patients who have the lowest baseline risk of lymphoma and who have the greatest life expectancy in the absence of a CD-related death. However, even in the worst scenario, the absolute individual risk is very low. Finally, in the extensive data available from registries as TREAT[25] and other recent safety studies[26,27], AZA is not associated with increased mortality as an independent factor, and there is no increase in mortality.

In short, the safety profile of AZA is well known, both by the wide experience in clinical use as by the great amount of available published data, and we can say that it is a relatively safe drug when used long term. Of course, these data are valid for AZA (or mercaptopurine) given alone. The association with other drugs, particularly biologics, could result in different risks, and long-term follow-up data are relatively few yet.

CONCLUSION AND RECOMMENDATIONS

When AZA alone (not associated with biological agent), adequately indicated, is maintaining remission in a patient with inflammatory bowel disease, we should not withdraw it, even after several years of treatment maintaining remission, except if significant adverse effects appear (grade recommendation: A; level of evidence: I). The withdrawal of AZA is one of the frequent mistakes in the treatment of IBD, as shown by various studies (see previously). As the professor Sacha'r said "no safe number of years has been determined after which these medications can be withdrawn without risk of relapse…", and "azathioprine works when you take it (and you take it enough amount of it), so do not stop AZA (and give it soon)"[28].

As regards to the use of AZA associated with biological agents, the questions are more and it is not possible to establish strong recommendations. The rationale to add AZA to biologics is to minimize antibody formation and thereby to enhance agent efficacy (or at least to prolong it) and reduce infusion reactions. Any case, we do not know yet the real impact of associating AZA to biologics in these items, and even less is known about the potential increase in adverse events, especially long term. The advantages of long term treatment maintaining the two drugs, in terms of efficacy and safety, must be demonstrated, especially when biologics are used as regularly scheduled infusions.

Only time (once again time…) will answer some of the questions raised about the use of AZA in inflammatory bowel disease. Until then, it is necessary to recognize current key role of AZA in this scenario to use it properly in our clinical practice, providing a significant benefit to our patients[29,30].

REFERENCES

1 **Candy S**, Wright J, Gerber M, Adams G, Gerig M, Goodman R. A controlled double blind study of azathioprine in the management of Crohn's disease. *Gut* 1995; **37**: 674-678
2 **Summers RW**, Switz DM, Sessions JT Jr, Becktel JM, Best WR, Kern F Jr, Singleton JW. National Cooperative Crohn's Disease Study: results of drug treatment. *Gastroenterology* 1979; **77**: 847-869
3 **Willoughby JM**, Beckett J, Kumar PJ, Dawson AM. Controlled trial of azathioprine in Crohn's disease. *Lancet* 1971; **2**: 944-947
4 **O'Donoghue DP**, Dawson AM, Powell-Tuck J, Bown RL, Lennard-Jones JE. Double-blind withdrawal trial of azathioprine as maintenance treatment for Crohn's disease. *Lancet* 1978; **2**: 955-957
5 **Rosenberg JL**, Levin B, Wall AJ, Kirsner JB. A controlled trial of azathioprine in Crohn's disease. *Am J Dig Dis* 1975;

20: 721-726
6 **Pearson DC**, May GR, Fick GH, Sutherland LR. Azathioprine and 6-mercaptopurine in Crohn disease. A meta-analysis. *Ann Intern Med* 1995; **123**: 132-142
7 **Pearson DC**, May GR, Fick G, Sutherland LR. Azathioprine for maintaining remission of Crohn's disease. *Cochrane Database Syst Rev* 2000; CD000067
8 **Fraser AG**, Orchard TR, Jewell DP. The efficacy of azathioprine for the treatment of inflammatory bowel disease: a 30 year review. *Gut* 2002; **50**: 485-489
9 **Holtmann MH**, Krummenauer F, Claas C, Kremeyer K, Lorenz D, Rainer O, Vogel I, Bocker U, Bohm S, Buning C, Duchmann R, Gerken G, Herfarth H, Lugering N, Kruis W, Reinshagen M, Schmidt J, Stallmach A, Stein J, Sturm A, Galle PR, Hommes DW, D'Haens G, Rutgeerts P, Neurath MF. Long-term effectiveness of azathioprine in IBD beyond 4 years: a European multicenter study in 1176 patients. *Dig Dis Sci* 2006; **51**: 1516-1524
10 **Timmer A**, McDonald JW, Macdonald JK. Azathioprine and 6-mercaptopurine for maintenance of remission in ulcerative colitis. *Cochrane Database Syst Rev* 2007; CD000478
11 **Ardizzone S**, Maconi G, Russo A, Imbesi V, Colombo E, Bianchi Porro G. Randomised controlled trial of azathioprine and 5-aminosalicylic acid for treatment of steroid dependent ulcerative colitis. *Gut* 2006; **55**: 47-53
12 **Rosenberg JL**, Wall AJ, Levin B, Binder HJ, Kirsner JB. A controlled trial of azathioprine in the management of chronic ulcerative colitis. *Gastroenterology* 1975; **69**: 96-99
13 **Kirk AP**, Lennard-Jones JE. Controlled trial of azathioprine in chronic ulcerative colitis. *Br Med J* (Clin Res Ed) 1982; **284**: 1291-1292
14 **Gisbert JP**, Nino P, Cara C, Rodrigo L. Comparative effectiveness of azathioprine in Crohn's disease and ulcerative colitis: prospective, long-term, follow-up study of 394 patients. *Aliment Pharmacol Ther* 2008; **28**: 228-238
15 **Vilien M**, Dahlerup JF, Munck LK, Norregaard P, Gronbaek K, Fallingborg J. Randomized controlled azathioprine withdrawal after more than two years treatment in Crohn's disease: increased relapse rate the following year. *Aliment Pharmacol Ther* 2004; **19**: 1147-1152
16 **Lemann M**, Mary JY, Colombel JF, Duclos B, Soule JC, Lerebours E, Modigliani R, Bouhnik Y. A randomized, double-blind, controlled withdrawal trial in Crohn's disease patients in long-term remission on azathioprine. *Gastroenterology* 2005; **128**: 1812-1818
17 **Bouhnik Y**, Lemann M, Mary JY, Scemama G, Tai R, Matuchansky C, Modigliani R, Rambaud JC. Long-term follow-up of patients with Crohn's disease treated with azathioprine or 6-mercaptopurine. *Lancet* 1996; **347**: 215-219
18 **Hawthorne AB**, Logan RF, Hawkey CJ, Foster PN, Axon AT, Swarbrick ET, Scott BB, Lennard-Jones JE. Randomised controlled trial of azathioprine withdrawal in ulcerative colitis. *BMJ* 1992; **305**: 20-22
19 **Lobel EZ**, Korelitz BI, Xuereb MA, Panagopoulos G. A search for the optimal duration of treatment with 6-mercaptopurine for ulcerative colitis. *Am J Gastroenterol* 2004; **99**: 462-465
20 **George J**, Present DH, Pou R, Bodian C, Rubin PH. The long-term outcome of ulcerative colitis treated with 6-mercaptopurine. *Am J Gastroenterol* 1996; **91**: 1711-1714
21 **Gisbert JP**, Gomollon F. Thiopurine-induced myelotoxicity in patients with inflammatory bowel disease: a review. *Am J Gastroenterol* 2008; **103**: 1783-1800
22 **Gisbert JP**, Luna M, Gonzalez-Lama Y, Pousa ID, Velasco M, Moreno-Otero R, Mate J. Liver injury in inflammatory bowel disease: long-term follow-up study of 786 patients. *Inflamm Bowel Dis* 2007; **13**: 1106-1114
23 **Kandiel A**, Fraser AG, Korelitz BI, Brensinger C, Lewis JD. Increased risk of lymphoma among inflammatory bowel disease patients treated with azathioprine and 6-mercaptopurine. *Gut* 2005; **54**: 1121-1125
24 **Lewis JD**, Schwartz JS, Lichtenstein GR. Azathioprine for maintenance of remission in Crohn's disease: benefits outweigh the risk of lymphoma. *Gastroenterology* 2000; **118**: 1018-1024
25 **Lichtenstein GR**, Feagan BG, Cohen RD, Salzberg BA, Diamond RH, Chen DM, Pritchard ML, Sandborn WJ. Serious infections and mortality in association with therapies for Crohn's disease: TREAT registry. *Clin Gastroenterol Hepatol* 2006; **4**: 621-630
26 **Lewis JD**, Gelfand JM, Troxel AB, Forde KA, Newcomb C, Kim H, Margolis DJ, Strom BL. Immunosuppressant medications and mortality in inflammatory bowel disease. *Am J Gastroenterol* 2008; **103**: 1428-1435; quiz 1436
27 **Toruner M**, Loftus EV Jr, Harmsen WS, Zinsmeister AR, Orenstein R, Sandborn WJ, Colombel JF, Egan LJ. Risk factors for opportunistic infections in patients with inflammatory bowel disease. *Gastroenterology* 2008; **134**: 929-936
28 **Sachar DB**. Ten common errors in the management of inflammatory bowel disease. *Inflamm Bowel Dis* 2003; **9**: 205-209
29 **Gisbert JP**, Gomollon F, Mate J, Pajares JM. [Questions and answers on the role of azathioprine and 6-mercaptopurine in the treatment of inflammatory bowel disease] *Gastroenterol Hepatol* 2002; **25**: 401-415
30 **García-Paredes J**, Gomollón F, Santamaría DMC, Abreu L, Vera M, Fazio VW, Sandborn WJ. Steroid dependency and steroid resistance in inflammatory bowel disease. *Drugs Today* 2001; **37** (Suppl E): 17-18

S- Editor Xiao LL **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5523

World J Gastroenterol 2008 September 28; 14(36): 5523-5527
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Javier P Gisbert, Professor; Fernando Gomollón, Dr., MD, PhD, *Series Editors*

Are we giving biologics too late? The case for early *versus* late use

Elena Ricart, Orlando García-Bosch, Ingrid Ordás, Julián Panés

Elena Ricart, Orlando García-Bosch, Ingrid Ordás, Julián Panés, Gastroenterology Department, Hospital Clinic, CIBER-EHD, C/Villarroel 170, Barcelona 08036, Spain
Author contributions: All authors contributed equally to this work.
Correspondence to: Elena Ricart, MD, Gastroenterology Department, Hospital Clinic, CIBER-EHD, C/Villarroel 170, Barcelona 08036, Spain. ericart@clinic.ub.es
Telephone: +34-93-2275418 Fax: +34-93-2279387
Received: August 18, 2008 Revised: August 26, 2008
Accepted: September 3, 2008
Published online: September 28, 2008

Abstract

Corticosteroids and immunomodulators have been the mainstay therapies for Crohn's disease. Corticosteroids are highly effective to control symptoms in the short-term, but they are not effective in maintaining remission, they heal the mucosa in a reduced proportion of cases, and long-time exposure is associated with an increased risk of infections and mortality. Immunomodulators, azathioprine and methotrexate, heal the mucosa in a higher proportion of patients that corticosteroids but their onset of action is slow and they benefit less than half of patients with Crohn's disease. In the last decade, medical therapy for Crohn's disease has experienced a remarkable change due to the introduction of biologic therapy, and particularly the use of anti-tumour necrosis factor-alpha agents. Infliximab, adalimumab, and certolizumab pegol have demonstrated efficacy for induction and maintenance of remission in active Crohn's disease. These agents have raised the bar for what is a suitable symptomatic response in Crohn's disease and modification of the natural history of the disease has become a major goal in the treatment of Crohn's disease. There are several data in the literature that suggest that early use of biologic therapy and achievement of mucosal healing contribute to disease course modification. However, many questions on early biological therapy for Crohn's disease remain still unanswered.

Key words: Biologic therapy; Crohn's disease; Corticosteroids; Immunomodulators

Ricart E, García-Bosch O, Ordás I, Panés J. Are we giving biologics too late? The case for early *versus* late use. *World J Gastroenterol* 2008; 14(36): 5523-5527 Available from: URL: http://www.wjgnet.com/1007-9327/14/5523.asp DOI: http://dx.doi.org/10.3748/wjg.14.5523

INTRODUCTION

To date, medical therapy for Crohn's disease has been restricted to treat the signs and symptoms of the disease, to prevent steroid dependence by using immunomodulating agents (azathioprine, 6-mercaptopurine, methotrexate), to maintain clinical remission, and to minimize medication toxicity. The introduction of biologic therapy, particularly the use of anti-tumor necrosis factor alpha (TNFα) agents (infliximab, adalimumab, certolizumab pegol), has provided a poweful instrument in the treatment and management of Crohn's disease that has lead to an expansion of goals in Crohn's disease therapy. These include a rapidly achieved and sustained clinical remission, complete and sustained healing of the bowel mucosa, reduction in steroid use, reduction in hospitalizations and surgeries, avoidance of complications of the disease (stenoses, fistulas), and improvement of patients' overall quality of life. Infliximab, adalimumab, and certolizumab pegol have demonstrated efficacy for induction and maintenance of remission in Crohn's disease. However, biologic agents are still used with reluctance, being the high cost and the uncertainty about long-term safety of these agents the most frequently cited reasons. An important question facing physicians that take care of Crohn's disease patients is at what point of the disease should biological therapy be used.

STEP-UP APPROACH

The standard treatment in Crohn's disease is based on a step-up approach in which "traditional" therapies thought to be more secure are used early and then successive therapies are added in case of lack of response or toxicity. Under this scheme, agents with low efficacy such as aminosalicylates may be used for long periods of time without any benefit. Patients who fail to respond to aminosalicylates are then treated with prednisone or budesonide if the disease is limited

to the ileum or the right colon. Corticosteroids are efficacious to induce remission in Crohn's disease patients but do not induce long-lasting remission[1]. About 40% of patients will develop a steroid-dependent or steroid-refractory course of the disease. In these cases, treatment with immunomodulating agents such as azathioprine, 6-mercaptopurine or methotrexate is started. Immunomodulation has long-term efficacy in inflammatory bowel disease but has limited value for induction of remission and benefits to less than half of the patients dependent or resistant to corticosteroids[2-6]. Under this therapeutic approach, population-based studies show that only 42% of Crohn's disease patients are symptom-free at 2 years after the initial diagnosis and 12% after 10 years, and that 10% of patients have chronically active disease in 2 years and 1% in 10 years[7]. Relapsing or chronically active disease leads to complications that often require surgical treatment that, unfortunately, does not cure or interrupt the progression of the disease[8]. Rates of surgical intervention remain high, with as many as one third of patients requiring surgey within a year of beginning oral corticosteroids[1] and with no significant decrease in the need of surgery in the last decades despite the earlier and increasing use of immunomodulators[9]. In the last years it has become also apparent that immunomodulators carry a significant safety risk for patients treated long term, including the risk of infection[10], lymphoma[11], cervical carcinoma[12], and nodular regenerative hyperplasia[13].

TOP-DOWN APPROACH

The ability of anti-TNFα agents to rapidly control disease symptoms and to heal the mucosa has raised the question of whether the early use of biological therapy in patients with newly diagnosed Crohn's disease can alter the course and natural history of the disease. In patients with early rheumatoid arthritis, initial combination therapy with high doses of prednisone and methotrexate or initial combination therapy with infliximab and methotrexate has shown to be more effective than sequential monotherapy or step-up combination therapy to prevent disease progression[14-18].

This concept, applied to Crohn's disease, has been tested in a trial carried out in Belgium, Holland and Germany by D'Haens *et al*[19]. This study included 133 patients with early Crohn's disease (defined as a diagnosis within the past 4 years) not previously treated with corticosteroids, immunomodulators or biological agents. Patients were randomly assigned to receive combined immunosuppression induction with azathioprine and three infusions of infliximab at weeks 0, 2, and 6 (n = 65) (top-down group), or to conventional therapy with two tapering courses of corticosteroids, and if indicated with azathioprine and then with infliximab (n = 64) (step-up group). Patients who were intolerant to azathioprine in both groups received methotrexate. Patients in the top-down group were given additional infliximab infusions in an "on-demand" basis and corticosteroids, if necessary, to control disease activity. The primary outcome was the combination of remission without corticosteroids and without bowel resection at weeks 26 and 52. At week 26, 39/65 (60%) of patients in the top-down group were in clinical remission without corticosteroids and without surgery compared to 23/64 (36%) in the step-up group (P = 0.0062) with an absolute difference of 24% (95% CI 7.3-40.8). At week 52, 40/65 (61.5%) in the top-down group were in remission compared to 27/64 (42.2%) in the step-up group (P = 0.0278), with an absolute difference of 19.4% (95% CI 2.4-36.3). After week 52, the proportion of patients in remission did no differ between the two groups. The median time to relapse was longer for patients in the top-down group (329 d, IQR 91-not reached) compared to patients in the step-up group (174.5 d, IQR 78.5-274, P = 0.031). Results from the IBDQs paralleled those of disease activity. At week 10, mean IBDQ score increased by 59.2 ± 36.6 points from baseline in the top-down group and by 37.4 ± 32.8 points in the step-up group (95% CI 8.7-34.9, P = 0.0014). Patients in the top-down group received significantly less methylprednisolone than patients in the step-up group. The 95th percentile of the daily methylprednisolone dose was 35 mg for patients in the step-up group and 0 mg for those in the top-down group. On the contrary, by the end of the trial 76% of patients in the step-up group were receiving an immunomodulator agent. After the completion of the induction course of infliximab in the top-down group, the proportion of patients on infliximab was similar in both groups. There were not important differences in the occurrence of adverse avents between the two groups, although the study was not primarly designed to address safety differences between the two strategies. The strongest argument for the top-down approach changing the natural history of Crohn's disease lies in the findings of the endoscopic studies performed in 49 patients of the study. At week 104, no ulcers were seen in 19/26 (73.1%) of patients in the top-down group compared with 7/23 (30.4%) in the step-up group (P = 0.0028). Endoscopic scores were 0.7 ± 1.5 and 3.1 ± 2.9, respectively (P < 0.001). This difference was marked despite the fact that there was no difference in disease activity scores between the top-down and step-up approaches at this timepoint. This leads to the notion that early introduction of biological therapy has direct benefits at an specific organ level (bowel) that far outreach the benefits of steroid sparing and overall clinical efficacy. The benefit of the early use of biological therapy has been demonstrated in rheumatoid arthritis, where the early introduction of biological therapy results in less joint damage on X-ray compared to a standard therapeutic approach using disease-modifying agents, regardless of similar clinical activity scores[16]. Mucosal healing has been associated with a reduction in hospitalizations and surgery for complications of Crohn's disease[20,21]. In the ACCENT I (A Crohn's disease Clinical study Evaluating Infliximab in a New long term Treatment regimen) trial[20], patients with short-term (week 10) and long-term (week 54) mucosal healing did no require hospitalization and patients with mucosal

healing at only one visit required fewer hospitalizations compared with patients without mucosal healing (18.8% *vs* 28%, respectively). If mucosal healing predicts a true change of natural history of the disease, these findings are of major significance.

This study has a number of aspects that should be pointed out. First, patients in the top-down group did not receive maintenance therapy with both azathioprine and infliximab. Rutgeerts *et al*[21] reported a significantly higher proportion of patients with mucosal healing in a maintenance infliximab group compared with those patients who received episodic treatment (50% *vs* 7%; $P = 0.007$). Thus, the study may have underestimated the benefit of combination therapy with an immunomodulator and infliximab administered on a regular basis. Second, the data suggest that infliximab might be used as a bridge to maintenance of remission with azathioprine. In a study by Lémann *et al*[22], 113 patients with steroid-dependent active Crohn's disease were randomized to azathioprine plus placebo or infliximab 5 mg/kg at weeks 0, 2, and 6. At weeks 24 and 54, the percentage of patients in remission and off steroids was higher in the infliximab group compared to the placebo group (57% *vs* 29%; $P = 0.003$ at week 24, and 40% *vs* 22%; $P = 0.04$ at week 54). However, episodic treatment is associated with immunogenicity that leads to hypersensibility reactions and loss of response to infliximab[23]. Third, in this study, azathioprine was started after two courses of corticosteroids, that may have underestimated the benefit of early introduction of conventional therapy with azathioprine. In a pediatric study by Markowitz *et al*[24], 55 children with newly diagnosed Crohn's disease were randomized to receive prednisone 40 mg/d with either 6-MP or placebo. Only 9% of the 6-MP treated group relapsed during a 18-mo follow-up period compared with 47% of the controls ($P = 0.007$). Candy *et al*[3] reported similar results in 63 adult patients with active Crohn's disease that were treated with a 12 wk diminishing dose of prednisolone and at the same time entered into a randomized, double blind 15 mo trial of either azathioprine (2.5 mg/kg) or placebo. Remission rates between the two groups were compared at 12 wk and at 15 mo. There was no significant difference in the proportion of patients who achieved and maintained remission by week 12, but at 15 mo there was a highly significant difference in the proportion of patients in remission (42% receiving azathioprine *vs* 7% receiving placebo, $P = 0.001$). Recent practice guidelines suggest that azathioprine should be introduced with the first course of corticosteroids[25]. Fourth, this study also underscores the fact that a proportion of patients will have a good clinical outcome at 12 mo irrespective of which treatment they receive. In other words, there is a proportion of patients with newly diagnosed Crohn's disease who do not require early intense treatment with biologic therapy.

BIOLOGICS IN EARLY CROHN'S DISEASE

Beyond achieving mucosal healing, the time of initiation of therapy might be crucial considering that a longer duration of the disease leads to more irreversible damage. There are several data in the literature that suggest higher response and remission rates to biologics in patients with a recent diagnosis of Crohn's disease compared to those patients with long-lasting disease. In a pediatric study that included 22 children, Lionetti *et al*[26] observed that the best response to infliximab was seen in children with a disease duration < 1 year. In addition, 5/6 children with early Crohn's disease had a complete closure of all fistulas compared with 2/7 children with a disease duration of more than 1 year. Kagathasan *et al*[27] showed a remarkably prolonged duration of response after a single infusion of infliximab in children with early compared to late Crohn's disease. In this study, among the 14/15 patients who responded, three of six children (50%) with early disease maintained clinical response through the 12-mo trial period, compared with none of eight children with late disease.

The CHARM (Crohn's trial of the fully Human antibody Adalimumab for remission Maintenance) study[28] was a double-blind, placebo-controlled phase 3 trial designed to determine the efficacy and safety of adalimumab 40 mg weekly *versus* every other week for maintenance of clinical remission in patients with moderate-to-severe Crohn's disease. A subanalysis of the CHARM study showed that disease duration was a significant contributor to the likehood of achieving remission. Remission rates at weeks 26 and 54 were highest when adalimumab was started in patients within the first 2 years after Crohn's disease diagnosis compared with patients diagnosed within > 5 years (59% *vs* 41%, respectively at week 26; 51% *vs* 35%, respectively at week 54). The PRECISE 2 (The Pegylated Antibody Fragment Evaluation in Crohn's disease: Safety and Efficacy 2) study was a randomized, double-blind, placebo-controlled trial that evaluated the efficacy of certolizumab pegol maintenance therapy in adults with moderate-to-severe Crohn's disease[29]. In a subanalysis of the study, a high rate of response and remission was observed at week 26 in patients with Crohn's disease diagnosed within the last 2 years and treated with certolizumab pegol compared with all patients included in the study (83.5% *vs* 62.8%, respectively; 61.5% *vs* 47.9%, respectively; $P < 0.02$)[30,31].

TOP-DOWN TREATMENT: LIMITATIONS

There is a growing evidence that suggests that the benefits of anti-TNF therapy extend beyond just the measure of clinical efficacy and that they may have the potential to alter the natural history of the disease. However, to date, several factors limit the use of biologic agents as primary therapy for Crohn's disease patients and early intense therapy needs to be balanced against the potential risks of increased infections and malignancy. Approximately 50% of all patients with inflammatory bowel disease will never require corticosteroids. Thus, first-line therapy with biologic therapies in this group would expose patients to toxicity or immunogenicity

without the benefit of potentially changing the natural history of their disease. The TREAT registry in North America has not shown, to date, an increased risk for malignancies or serious infections related to the use of infliximab in Crohn's disease[32] but clinicians should be aware that opportunistic infections such as tuberculosis, hystoplasmosis or *Pneumocytes carinii* do develop in anti-TNFα-treated patients. Nevertheless, the TREAT registry also shows that in the overall population of the registry, steroid used but not infliximab treatment was an independent predictive factor for infectious complications, and this point has been confirmed in the prospective European ENCORE registry[33]. The recently reported cases of hepatosplenic T-cell lymphoma[34], a rare and aggressive tumour, which most frequently develops in young male adults treated with infliximab and concomitant immunosuppresive therapy, has raised the question of whether it is the best strategy to discontinue the immunomodulators after certain period of combined therapy, or to stop the biologic therapy and try to maintain remission with immunomodulators alone[35]. Clinicians should also balance the side effects of the new biological therapies against the well-known and frequent toxicity of both corticosteroids and immunomodulators.

CONCLUSION

At present, Crohn's disease therapy is based in a sequential step-up strategy and biologic therapies are placed on a late position in this treatment strategy, mirroring the design of pivotal randomized controlled trials. However, the failure of many patients to respond to conventional therapy and the assessment that step-up strategy does not significantly alter the natural course of the disease has encouraged clinicians and investigators to question whether this late position is the most appropriate for biologics. Challenge is to identify those Crohn's disease patients who will develop a complicated course of the disease in whom the introduction of early and more intensive treatment from the beginning of the disease can be justified. In a recent report by Beaugerie *et al*[36], young age at time of disease onset, presence of perianal disease, early need for corticosteroids, and isolated small bowel involvement were identified as clinical factors associated with poor clinical outcomes and disability. Combination of genetic and serologic profiles may have an additive value in stratifying the disease outcome[37,38] and this may help clinicians categorize patients into subgroups to better guide therapeutic decision-making.

REFERENCES

1 **Faubion WA Jr**, Loftus EV Jr, Harmsen WS, Zinsmeister AR, Sandborn WJ. The natural history of corticosteroid therapy for inflammatory bowel disease: a population-based study. *Gastroenterology* 2001; **121**: 255-260

2 **Present DH**, Korelitz BI, Wisch N, Glass JL, Sachar DB, Pasternack BS. Treatment of Crohn's disease with 6-mercaptopurine. A long-term, randomized, double-blind study. *N Engl J Med* 1980; **302**: 981-987

3 **Candy S**, Wright J, Gerber M, Adams G, Gerig M, Goodman R. A controlled double blind study of azathioprine in the management of Crohn's disease. *Gut* 1995; **37**: 674-678

4 **Bouhnik Y**, Lemann M, Mary JY, Scemama G, Tai R, Matuchansky C, Modigliani R, Rambaud JC. Long-term follow-up of patients with Crohn's disease treated with azathioprine or 6-mercaptopurine. *Lancet* 1996; **347**: 215-219

5 **Feagan BG**, Rochon J, Fedorak RN, Irvine EJ, Wild G, Sutherland L, Steinhart AH, Greenberg GR, Gillies R, Hopkins M. Methotrexate for the treatment of Crohn's disease. The North American Crohn's Study Group Investigators. *N Engl J Med* 1995; **332**: 292-297

6 **Feagan BG**, Fedorak RN, Irvine EJ, Wild G, Sutherland L, Steinhart AH, Greenberg GR, Koval J, Wong CJ, Hopkins M, Hanauer SB, McDonald JW. A comparison of methotrexate with placebo for the maintenance of remission in Crohn's disease. North American Crohn's Study Group Investigators. *N Engl J Med* 2000; **342**: 1627-1632

7 **Munkholm P**, Langholz E, Davidsen M, Binder V. Disease activity courses in a regional cohort of Crohn's disease patients. *Scand J Gastroenterol* 1995; **30**: 699-706

8 **Cosnes J**, Cattan S, Blain A, Beaugerie L, Carbonnel F, Parc R, Gendre JP. Long-term evolution of disease behavior of Crohn's disease. *Inflamm Bowel Dis* 2002; **8**: 244-250

9 **Cosnes J**, Nion-Larmurier I, Beaugerie L, Afchain P, Tiret E, Gendre JP. Impact of the increasing use of immunosuppressants in Crohn's disease on the need for intestinal surgery. *Gut* 2005; **54**: 237-241

10 **Toruner M**, Loftus EV Jr, Harmsen WS, Zinsmeister AR, Orenstein R, Sandborn WJ, Colombel JF, Egan LJ. Risk factors for opportunistic infections in patients with inflammatory bowel disease. *Gastroenterology* 2008; **134**: 929-936

11 **Kandiel A**, Fraser AG, Korelitz BI, Brensinger C, Lewis JD. Increased risk of lymphoma among inflammatory bowel disease patients treated with azathioprine and 6-mercaptopurine. *Gut* 2005; **54**: 1121-1125

12 **Kane S**, Khatibi B, Reddy D. Higher incidence of abnormal Pap smears in women with inflammatory bowel disease. *Am J Gastroenterol* 2008; **103**: 631-636

13 **Vernier-Massouille G**, Cosnes J, Lemann M, Marteau P, Reinisch W, Laharie D, Cadiot G, Bouhnik Y, De Vos M, Boureille A, Duclos B, Seksik P, Mary JY, Colombel JF. Nodular regenerative hyperplasia in patients with inflammatory bowel disease treated with azathioprine. *Gut* 2007; **56**: 1404-1409

14 **Bathon JM**, Martin RW, Fleischmann RM, Tesser JR, Schiff MH, Keystone EC, Genovese MC, Wasko MC, Moreland LW, Weaver AL, Markenson J, Finck BK. A comparison of etanercept and methotrexate in patients with early rheumatoid arthritis. *N Engl J Med* 2000; **343**: 1586-1593

15 **Breedveld FC**, Emery P, Keystone E, Patel K, Furst DE, Kalden JR, St Clair EW, Weisman M, Smolen J, Lipsky PE, Maini RN. Infliximab in active early rheumatoid arthritis. *Ann Rheum Dis* 2004; **63**: 149-155

16 **St Clair EW**, van der Heijde DM, Smolen JS, Maini RN, Bathon JM, Emery P, Keystone E, Schiff M, Kalden JR, Wang B, Dewoody K, Weiss R, Baker D. Combination of infliximab and methotrexate therapy for early rheumatoid arthritis: a randomized, controlled trial. *Arthritis Rheum* 2004; **50**: 3432-3443

17 **Goekoop-Ruiterman YP**, de Vries-Bouwstra JK, Allaart CF, van Zeben D, Kerstens PJ, Hazes JM, Zwinderman AH, Ronday HK, Han KH, Westedt ML, Gerards AH, van Groenendael JH, Lems WF, van Krugten MV, Breedveld FC, Dijkmans BA. Clinical and radiographic outcomes of four different treatment strategies in patients with early rheumatoid arthritis (the BeSt study): a randomized, controlled trial. *Arthritis Rheum* 2005; **52**: 3381-3390

18 **Goekoop-Ruiterman YP**, de Vries-Bouwstra JK, Allaart

CF, van Zeben D, Kerstens PJ, Hazes JM, Zwinderman AH, Peeters AJ, de Jonge-Bok JM, Mallee C, de Beus WM, de Sonnaville PB, Ewals JA, Breedveld FC, Dijkmans BA. Comparison of treatment strategies in early rheumatoid arthritis: a randomized trial. *Ann Intern Med* 2007; **146**: 406-415

19 **D'Haens G**, Baert F, van Assche G, Caenepeel P, Vergauwe P, Tuynman H, De Vos M, van Deventer S, Stitt L, Donner A, Vermeire S, Van de Mierop FJ, Coche JC, van der Woude J, Ochsenkuhn T, van Bodegraven AA, Van Hootegem PP, Lambrecht GL, Mana F, Rutgeerts P, Feagan BG, Hommes D. Early combined immunosuppression or conventional management in patients with newly diagnosed Crohn's disease: an open randomised trial. *Lancet* 2008; **371**: 660-667

20 **Hanauer SB**, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, Rachmilewitz D, Wolf DC, Olson A, Bao W, Rutgeerts P. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet* 2002; **359**: 1541-1549

21 **Rutgeerts P**, Diamond RH, Bala M, Olson A, Lichtenstein GR, Bao W, Patel K, Wolf DC, Safdi M, Colombel JF, Lashner B, Hanauer SB. Scheduled maintenance treatment with infliximab is superior to episodic treatment for the healing of mucosal ulceration associated with Crohn's disease. *Gastrointest Endosc* 2006; **63**: 433-442; quiz 464

22 **Lemann M**, Mary JY, Duclos B, Veyrac M, Dupas JL, Delchier JC, Laharie D, Moreau J, Cadiot G, Picon L, Bourreille A, Sobahni I, Colombel JF. Infliximab plus azathioprine for steroid-dependent Crohn's disease patients: a randomized placebo-controlled trial. *Gastroenterology* 2006; **130**: 1054-1061

23 **Baert F**, Noman M, Vermeire S, Van Assche G, D' Haens G, Carbonez A, Rutgeerts P. Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. *N Engl J Med* 2003; **348**: 601-608

24 **Markowitz J**, Grancher K, Kohn N, Lesser M, Daum F. A multicenter trial of 6-mercaptopurine and prednisone in children with newly diagnosed Crohn's disease. *Gastroenterology* 2000; **119**: 895-902

25 **Lichtenstein GR**, Abreu MT, Cohen R, Tremaine W. American Gastroenterological Association Institute technical review on corticosteroids, immunomodulators, and infliximab in inflammatory bowel disease. *Gastroenterology* 2006; **130**: 940-987

26 **Lionetti P**, Bronzini F, Salvestrini C, Bascietto C, Canani RB, De Angelis GL, Guariso G, Martelossi S, Papadatou B, Barabino A. Response to infliximab is related to disease duration in paediatric Crohn's disease. *Aliment Pharmacol Ther* 2003; **18**: 425-431

27 **Kugathasan S**, Werlin SL, Martinez A, Rivera MT, Heikenen JB, Binion DG. Prolonged duration of response to infliximab in early but not late pediatric Crohn's disease. *Am J Gastroenterol* 2000; **95**: 3189-3194

28 **Colombel JF**, Sandborn WJ, Rutgeerts P, Enns R, Hanauer SB, Panaccione R, Schreiber S, Byczkowski D, Li J, Kent JD, Pollack PF. Adalimumab for maintenance of clinical response and remission in patients with Crohn's disease: the CHARM trial. *Gastroenterology* 2007; **132**: 52-65

29 **Schreiber S**, Khaliq-Kareemi M, Lawrance IC, Thomsen OO, Hanauer SB, McColm J, Bloomfield R, Sandborn WJ. Maintenance therapy with certolizumab pegol for Crohn's disease. *N Engl J Med* 2007; **357**: 239-250

30 **Schreiber S**, Renisch W, Colombel JF, Sandborn WJ, Hommes DW, Li J, Kent JD, Pollack PF. Early Crohn's disease shows high levels of remission to therapy with adalimumab: Sub-Analysis of CHARM. *Gastroenterology* 2007; **132**: A147

31 **Schreiber S**, Hanauer SB, Lichtenstein GR, Sandborn WJ. Superior efficacy of certolizumab pegol in early Crohn's disease is independent of CRP status. *Gastroenterology* 2007; **132**: A510

32 **Lichtenstein GR**, Feagan BG, Cohen RD, Salzberg BA, Diamond RH, Chen DM, Pritchard ML, Sandborn WJ. Serious infections and mortality in association with therapies for Crohn's disease: TREAT registry. *Clin Gastroenterol Hepatol* 2006; **4**: 621-630

33 **Panes J**, Colombel JF, D'Haens G, Rutgeerts P, Ekbom A, Hommes DW, Barai U, van Hoogstraten, Wiekowski M, Antoni C. Corticosteroids pose an increased risk for serious infections: An interim safety analysis of the ENCORE registry. *JCC* 2008; **1**: A23

34 **Mackey AC**, Green L, Liang LC, Dinndorf P, Avigan M. Hepatosplenic T cell lymphoma associated with infliximab use in young patients treated for inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 2007; **44**: 265-267

35 **Van Assche G**, Magdelaine-Beuzelin C, D'Haens G, Baert F, Noman M, Vermeire S, Ternant D, Watier H, Paintaud G, Rutgeerts P. Withdrawal of immunosuppression in Crohn's disease treated with scheduled infliximab maintenance: a randomized trial. *Gastroenterology* 2008; **134**: 1861-1868

36 **Beaugerie L**, Seksik P, Nion-Larmurier I, Gendre JP, Cosnes J. Predictors of Crohn's disease. *Gastroenterology* 2006; **130**: 650-656

37 **Dubinsky MC**, Lin YC, Dutridge D, Picornell Y, Landers CJ, Farrior S, Wrobel I, Quiros A, Vasiliauskas EA, Grill B, Israel D, Bahar R, Christie D, Wahbeh G, Silber G, Dallazadeh S, Shah P, Thomas D, Kelts D, Hershberg RM, Elson CO, Targan SR, Taylor KD, Rotter JI, Yang H. Serum immune responses predict rapid disease progression among children with Crohn's disease: immune responses predict disease progression. *Am J Gastroenterol* 2006; **101**: 360-367

38 **Mow WS**, Vasiliauskas EA, Lin YC, Fleshner PR, Papadakis KA, Taylor KD, Landers CJ, Abreu-Martin MT, Rotter JI, Yang H, Targan SR. Association of antibody responses to microbial antigens and complications of small bowel Crohn's disease. *Gastroenterology* 2004; **126**: 414-424

S- Editor Xiao LL **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5528

World J Gastroenterol 2008 September 28; 14(36): 5528-5531
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Javier P Gisbert, Professor; Fernando Gomollón, Dr., MD, PhD, *Series Editors*

Are we giving biologics too much time? When should we stop treatment?

Edouard Louis, J Belaiche, C Reenaers

Edouard Louis, J Belaiche, C Reenaers, Department of Gastroenterology CHU Liège, and GIGA Research University of Liège, Belgium
Author contributions: Louis E, Belaiche J and Reenaers C took part to the redaction of the manuscript.
Correspondence to: Edouard Louis, Department of Gastroenterology, CHU Liège, Domaine du Sart Tilman 4000 Liège, Belgium. edouard.louis@ulg.ac.be
Telephone: +32-43-667256 Fax: +32-43-667889
Received: August 18, 2008 Revised: August 26, 2008
Accepted: September 3, 2008
Published online: September 28, 2008

Abstract

The optimal duration of biological treatment, particularly anti-TNF, in inflammatory bowel disease (IBD) is a very important question both for patients and physicians. There is no published evidence to clearly and definitely answer this question. However data on natural history of IBD, long term safety of biologics, immunosuppressors (IS) cessation and some preliminary studies on biologics cessation may help us to discuss this topic. The decision to stop a biological treatment is currently based on a compromise between the benefits and risks associated with the prolongation of this treatment. IBD, more particularly CD, are characterized by the development of complications and the need for recurrent hospitalizations and surgeries in approximately 2/3 of cases. In these patients potentially in need of biological treatments, it is probable that, as it has been demonstrated for IS, the longer a stable remission has be achieved under treatment, the lower the risk of relapse is after treatment cessation. Further prospective studies should now aim at disclosing patient characteristics associated with a low risk of relapse to implement this strategy.

Key words: Inflammatory bowel disease; Immunosuppressors; Biological treatment

Louis E, Belaiche J, Reenaers C. Are we giving biologics too much time? When should we stop treatment? *World J Gastroenterol* 2008; 14(36): 5528-5531 Available from: URL: http://www.wjgnet.com/1007-9327/14/5528.asp DOI: http://dx.doi.org/10.3748/wjg.14.5528

INTRODUCTION

The question of treatment duration in inflammatory bowel disease (IBD) is certainly a very important one and is one of the greatest preoccupations of patients. When starting a new treatment in IBD, one of the first questions of the patients is usually: "when will I be able to stop this treatment"? While the question is very important, we actually have very little data to give a clear answer, because the controlled data we have with pivotal trials usually give us efficacy data for remission and response induction and for remission and response maintenance over a one year period[1-3].

However we have most often indirect elements to help us and discuss this point of optimal duration of biological treatment in IBD: these elements are the natural history of the disease, the available data with immunosuppressive drugs, the long term safety of biologics, and a few investigator-initiated studies having started to address this question. Beyond that, the cessation of a biological treatment in IBD must be decided on a case-by-case basis and adapted strategies must be proposed.

NATURAL HISTORY OF IBD

IBD are chronic relapsing diseases. There is probably a difference between UC and CD, the latter being more often a chronic active disease. In CD, population-based and cohort studies have showed that a small half of the patients have little evolutive disease with low prevalence of relapses, hospitalizations, or complications[4-7]. These patients probably do not need biologics and if a biological treatment has been used, an arrest must certainly be discussed as soon as the flare has been controlled. The other patients will develop complications including strictures and internal or perianal fistulas over the course of the disease[8,9]. These will lead to hospitalizations and surgeries and will considerably interfere with patients every day life and long term projects. For these patients a sustained control of the disease process is strongly warranted and an effective treatment can only be stopped if reasonable evidence shows absence of activity of this process. There is probably a difference between early disease and long-lasting disease, the reversal and control of the disease process being more difficult and unstable in the latter situation. Long-lasting disease are

indeed characterized by anatomical damages including mucosal and submucosal architectural changes, fibrosis, strictures and complex fistulas that will favour clinical relapse and that will render an asymptomatic remission more difficult to achieve. Furthermore, immunological status of the patients may change over the course of the disease[10]. In parallel, immunization against luminal material may increase, enhancing the potential reactivation of the immune process[11,12]. As a correlate, a stable remission will usually be more difficult to obtain in long-lasting diseases[13] and these diseases will usually be more treatment-dependent. This certainly represents an argument for earlier treatment with biologics in CD. These patients being treated earlier with biologics and in whom a more complete reversal of the immune process and the tissue lesions can be achieved are also probably better candidates for treatment cessation.

The problem of ulcerative colitis is a little bit different. There is usually less tissue damage in UC, the disease affecting only the mucosa. Strictures and fistulas are unusual and the biggest long term complication is cancer development. This risk of cancer is linked to several factors, including disease extent and chronic uncontrolled inflammation. In UC, flares can be separated by long period of full remission with both endoscopic and histological normalisation of the mucosa. Therefore, apart from patients with chronic active disease and incomplete mucosal healing, biological treatment cessation could be attempted when the flare has been controlled and a mucosal healing has been achieved.

AVAILABLE DATA WITH IMMUNOSUPPRESSIVE DRUGS

Little data exist on immunosuppressive drug cessation in CD. In an observational GETAID study of relapse after azathioprine cessation in CD, it was shown that the longer the duration of remission under azathioprine, the lowest the risk of relapse was[14]. Particularly, the risk of relapse seemed particularly low after 4 years of sustained remission. The same group then embarked a placebo-controlled trial of azathioprine prolonged treatment beyond 42 mo of sustained remission[15]. This trial showed that the strategy of treatment cessation was not equivalent to treatment prolongation. Particularly, 18-mo relapse rate were around 20% in the cessation group as compared to 10% in the prolongation group. This study indicates that even in patients in stable remission, the cessation of an immunosuppressive drug is associated with an increased risk of relapse. The risk of relapse in the cessation group remains however reasonably low and one could consider it on a case-by-case basis after discussion with the patients. No such data are available with methotrexate in CD or more generally in ulcerative colitis.

LONG TERM SAFETY OF BIOLOGICS

While short and mid term safety and tolerance of biologics is usually very good, the fear of long term complication is generally the reason why both patients and physicians would like to stop the drug when the disease has been completely stabilized. This fear is based on the mechanism of action of these drugs. For IBD, we currently only have anti-TNF treatments. These drugs block the tumor necrosis factor alpha which is a pivotal cytokine in some anti-microbial and anti-tumoral physiological processes. Indeed, the best documented side-effects are the increased risk of tuberculosis[16] and of other infections, mainly with intra-cellular pathogens (mycobacteria, listeria, histoplasmosis…), as well as a probable slight increase in the risk of lymphoma[17]. The active and systematic search for latent tuberculosis has already significantly decreased the incidence of active tuberculosis under anti-TNF treatment. Furthermore, a recent meeting of the European Crohn and Colitis Organisation on infections and biologics has proposed a series of guidelines, including vaccination against herpes zoster, hepatitis B, influenza and streptococcus pneumoniae as well as avoiding some aliments potentially containing germs as lysteria (i.e. unpasteurized milk or insufficiently cooked meat). These guidelines, not yet published, should also in a near future help and diminish fatal complications linked to biologics. Another measure that gains more and more support is the avoidance of long term combined treatment with immunosuppressors. While there is currently very little evidence for a cumulative benefit of these drugs[1,2], combined therapies were associated in a retrospective study with a very significant increase in the risk of opportunistic infections with a relative risk of 12 when two treatments were combined[18]. Furthermore in a recently reported pediatric series of nearly universally fatal hepato-splenic T cell lymphoma in infliximab treated patients, all the patients affected had been treated with combined therapy with thiopurines[19]. All together, these measures should lower the risk profile associated with biologics and allow the physician to prescribe them for enough time to achieve stable and durable remission of the disease.

AVAILABLE STUDIES ON BIOLOGICAL TREATMENT CESSATION IN IBD

Early data with infliximab in CD were only short term induction data[20]. Only one single infusion was used at that time and it is striking to note the some patients had a very prolonged clinical response or even remission after such isolated infusion[21]. These data already suggested that prolonged treatment was probably not necessary in all the treated patients. Since then however, it has become clear that such one-shot treatment was not a good option for the majority of patients because the median time to relapse was 10 wk and because re-use of infliximab more than 4 mo after a single infusion was associated with high risk of allergic reaction. More recently the "bridge" study of the GETAID explored the idea of a 3-dose infliximab induction given in parallel with immunosuppressor that would then maintain the remission[22]. The results of this study were rather disappointing. While the short term effect of infliximab was very strong, the maintenance effect with immuno-

suppressor was globally rather weak. After one year, the overall sustained remission rate in these patients was low and actually close to the one of patients receiving a placebo induction. Only in the patients who were immunosuppressor-naïve at the time of infliximab induction, the benefit was more consistent with a reasonable 40% remission rate after one year. This study clearly shows that in patients who have failed under immunosuppressors, a longer period of anti-TNF treatment is necessary to obtain a durable remission and allow treatment cessation. This has been explored in a recent GETAID study, not yet fully completed. In this cohort study, over 100 patients with a stable remission on combined immunosuppressor-infliximab therapy for more than one year had their infliximab stopped, while pursuing immunosuppressor treatment. An interim analysis indicates that after one year more than half of the patients are still in sustained remission. A multivariate analysis of predictive factors for such sustained remission should allow to better identify the subgroup of patients in whom such strategy may be proposed.

PRACTICAL CONSIDERATION FOR STOPPING BIOLOGIC TREATMENT IN IBD

Usually, a biological treatment is started in patients who do no longer respond to conventional therapies. In patients responding to the treatment, it is certainly not wise to contemplate a treatment cessation as long as a complete clinical remission has not been achieved. In patients who have been in clinical remission for a sufficient period of time, it is probably useful to assess biological as well as endoscopic signs of disease activity. In several models and clinical situations, C-reactive protein (CRP) serum concentration has been associated with the risk of relapse[23]. More sophisticated serum or stool markers have also been proposed, but their added value as compared to CRP has not been clearly demonstrated[24-26]. A stable value of CRP within normal range should therefore also be obtained before biologics cessation. Correlation between clinical indexes of activity or biological markers of inflammation and mucosal healing is not very strong[27]. Mucosal healing under anti-TNF treatment has been associated with a decrease in relapse rate, hospitalisation and surgeries[28]. A third condition for biologics cessation is thus the existence of a mucosal healing in patients with ileo-colonic disease accessible to endoscopic control. For patients with proximal small bowel disease, there is not universally accepted exploration to assess the control of inflammation at the tissue level. However, entero-MRI could be a good candidate[29]. An absence of mucosal lesion (not always easy to detect), and of contrast enhancement of the bowel wall or the mesenterium could be interpreted as tissue healing.

CONCLUSION

Globally, the decision to stop or carry on with biological treatment in IBD is based on an estimated benefit-risk ratio. The patient must certainly be informed at the highest levels on both advantages and risks linked to any therapeutic strategy that is proposed. In patients with unstable chronic active disease, stopping an effective treatment will put the patients at risk of worsening and complications development and should probably not be attempted. However in patients stabilized for a reasonably long period of time, a careful assessment of the clinical, biological and endoscopic situation may help to take a thoughtful decision in collaboration with the patient himself.

REFERENCES

1 **Hanauer SB**, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, Rachmilewitz D, Wolf DC, Olson A, Bao W, Rutgeerts P. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet* 2002; **359**: 1541-1549

2 **Colombel JF**, Sandborn WJ, Rutgeerts P, Enns R, Hanauer SB, Panaccione R, Schreiber S, Byczkowski D, Li J, Kent JD, Pollack PF. Adalimumab for maintenance of clinical response and remission in patients with Crohn's disease: the CHARM trial. *Gastroenterology* 2007; **132**: 52-65

3 **Schreiber S**, Khaliq-Kareemi M, Lawrance IC, Thomsen OO, Hanauer SB, McColm J, Bloomfield R, Sandborn WJ. Maintenance therapy with certolizumab pegol for Crohn's disease. *N Engl J Med* 2007; **357**: 239-250

4 **Munkholm P**, Langholz E, Davidsen M, Binder V. Disease activity courses in a regional cohort of Crohn's disease patients. *Scand J Gastroenterol* 1995; **30**: 699-706

5 **Wolters FL**, Russel MG, Sijbrandij J, Ambergen T, Odes S, Riis L, Langholz E, Politi P, Qasim A, Koutroubakis I, Tsianos E, Vermeire S, Freitas J, van Zeijl G, Hoie O, Bernklev T, Beltrami M, Rodriguez D, Stockbrugger RW, Moum B. Phenotype at diagnosis predicts recurrence rates in Crohn's disease. *Gut* 2006; **55**: 1124-1130

6 **Beaugerie L**, Seksik P, Nion-Larmurier I, Gendre JP, Cosnes J. Predictors of Crohn's disease. *Gastroenterology* 2006; **130**: 650-656

7 **Loly C**, Belaiche J, Louis E. Predictors of severe Crohn's disease. *Scand J Gastroenterol* 2008; 1-8

8 **Louis E**, Collard A, Oger AF, Degroote E, Aboul Nasr El Yafi FA, Belaiche J. Behaviour of Crohn's disease according to the Vienna classification: changing pattern over the course of the disease. *Gut* 2001; **49**: 777-782

9 **Cosnes J**, Cattan S, Blain A, Beaugerie L, Carbonnel F, Parc R, Gendre JP. Long-term evolution of disease behavior of Crohn's disease. *Inflamm Bowel Dis* 2002; **8**: 244-250

10 **Kugathasan S**, Saubermann LJ, Smith L, Kou D, Itoh J, Binion DG, Levine AD, Blumberg RS, Fiocchi C. Mucosal T-cell immunoregulation varies in early and late inflammatory bowel disease. *Gut* 2007; **56**: 1696-1705

11 **Dubinsky MC**, Kugathasan S, Mei L, Picornell Y, Nebel J, Wrobel I, Quiros A, Silber G, Wahbeh G, Katzir L, Vasiliauskas E, Bahar R, Otley A, Mack D, Evans J, Rosh J, Hemker MO, Leleiko N, Crandall W, Langton C, Landers C, Taylor KD, Targan SR, Rotter JI, Markowitz J, Hyams J. Increased Immune Reactivity Predicts Aggressive Complicating Crohn's Disease in Children. *Clin Gastroenterol Hepatol* 2008; **6:** 1105-1111

12 **Rieder F**, Schleder S, Wolf A, Dirmeier A, Strauch U, Obermeier F, Spector L, Fire E, Yarden J, Rogler G, Dotan N, Klebl F. Specific levels and combinations of the anti-glycan antibodies anti-L, anti-C, ALCA, ACCA, gASCA and AMCA contribute to diagnosis and differential diagnosis of patients with Crohn's disease and are associated with complicated disease and surgery. *Gastroenterology* 2008; **134**; A53: 392

13 **Schreiber S**, Hanauer S, Lichtenstein G, Sandborn W. Superior efficacy of certolizumab pegol in early Crohn's disease is independent of CRP status. *Gastroenterology* 2007;

132: A510: T1298
14 **Bouhnik Y**, Lemann M, Mary JY, Scemama G, Tai R, Matuchansky C, Modigliani R, Rambaud JC. Long-term follow-up of patients with Crohn's disease treated with azathioprine or 6-mercaptopurine. *Lancet* 1996; **347**: 215-219
15 **Lemann M**, Mary JY, Colombel JF, Duclos B, Soule JC, Lerebours E, Modigliani R, Bouhnik Y. A randomized, double-blind, controlled withdrawal trial in Crohn's disease patients in long-term remission on azathioprine. *Gastroenterology* 2005; **128**: 1812-1818
16 **Keane J**, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, Siegel JN, Braun MM. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N Engl J Med* 2001; **345**: 1098-1104
17 **Siegel C**, Sadie M, Marden S, Persing SM, Larson RJ, Sands BE. Risk of lymphoma associated with anti-TNF agents for the treatment of Crohn's disease: a meta-analysis. *Gastroenterology* 2008; **134**: A144: 970
18 **Toruner M**, Loftus EV Jr, Harmsen WS, Zinsmeister AR, Orenstein R, Sandborn WJ, Colombel JF, Egan LJ. Risk factors for opportunistic infections in patients with inflammatory bowel disease. *Gastroenterology* 2008; **134**: 929-936
19 **Mackey AC**, Green L, Liang LC, Dinndorf P, Avigan M. Hepatosplenic T cell lymphoma associated with infliximab use in young patients treated for inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 2007; **44**: 265-267
20 **Targan SR**, Hanauer SB, van Deventer SJ, Mayer L, Present DH, Braakman T, DeWoody KL, Schaible TF, Rutgeerts PJ. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *N Engl J Med* 1997; **337**: 1029-1035
21 **Rutgeerts P**, D'Haens G, Targan S, Vasiliauskas E, Hanauer SB, Present DH, Mayer L, Van Hogezand RA, Braakman T, DeWoody KL, Schaible TF, Van Deventer SJ. Efficacy and safety of retreatment with anti-tumor necrosis factor antibody (infliximab) to maintain remission in Crohn's disease. *Gastroenterology* 1999; **117**: 761-769
22 **Lemann M**, Mary JY, Duclos B, Veyrac M, Dupas JL, Delchier JC, Laharie D, Moreau J, Cadiot G, Picon L, Bourreille A, Sobahni I, Colombel JF. Infliximab plus azathioprine for steroid-dependent Crohn's disease patients: a randomized placebo-controlled trial. *Gastroenterology* 2006; **130**: 1054-1061
23 **Vermeire S**, Van Assche G, Rutgeerts P. C-reactive protein as a marker for inflammatory bowel disease. *Inflamm Bowel Dis* 2004; **10**: 661-665
24 **Louis E**, Belaiche J, van Kemseke C, Franchimont D, de Groote D, Gueenen V, Mary JY. A high serum concentration of interleukin-6 is predictive of relapse in quiescent Crohn's disease. *Eur J Gastroenterol Hepatol* 1997; **9**: 939-944
25 **Louis E**, Belaiche J, Van Kemseke C, Schaaf N, Mahieu P, Mary JY. Soluble interleukin-2 receptor in Crohn's disease. Assessment of disease activity and prediction of relapse. *Dig Dis Sci* 1995; **40**: 1750-1756
26 **Costa F**, Mumolo MG, Ceccarelli L, Bellini M, Romano MR, Sterpi C, Ricchiuti A, Marchi S, Bottai M. Calprotectin is a stronger predictive marker of relapse in ulcerative colitis than in Crohn's disease. *Gut* 2005; **54**: 364-368
27 **Cellier C**, Sahmoud T, Froguel E, Adenis A, Belaiche J, Bretagne JF, Florent C, Bouvry M, Mary JY, Modigliani R. Correlations between clinical activity, endoscopic severity, and biological parameters in colonic or ileocolonic Crohn's disease. A prospective multicentre study of 121 cases. The Groupe d'Etudes Therapeutiques des Affections Inflammatoires Digestives. *Gut* 1994; **35**: 231-235
28 **Rutgeerts P**, Vermeire S, Van Assche G. Mucosal healing in inflammatory bowel disease: impossible ideal or therapeutic target? *Gut* 2007; **56**: 453-455
29 **Ryan ER**, Heaslip IS. Magnetic resonance enteroclysis compared with conventional enteroclysis and computed tomography enteroclysis: a critically appraised topic. *Abdom Imaging* 2008; **33**: 34-37

S- Editor Xiao LL **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5532

World J Gastroenterol 2008 September 28; 14(36): 5532-5539
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Javier P Gisbert, Professor; Fernando Gomollón, Dr., MD, PhD, *Series Editors*

Timing of surgery in Crohn's disease: A key issue in the management

Rafael Alós, Joaquín Hinojosa

Rafael Alós, Servicio de Cirugía General, Unidad de Coloproctología, Hospital de Sagunto, Valencia, Spain
Joaquín Hinojosa, Unidad de Digestivo, Servicio de Medicina Interna, Hospital de Sagunto, Valencia, Spain
Author contributions: Alós R and Hinojosa J contributed equally to this work.
Correspondence to: Rafael Alós, Servicio de Cirugía General, Unidad de Coloproctología, Hospital de Sagunto, Valencia, Spain. alos_raf@gva.es
Telephone: +34-96-2659414 Fax: +34-96-2659420
Received: August 18, 2008 Revised: August 26, 2008
Accepted: September 3, 2008
Published online: September 28, 2008

Abstract

The timing of the decision for operation in Crohn's disease is based on an evaluation of the several factors such as the failure of medical treatment, complications due to the Crohn's disease or to the farmacological therapy, development of dysplasia or cancer and growth retardation. A complete evaluation of these factors should result in operation timed to the patient's best advantage, achieving maximal relief of symptoms with improvement of quality of life. Given the complexity and heterogeneity of the disease and the different options for treatment, is difficult to systematize when the optimal moment for the surgery is arrived. A very important factor in the management of Crohn's disease is the multidisciplinary approach and the patient preference should be a significant factor in determining the choice of therapy. The surgery should be considered such another option in the sequential treatment of Crohn's disease. We have analyzed the factors that are involved in the decision taking of the surgical treatment regarding to the experience and the published literature. When did the medical therapy fail? when is the appropriate moment to operate on the patient? Or which complications of Crohn's disease need a surgery? These are some of the questions we will try to answer.

Key words: Crohn's disease; Surgical treatment; Medical therapy

Alós R, Hinojosa J. Timing of surgery in Crohn's disease: A key issue in the management. *World J Gastroenterol* 2008; 14(36): 5532-5539 Available from: URL: http://www.wjgnet.com/1007-9327/14/5532.asp DOI: http://dx.doi.org/10.3748/wjg.14.5532

INTRODUCTION

Over fifty years ago, the introduction of corticosteroids in management of active inflammatory bowel disease meant a radical shift in patient management, both from the medical and the surgical standpoints[1]. Steroids brought about a drop in mortality rates and reduced the need of surgery in flare-ups[2]. Some forty years later another outstanding leap forward took place in terms of patient management with the introduction of biological therapies[2,3]. However, despite these advances, it is estimated that approximately 80% of patients with Crohn's disesase (CD) will require surgery at some point during their lifetime, especially when the disease is located in the ileocecal region[4]. In general, the indication for surgery in CD depends on a number of factors-complications, clinical course, relapse and location. Broadly speaking we could say that surgery is timely in any of the following situations: (a) failure of medical treatment, (b) onset of specific complications related to the disease or to pharmacological treatment, (c) dysplasia or cancer and (d) stagnated or retarded growth in children. In some cases it is fairly straightforward to decide when is the best time for surgery- free perforation or abdominal fistula, for instance. However, CD invariably poses a genuine challenge to the team treating the patient. The broad clinical heterogeneity of the disease, the various complications that arise and evolutionary possibilities are all so diverse that it is hard to generalise or adopt a systematic approach when it comes to determining when to operate. Each case should be assessed individually and that is why it is vital to have multidisciplinary teams involved in decision-making together with patients. With these teams it is still possible to improve outcomes[5], because all the physicians involved have common goals in mind, namely to avoid diagnostic delays, to establish the ideal moment for surgery, to attain the best possible pre-operative conditions and to optimise medical-surgical outcomes; given that this is a chronic disease affecting young

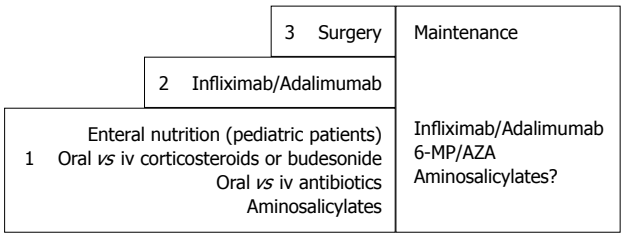


Figure 1 Suggested steps in active (left boxes) and inactive (right box) Crohn's disease. 6-MP: 6 Mercaptopurine. AZA: Azathioprine.

patients, medical-surgical management is crucial.

Recent advances in biological therapies have allowed substantial clinical improvements and better monitoring of patients-especially in fistulizing perianal disease-reducing hospitalizations, number of operations and prolonging the time that elapses between diagnosis and surgery[6]. Colombel *et al*[7] and Marchal *et al*[8] suggest that these drugs do not increase post-operative complications in patients with CD, although the same cannot be said in the case of proctocolectomy in ulcerative colitis where the risk of post-op complications is far higher if Infliximab is administered[9,10]. So, further studies are required to determine the risk of post-operative complications when these drugs are used to treat CD. On the other hand, although a recent meta-analysis of anti-TNF versus placebo did not demonstrate that there is a higher risk of death, tumours or severe infections[11], one must not forget that biological therapy is not innocuous. The risks/benefits should always be weighed up, bearing in mind that the goal of pharmacological treatment is not to avoid surgery but rather to improve quality of life. If the latter cannot be achieved then surgery will have to be considered as the next step in treatment (Figure 1).

More than half of patients with CD present with affected terminal ileum, with or without extension to the proximal colon, and around 90% will require surgery at some point in their lifetime. Far less frequent-around 10% of all CD patients-are cases presenting with affected proximal areas of the small intestine and the duodenum whereas location in the colon and/or anorectum oscillates around 30%[12,13]. In these other regions-i.e. not the terminal ileum-the probability of patients requiring surgery is estimated at 60%. According to the classical study by Farmer *et al*[12] and the more recent review by Bernell *et al*[4] overall it is estimated that 74% of patients will require initial surgery within the first 13 years from onset of the disease and that around 50% of relapses will require further surgery within the first 10 years of follow-up after the first operation.

Medical treatment failure and/or patients' impaired quality of life are ultimately the reasons leading to surgical treatment. But what do we mean by medical treatment failure? When precisely is it best to conduct surgery? In the face of which complications is it necessary to operate? What other reasons may lead us to decide to opt for surgery? Or, when is quality of life so severely impaired that surgery is called for? These questions remain mooted points in many cases. Answers to these questions will depend on the consensus and protocols of multi-disciplinary teams, but patients must always be informed and involved in decision-making.

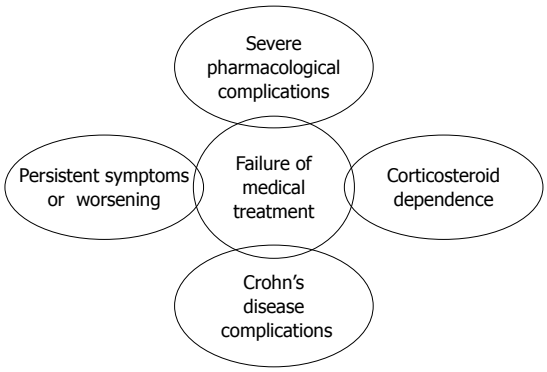


Figure 2 Clinical situations when medical treatment has failed and there is indication for surgery.

WHAT DO WE MEAN BY MEDICAL TREATMENT FAILURE?

Once the disease becomes medically untreatable it is necessary to resort to surgery. Failure of medical treatment or "untreatability" of CD is hard to define[14] and the concept does not bear the same meaning for all physicians or patients. We may consider four scenarios in which surgery is indicated (Figure 2).

(a) Persistence or worsening of symptoms despite correct treatment. In many cases, recognising medical treatment failure on these grounds requires experience and the right clinical judgement. In addition, there are times when it is necessary to consider each patient's individual circumstances since if partial clinical improvement occurs, residual symptoms may or may not be tolerated by patients, in which case subjectivity is a primary factor in deciding to operate. Although biological therapy has meant a remarkable step forward in management of situations involving patients refractory to corticosteroids with mild to severe flare-ups, it is crucial to define response times in order to determine the optimal time for surgery in these particular clinical cases. Non-response to induction with Infliximab or Adalimumab should be considered as therapeutic failure.

(b) Onset of unacceptable drug-related complications and there is no other efficacious medical alternative. This occurs especially with steroids which may trigger severe adverse effects-aseptic femoral necrosis, cataracts, compression-related fractures, *etc*. In rarer cases the same applies with azathioprine or 6-mercaptopurine which may produce medullar aplasia. With the introduction of biological agents, the chances of septic complications or reactivation of latent infections (tuberculosis, viral hepatitis, *etc*) are high, so extreme caution is necessary[15]. As to the presence of abdominal or perirectal septic foci these must always be drained before administering Infliximab otherwise severe sepsis may occur[15].

(c) In cases of steroid-dependence. These drugs

are used to tackle acute crises and not for maintenance purposes; so if after remission of flare-ups the drugs cannot be discontinued after two successive attempts over a six month period, or after three attempts over one year, the case should be considered as untreatable as a result of steroid-dependence. One of the two existing population-based studies included 173 patients with CD, of which 74 (43%) required treatment with steroids and 21 (28%) of these presented with steroid-dependence after a year[16]. This roughly represents 12% of all patients with CD and the results are similar to those of an earlier trial[17]. In children, CD is more aggressive and these percentages are higher-more than 60% require steroids and 16% of the total number of patients are steroid-dependent[17].

(d) Onset of complications associated to the disease that compromise patient's quality of life.

WHEN IS THE OPTIMAL TIME FOR SURGERY?

There is a good deal of controversy surrounding the issue. Those advocating early surgery argue that if medical treatment does not achieve substantial improvement there is no reason to await the onset of a serious, potentially life-threatening complication, or to increase surgery-related risk. Along these lines, one particular study adopted an active surgical approach in 136 patients with mean follow-up of 16.6 years concluding that morbidity and mortality are lower when following this approach, with good functional outcomes and complete remission of symptoms[18]. Another retrospective study with 74 patients who had undergone various first and subsequent operations for CD between 1975 and 2000 showed that the number of operations increased every 5-year[19] period, suggesting that the threshold to establish an indication for surgery has dropped with the passing of time (years) in that particular setting. On the other hand, authors critical of early surgery argue that since relapse and re-operation rates are high, the chances of short bowel syndrome are very low. From our point of view, this argument does not hold because since small resections and strictureplasties are being conducted this syndrome is highly unlikely to occur. In an attempt to identify the factors that increase the probability of surgery in the early stages of CD, Sands *et al*[20] reached the conclusion that the following have a significant impact: smoking, affection of the small intestine only, nausea and vomiting or abdominal pain as the main form of presentation, neutrophilia and use of steroids within the fist six months after diagnosis. In addition, the authors note that the percentage of early interventions is high (20%) and suggest that treatment with rapid action drugs should be initiated in order to change the disease's natural course hence reducing the number of patients requiring surgery. Contrarily, the same authors conclude that when only the colon is affected, blood in faeces, the use of 5-ASA and lymphocytosis are linked to later interventions, although the only independent factor turned out to be affection of the colon alone. More recently, Morimoto *et al*[21] noted that the only independent risk factors determining whether a patient should be operated are gender (female), and that the disease is located exclusively in the ileum. So, the presence or absence of one or more of the factors described may help us decide how early surgery should be conducted.

Table 1 Influencing factors in the choice of optimal time to operate on a patient

Severity of symptoms
Type of symptoms
Failure of medical treatment
Adverse effects of medical therapy
Risk of complications due to Crohn's disease
Established complications
Obvious benefits (or no disadvantages) of surgery
Gastroenterologist
Surgeon
Patient and family

On the other hand, the use of drugs that may potentially increase the risk of surgery-related complications should not be an obstacle to surgery if this becomes necessary at some point. In this regard there are few publications addressing steroid-related risk in postoperative CD complications although experience suggests that prolonged preoperative use is an adverse factor in itself. Two recent articles agree in pointing out that there is a higher risk of dehiscences and septic complications if these are administered pre-operatively over more than three months[22,23]. However, the same authors note that there are other more significant factors that come into play in the onset of post-op septic complications, as is the case with malnourished patients. Regarding treatment with Infliximab, as discussed above, it is highly likely that it has no role in CD post-op complications[7,8] although further studies are needed. The use of other immunosuppressants does not influence surgery-related complications either[7].

On the basis of what has been discussed so far, in order to determine the best time for surgery we should assess the severity and type of symptoms, failure of medical treatment, the onset of adverse effects and surgical risk/benefit. All this together will enable gastroenterologists, surgeons and patients to agree on optimal time for surgery with a view to ensuring that the operation will resolve symptoms with no or minimal disadvantages for patients (Table 1). To assess the importance of patients' views in decision-making we note the study by Scott and Hughes[24] in which they interviewed 70 patients who underwent ileocolic resection to ascertain when surgery should have taken place-earlier, later, or at the time it was conducted. None of the patients declared preference for a later operation, and 77% would have preferred earlier surgery, with a median of at least 12 mo sooner. Almost 100% of interviewees said that the main reason for wanting to bring surgery forward was severity of their symptoms.

Identifying the best time for surgery is not always an easy task. To be as sensible as possible, we should always remember that it is as inappropriate to indicate surgery before having resorted to all the drugs available-selected on the basis of clinical status-as unnecessarily delaying surgery until the onset of a serious complication.

IN THE FACE OF WHICH COMPLICATIONS IS SURGERY REQUIRED?

One of the features of CD is that it may give rise to a number of complications throughout its clinical course (Table 2) that may require surgery.

Intestinal obstruction: This is the most frequent indication for surgery when CD is located in the ileocecal area. This may also occur when CD is located in the jejunoileal area with one or multiple stenoses and, in these cases it may progress with greater severity and a higher probability of large resections[25]. Approximately 25% of all CD operated patients have undergone surgery due to intestinal obstruction[13] and commonly further interventions are required following a number of sub-occlusion flare-ups, although urgent operations are the exception. If occlusion occurs as a result of inflammatory events with edema in the intestinal wall and there is some response to crisis and maintenance medical therapy then there will be no need for surgery. However, if obstruction is due to fibrosis or reactive scarring changes, an operation is called for once absence of activity has been confirmed. In general terms obstruction is never complete, and responds to conservative treatment with gastric decompression and therapy with fluids and steroids, so surgery can be scheduled in the best possible conditions. In chronic situations, which should be avoided, a large prestenotic dilation of the intestine will appear, palpable as an abdominal tumour, which can cause severe nutrition-related problems.

Although there is no rule of thumb, we may affirm that surgery is indicated when two sub-occlusion flare-ups occur over a period of one year, requiring hospitalisation, or if the steroid treatment prescribed after a crisis cannot be discontinued at three months, or if dosages higher than 15 mg of Prednisone/day are required in the period three to six months following the occlusive crisis[26]. Delaying surgery in this particular case will lead to patient decline and further complications such as perforation, with an increase in surgery-related morbidity and mortality. In certain cases-single stenosis not larger than 8 cm in length, especially following post-op relapses-if the area is accessible with an endoscope, surgery can be replaced by endoscopic dilation either alone or associated with local steroid infiltration[27,28].

Abdominal abscess: Abdominal abscess is characterised by the presence of an inflammatory "mass" that always originates in micro-perforations of the intestinal wall; the mass may be an abscess, phlegmon or a combination of both. Excluding perianal disease, this complication is almost exclusive to the ileocecal region. More than a quarter of patients with ileocecal CD need to undergo surgery for this reason. Of these abscesses or phlegmons, 40% are associated with fistulae. In general terms, these are septic patients whose initial treatment, as well as broad spectrum anti-biotherapy and anaerobicides, involved CT or ultrasound-guided percutaneous drainage which allows improving patients' clinical status. The success rate of drainage exceeds 90% and avoids short-term surgery in 50% of patients[29], whereas those who will ultimately need to undergo an operation will only require single-stage elective surgery, under far more favourable conditions[30,31]. If even despite drainage, septic status shows no improvement, urgent surgery should be considered. Likewise, surgery is indicated in the event of multiple abscesses for which radiological drainage is not feasible. Normally when an inflammatory "mass" is detected through palpation, it means that the disease has attained such degree of severity that it is very unlikely to respond to medical treatment. Some authors have even reported initial improvement after percutaneous drainage of an abscess in small series of patients, but even so, surgery becomes necessary sooner or later[32].

Abdominal fistulae: Occur in their majority when CD

Table 2 Complications of Crohn's disease and treatment approaches

Complication	Treatment
Acute intestinal obstruction	Conservative
Recurrent intestinal obstruction	Elective surgery
Abdominal abscess	Percutaneous drainage Total recovery: medical therapy No improvement: urgent surgery Improvement: elective surgery
Abdominal fistula	Enteroenteric Asymptomatic: conservative Symptomatic: elective surgery Enterourologic o enterogynecologic Elective surgery Enterocutaneous Improve nutritional status and elective surgery
Free perforation or massive bleeding	Urgent surgery
Severe acute colitis	Medical therapy No response in 3-5 d: urgent surgery Obvious improvement: medical therapy
Toxic megacolon	Complication of acute colitis: urgent surgery Debut: medical therapy No response in 1-3 days: urgent surgery Response: elective surgery
Perianal disease	Individual treatment and interdisciplinary approach Surgery Failure medical therapy Symptomatic lesions Complications

is located in the ileocecal region. They may be internal, between neighbouring organs-the most frequent- or external, towards any cutaneous abdominal area-these are almost always post-operative. In the case of a spontaneous enterocutaneous fistula, or one that occurs following abscess drainage, there is consensus in so far that surgery is indicated because closure does not occur spontaneously[33,34], although some authors report good results in isolated cases with the use of Infliximab[35]. In the event of malnutrition and sepsis these have to be addressed before the operation since surgery on a fistula of this kind is not an urgent matter. If the fistula is post-operative it must be managed like that of a normal patient. If it occurs prematurely, within the first seven days post-op, in all likelihood it will require surgery; however, if it occurs at a later date but within sixty days one should adopt an expectant approach to treatment,-with enteral or parenteral nutrition-because they do tend to close provided there is no associated obstructive process. A special situation we have had to deal with sometimes, involves CD patients with long course of the disease, presenting with multiple enterocutaneous fistulae and with variable nutritional status, complexity of fistulae, degree of sepsis and food tolerance. In these cases, the decision whether to operate or not has to be assessed individually, on the basis of the factors mentioned.

Internal or enteroenteric fistulae are the most frequent, and alone they are not an indication for surgery, except if they exclude a large segment of the intestine and they are symptomatic, as in ileosigmoid or gastrocolic fistulae[36,37]. Less frequent are fistulae in the vagina, bladder, urethra and ureter; here the indication is scheduled surgery as the definitive solution[38].

Free perforation: May occur both in the case of CD in the small intestine or CD in the colon, although it is often associated with toxic colitis or megacolon[39]. Incidence is low, oscillating between 1%-3%. In this situation, peritonitis occurs, requiring resuscitation and urgent surgery. Some authors have described rupture of an abscess in the abdominal cavity, and obviously this would also require urgent surgery[40].

Massive bleeding: Occurs in 1%-13% of patients with CD. The first measure is to determine the site where haemorrhaging originates, *via* examination such as endoscopy, arteriography *etc*. The first option is conservative treatment, although probabilities of surgery exceed 60% as shown by the scant series of patients published in the literature[41,42]. If the bleeding can be stopped it is quite possible that further bleeding will occur, in which case surgery is recommended[43]. Ideally, it should be possible to ascertain the site of haemorrhaging perioperatively in order to avoid massive resections in this type of patient. To this aim, some authors have used selective arteriography injecting methylene blue tincture to highlight the bleeding intestinal segment[44].

Severe acute colitis: Here treatment criteria are similar to those for severe acute flare-ups in ulcerative colitis. Active treatment is required with intravenous fluids, thromboembolic prophylaxis, steroids and broad spectrum antibiotics, and considering sequential introduction of Infliximab in the event of steroid-resistance[45]. Urgent surgery will be conducted in the event of worsening of status in the 24-h period following treatment or within five days if there is no clear recovery[46]. Joint monitoring-by physicians and surgeons-and the approach outlined above will significantly reduce surgery-related mortality. By analogy with ulcerative colitis, we can affirm that predictive factors for surgery in the severe acute case should be identical to those in CD. Along these lines, Travis *et al*[47] noted that the presence of gas in three or more loops of the small intestine, and more than eight motions or stools daily, or a PCR above 45 mg/L predicts poor response to medical treatment, whereas Lindgren *et al*[48] conclude that persistently high body temperature, rectal bleeding or diarrhoea and elevated PCR increases the probability that surgery will be required. The presence of these factors should alert us to the need to conduct stricter surveillance.

Toxic megacolon: Occurs in 4%-6% of all patients with CD in the colon and although it can be controlled initially with medical treatment, in almost all cases delayed surgery should be considered since half of patients will present with a new megacolon flare-up and the other half will be poorly controlled. It occurs more frequently in the case of severe acute flare-up in extensive colitis; in this case, urgent surgery is indicated[49]. It is rarer as a debut manifestation and if this were the case, intensive medical treatment similar to that applicable to a severe flare-up should be initiated, but if there is no clear improvement, surgery should be indicated after 24-48 h. Immediate action-which reduces mortality rates to 2%-8%[50]-will prevent the onset of multiple organ dysfunction syndrome and perforation, which yields high mortality rates-close to 40%.

Perianal Crohn's disease: This is sometimes a complex problem which is hard to manage. Treatment must always be designed on a case-by-case basis and symptoms and complications should be treated surgically[51]. Here the major problems are perianal fistulae and sepsis. In the event of perianal abscess, urgent surgical drainage is always the approach to take. In an attempt to summarise such a complex problem, if the rectum presents no activity or is healthy and the fistulae are straightforward, scheduled fistulotomy is indicated; if they are complex medical treatment is preferable and rarely scheduled surgery for an advanced flap. If the rectum is diseased or there is genuine perianal sepsis, seton drainage appears to be the method favoured by most authors[51,52]. All septic foci should be drained and drainage seton sutures should be placed along with treatment using Infliximab, since results are better than with Infliximab alone[53].

In very severe cases that fail to respond to medical treatment, a relatively urgent ileostomy will be needed, in an attempt to control sepsis. Normally, if the rectum is affected and sepsis cannot be controlled, a proctectomy will be required[54].

WHAT OTHER REASONS MAY LEAD US TO OPT FOR SURGERY?

Dysplasia and cancer. Carcinoma-related risk in colon CD, with long course of the disease, seems to be similar to that associated to ulcerative colitis. A recent meta-analysis suggests that the relative risk of cancer in colon CD is equal to 4.5[55], so a monitoring programme including colonoscopy and biopsies will be beneficial *a priori*. The same criteria as in ulcerative colitis apply regarding monitoring and indication for prophylactic colectomy. So, surgery is indicated in the event of multi-focal low degree dysplasia, or high degree dysplasia confirmed by two different pathologists[56]. As with ulcerative colitis, the efficacy of monitoring programmes is controversial[57]. If CD is located in the ileum the risk of cancer is no higher than that of the normal population[55]. Under special circumstances risk appears to increase significantly, as is the case of patients diagnosed with CD before the age of 30 and with affected regions of the colon-here the relative risk is 20[58]. Once the carcinoma is established it may be difficult to determine why it triggers the symptoms that may be confounded with the disease, especially if it located in the ileum. Once diagnosed, surgery is called for following the same oncological principles that apply to any kind of neoplastic tumour. At any event, a significant percentage of cancers are found intraoperatively or during histological examination of the surgical specimen[26].

Retarded or stagnated growth occurs as a result of the complex interaction between nutritional status, inflammation, severity of the disease and genotype[59]. In addition, if treatment is based on the continued use of steroids, bone epiphysis will close prematurely. So poorly controlled CD, or that which requires steroid treatment, should be operated on as soon as possible, and in all cases before puberty.

WHEN IS QUALITY OF LIFE SO SEVERELY IMPAIRED THAT SURGERY IS NEEDED?

This question is extremely difficult to address given that no studies have come up with an answer. In addition, the evolution of CD with flare-ups, remissions, and exacerbations means that quality of life (QL) will vary over time. However, measuring QL is a useful tool to gain insight into response to pharmacological treatment, impact of the disease, assessment of healthcare services, contribution of surgical treatment, *etc*. One of the aims of treatment-whether medical or surgical-is to improve QL of CD patients. A plethora of factors impact QL, such as the fear of relapse, the fear of not enjoying good health and being unable to work, the impact of the disease on body image, infertility, *etc*. Moreover, determining when QL is so severely affected that surgery has be resorted to is also difficult to establish given that each patient may have very different perceptions in terms of how his or her own QL has altered. An organisational change is needed, with the introduction of health education programmes for patients[60], and dedicated units should be set up to provide comprehensive assistance[61]. This would improve the quality of healthcare assistance, while optimising healthcare resources[62]. According to the study conducted by Casellas *et al*[63], QL improves in a similar manner both if CD remission is attained *via* medical or surgical treatment, so it may be concluded that the important issue is to achieve remission, regardless of the method of choice.

In some situations, QL is severely affected. For instance, when CD is diffusely located in the small intestine, or when-regardless of location-it leads to nutritional problems or growth impairment, its impact on patients' social life is so great that surgery will have to be considered[64,65]. In general terms, the factors that patients rank highest as life-limiting factors are: number of motions or stools, appetite and dietary options, sleep, depending on others, mental health and psychosexual morbidity. To assess and measure these factors, a QL questionnaire, specifically designed for inflammatory bowel disease, is used[66]. The score obtained is closely linked to flare-ups of the disease, so that during remission phases patients refer maximum scores-similar to scores during phases when the disease is under control-whereas greater or lesser activity alters scores, worsening QL proportionally to the magnitude of the flare-up[63].

CD and ulcerative colitis should not be assessed on the basis of clinical criteria alone. We should use a QL questionnaire to understand patients' genuine status of wellbeing. All factors-both clinical and QL related-taken together will help us decide whether to continue with medical treatment or to indicate surgery.

CONCLUSION

Deciding whether to operate on a patient with CD may be relatively easy although in a variety of cases it can be a difficult call. In these complex cases, an organization based on multi-disciplinary teams, while also bearing in mind patients' opinions, plays an extremely relevant role in order to attain the best possible outcomes. The goal of all those involved is to achieve better quality of life for patients. Hence, surgery should be seen as another treatment option and not as an approach to avoid at all costs. Deciding when is the best time for surgery will essentially depend on a number of factors such as medical "untreatability", symptom severity, types of complications, drug-related adverse effects and adequate risk/benefit assessment of surgery at a given

point in time. An overall view of clinical status along with analysis of each of these factors from an integral standpoint will enable us to adopt the most appropriate decision.

REFERENCES

1 **Truelove SC**, Witts LJ. Cortisone in ulcerative colitis; preliminary report on a therapeutic trial. *Br Med J* 1954; **2**: 375-378
2 **Rutgeerts PJ**. An historical overview of the treatment of Crohn's disease: why do we need biological therapies? *Rev Gastroenterol Disord* 2004; **4** Suppl 3: S3-S9
3 **van Dullemen HM**, van Deventer SJ, Hommes DW, Bijl HA, Jansen J, Tytgat GN, Woody J. Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). *Gastroenterology* 1995; **109**: 129-135
4 **Bernell O**, Lapidus A, Hellers G. Risk factors for surgery and recurrence in 907 patients with primary ileocaecal Crohn's disease. *Br J Surg* 2000; **87**: 1697-1701
5 **Nicholls J**. The inflammatory bowel disease unit and the multidisciplinary team meeting. *Colorectal Dis* 2007; **9**: 477
6 **Lichtenstein GR**, Yan S, Bala M, Blank M, Sands BE. Infliximab maintenance treatment reduces hospitalizations, surgeries, and procedures in fistulizing Crohn's disease. *Gastroenterology* 2005; **128**: 862-869
7 **Colombel JF**, Loftus EV Jr, Tremaine WJ, Pemberton JH, Wolff BG, Young-Fadok T, Harmsen WS, Schleck CD, Sandborn WJ. Early postoperative complications are not increased in patients with Crohn's disease treated perioperatively with infliximab or immunosuppressive therapy. *Am J Gastroenterol* 2004; **99**: 878-883
8 **Marchal L**, D'Haens G, Van Assche G, Vermeire S, Noman M, Ferrante M, Hiele M, Bueno De Mesquita M, D'Hoore A, Penninckx F, Rutgeerts P. The risk of post-operative complications associated with infliximab therapy for Crohn's disease: a controlled cohort study. *Aliment Pharmacol Ther* 2004; **19**: 749-754
9 **Shen B**. Impact of preoperative infliximab use on postoperative infectious complications in ulcerative colitis: the price we have to pay? *Inflamm Bowel Dis* 2008; **14**: 1019-1021
10 **Selvasekar CR**, Cima RR, Larson DW, Dozois EJ, Harrington JR, Harmsen WS, Loftus EV Jr, Sandborn WJ, Wolff BG, Pemberton JH. Effect of infliximab on short-term complications in patients undergoing operation for chronic ulcerative colitis. *J Am Coll Surg* 2007; **204**: 956-962; discussion 962-963
11 **Peyrin-Biroulet L**, Deltenre P, de Suray N, Branche J, Sandborn WJ, Colombel JF. Efficacy and safety of tumor necrosis factor antagonists in Crohn's disease: meta-analysis of placebo-controlled trials. *Clin Gastroenterol Hepatol* 2008; **6**: 644-653
12 **Farmer RG**, Whelan G, Fazio VW. Long-term follow-up of patients with Crohn's disease. Relationship between the clinical pattern and prognosis. *Gastroenterology* 1985; **88**: 1818-1825
13 **Michelassi F**, Balestracci T, Chappell R, Block GE. Primary and recurrent Crohn's disease. Experience with 1379 patients. *Ann Surg* 1991; **214**: 230-238; discussion 238-240
14 **Hanauer SB**, Meyers S. Management of Crohn's disease in adults. *Am J Gastroenterol* 1997; **92**: 559-566
15 **Lopez-San Roman A**, Obrador A, Fortun J, Munoz P, Gassull MA. [Recommendations on tuberculosis and treatment of inflammatory bowel disease with infliximab. 2006 update] *Gastroenterol Hepatol* 2006; **29**: 81-84
16 **Faubion WA Jr**, Loftus EV Jr, Harmsen WS, Zinsmeister AR, Sandborn WJ. The natural history of corticosteroid therapy for inflammatory bowel disease: a population-based study. *Gastroenterology* 2001; **121**: 255-260
17 **Tung J**, Loftus EV Jr, Freese DK, El-Youssef M, Zinsmeister AR, Melton LJ 3rd, Harmsen WS, Sandborn WJ, Faubion WA Jr. A population-based study of the frequency of corticosteroid resistance and dependence in pediatric patients with Crohn's disease and ulcerative colitis. *Inflamm Bowel Dis* 2006; **12**: 1093-1100
18 **Nordgren SR**, Fasth SB, Oresland TO, Hulten LA. Long-term follow-up in Crohn's disease. Mortality, morbidity, and functional status. *Scand J Gastroenterol* 1994; **29**: 1122-1128
19 **Edna TH**, Bjerkeset T, Skreden K. Abdominal surgery for Crohn's disease during 30 years in Middle Norway. *Hepatogastroenterology* 2004; **51**: 481-484
20 **Sands BE**, Arsenault JE, Rosen MJ, Alsahli M, Bailen L, Banks P, Bensen S, Bousvaros A, Cave D, Cooley JS, Cooper HL, Edwards ST, Farrell RJ, Griffin MJ, Hay DW, John A, Lidofsky S, Olans LB, Peppercorn MA, Rothstein RI, Roy MA, Saletta MJ, Shah SA, Warner AS, Wolf JL, Vecchio J, Winter HS, Zawacki JK. Risk of early surgery for Crohn's disease: implications for early treatment strategies. *Am J Gastroenterol* 2003; **98**: 2712-2718
21 **Morimoto N**, Kato J, Kuriyama M, Fujimoto T, Nasu J, Miyaike J, Morita T, Okada H, Suzuki S, Shiode J, Yamamoto H, Sakaguchi K, Shiratori Y. Risk factors and indications for first surgery in Crohn's disease patients. *Hepatogastroenterology* 2007; **54**: 2011-2016
22 **Yamamoto T**, Allan RN, Keighley MR. Risk factors for intra-abdominal sepsis after surgery in Crohn's disease. *Dis Colon Rectum* 2000; **43**: 1141-1145
23 **Alves A**, Panis Y, Bouhnik Y, Pocard M, Vicaut E, Valleur P. Risk factors for intra-abdominal septic complications after a first ileocecal resection for Crohn's disease: a multivariate analysis in 161 consecutive patients. *Dis Colon Rectum* 2007; **50**: 331-336
24 **Scott NA**, Hughes LE. Timing of ileocolonic resection for symptomatic Crohn's disease--the patient's view. *Gut* 1994; **35**: 656-657
25 **Higuero T**, Merle C, Thiefin G, Coussinet S, Jolly D, Diebold MD, Zeitoun P, Cadiot G. Jejunoileal Crohn's disease: a case-control study. *Gastroenterol Clin Biol* 2004; **28**: 160-166
26 **Lledo S**. Crohn's Disease. Clinical Guides of the Spanish Association of Surgeons. 1th ed. Madrid: Aran Editions SA, 2000: 205-230
27 **Ferlitsch A**, Reinisch W, Puspok A, Dejaco C, Schillinger M, Schofl R, Potzi R, Gangl A, Vogelsang H. Safety and efficacy of endoscopic balloon dilation for treatment of Crohn's disease strictures. *Endoscopy* 2006; **38**: 483-487
28 **Ajlouni Y**, Iser JH, Gibson PR. Endoscopic balloon dilatation of intestinal strictures in Crohn's disease: safe alternative to surgery. *J Gastroenterol Hepatol* 2007; **22**: 486-490
29 **Garcia JC**, Persky SE, Bonis PA, Topazian M. Abscesses in Crohn's disease: outcome of medical versus surgical treatment. *J Clin Gastroenterol* 2001; **32**: 409-412
30 **Gervais DA**, Hahn PF, O'Neill MJ, Mueller PR. Percutaneous abscess drainage in Crohn disease: technical success and short- and long-term outcomes during 14 years. *Radiology* 2002; **222**: 645-651
31 **Lee H**, Kim YH, Kim JH, Chang DK, Son HJ, Rhee PL, Kim JJ, Paik SW, Rhee JC. Nonsurgical treatment of abdominal or pelvic abscess in consecutive patients with Crohn's disease. *Dig Liver Dis* 2006; **38**: 659-664
32 **Neufeld D**, Keidar A, Gutman M, Zissin R. Abdominal wall abscesses in patients with Crohn's disease: clinical outcome. *J Gastrointest Surg* 2006; **10**: 445-449
33 **Hill GL**, Bourchier RG, Witney GB. Surgical and metabolic management of patients with external fistulas of the small intestine associated with Crohn's disease. *World J Surg* 1988; **12**: 191-197
34 **Poritz LS**, Gagliano GA, McLeod RS, MacRae H, Cohen Z. Surgical management of entero and colocutaneous fistulae in Crohn's disease: 17 year's experience. *Int J Colorectal Dis* 2004; **19**: 481-485; discussion 486
35 **Present DH**, Rutgeerts P, Targan S, Hanauer SB, Mayer L,

van Hogezand RA, Podolsky DK, Sands BE, Braakman T, DeWoody KL, Schaible TF, van Deventer SJ. Infliximab for the treatment of fistulas in patients with Crohn's disease. *N Engl J Med* 1999; **340**: 1398-1405
36 **McNamara MJ**, Fazio VW, Lavery IC, Weakley FL, Farmer RG. Surgical treatment of enterovesical fistulas in Crohn's disease. *Dis Colon Rectum* 1990; **33**: 271-276
37 **Khanna MP**, Gordon PH. Gastrocolic fistulization in Crohn's disease: a case report and a review of the literature. *Can J Surg* 2000; **43**: 53-56
38 **Solem CA**, Loftus EV Jr, Tremaine WJ, Pemberton JH, Wolff BG, Sandborn WJ. Fistulas to the urinary system in Crohn' s disease: clinical features and outcomes. *Am J Gastroenterol* 2002; **97**: 2300-2305
39 **Hurst RD**, Molinari M, Chung TP, Rubin M, Michelassi F. Prospective study of the features, indications, and surgical treatment in 513 consecutive patients affected by Crohn's disease. *Surgery* 1997; **122**: 661-667; discussion 667-668
40 **Mowatt JI**, Burnstein MJ. Free perforation of small bowel Crohn's disease: A case report and review. *Can J Gastroenterol* 1993; **7**: 300-302
41 **Cirocco WC**, Reilly JC, Rusin LC. Life-threatening hemorrhage and exsanguination from Crohn's disease. Report of four cases. *Dis Colon Rectum* 1995; **38**: 85-95
42 **Kostka R**, Lukas M. Massive, life-threatening bleeding in Crohn's disease. *Chir Belg* 2005; **105**: 168-174
43 **Veroux M**, Angriman I, Ruffolo C, Barollo M, Buffone A, Madia C, Caglia P, Fiamingo P, D'Amico D. Severe gastrointestinal bleeding in Crohn's disease. *Ann Ital Chir* 2003; **74**: 213-215; discussion 216
44 **Remzi FH**, Dietz DW, Unal E, Levitin A, Sands MJ, Fazio VW. Combined use of preoperative provocative angiography and highly selective methylene blue injection to localize an occult small-bowel bleeding site in a patient with Crohn's disease: report of a case. *Dis Colon Rectum* 2003; **46**: 260-263
45 **Travis SP**, Stange EF, Lemann M, Oresland T, Chowers Y, Forbes A, D'Haens G, Kitis G, Cortot A, Prantera C, Marteau P, Colombel JF, Gionchetti P, Bouhnik Y, Tiret E, Kroesen J, Starlinger M, Mortensen NJ. European evidence based consensus on the diagnosis and management of Crohn's disease: current management. *Gut* 2006; **55** Suppl 1: i16-i35
46 **Fazio VW**, Wu JS. Surgical therapy for Crohn's disease of the colon and rectum. *Surg Clin North Am* 1997; **77**: 197-210
47 **Travis SP**, Farrant JM, Ricketts C, Nolan DJ, Mortensen NM, Kettlewell MG, Jewell DP. Predicting outcome in severe ulcerative colitis. *Gut* 1996; **38**: 905-910
48 **Lindgren SC**, Flood LM, Kilander AF, Lofberg R, Persson TB, Sjodahl RI. Early predictors of glucocorticosteroid treatment failure in severe and moderately severe attacks of ulcerative colitis. *Eur J Gastroenterol Hepatol* 1998; **10**: 831-835
49 **Berg DF**, Bahadursingh AM, Kaminski DL, Longo WE. Acute surgical emergencies in inflammatory bowel disease. *Am J Surg* 2002; **184**: 45-51
50 **Sheth SG**, LaMont JT. Toxic megacolon. *Lancet* 1998; **351**: 509-513
51 **Sandborn WJ**, Fazio VW, Feagan BG, Hanauer SB. AGA technical review on perianal Crohn's disease. *Gastroenterology* 2003; **125**: 1508-1530
52 **Caprilli R**, Gassull MA, Escher JC, Moser G, Munkholm P, Forbes A, Hommes DW, Lochs H, Angelucci E, Cocco A, Vucelic B, Hildebrand H, Kolacek S, Riis L, Lukas M, de Franchis R, Hamilton M, Jantschek G, Michetti P, O'Morain C, Anwar MM, Freitas JL, Mouzas IA, Baert F, Mitchell R, Hawkey CJ. European evidence based consensus on the diagnosis and management of Crohn's disease: special situations. *Gut* 2006; **55** Suppl 1: i36-i58
53 **Regueiro M**, Mardini H. Treatment of perianal fistulizing Crohn's disease with infliximab alone or as an adjunct to exam under anesthesia with seton placement. *Inflamm Bowel Dis* 2003; **9**: 98-103
54 **Regimbeau JM**, Panis Y, Marteau P, Benoist S, Valleur P. Surgical treatment of anoperineal Crohn's disease: can abdominoperineal resection be predicted? *J Am Coll Surg* 1999; **189**: 171-176
55 **Canavan C**, Abrams KR, Mayberry J. Meta-analysis: colorectal and small bowel cancer risk in patients with Crohn's disease. *Aliment Pharmacol Ther* 2006; **23**: 1097-1104
56 **Zisman TL**, Rubin DT. Colorectal cancer and dysplasia in inflammatory bowel disease. *World J Gastroenterol* 2008; **14**: 2662-2669
57 **Eaden JA**, Ward BA, Mayberry JF. How gastroenterologists screen for colonic cancer in ulcerative colitis: an analysis of performance. *Gastrointest Endosc* 2000; **51**: 123-128
58 **Ekbom A**, Helmick C, Zack M, Adami HO. Increased risk of large-bowel cancer in Crohn's disease with colonic involvement. *Lancet* 1990; **336**: 357-359
59 **Shamir R**, Phillip M, Levine A. Growth retardation in pediatric Crohn's disease: pathogenesis and interventions. *Inflamm Bowel Dis* 2007; **13**: 620-628
60 **Waters BM**, Jensen L, Fedorak RN. Effects of formal education for patients with inflammatory bowel disease: a randomized controlled trial. *Can J Gastroenterol* 2005; **19**: 235-244
61 **Fontanet G**, Casellas F, Malagelada JR. [The Crohn-Colitis care unit: analysis of the first 3 years of activity] *Gac Sanit* 2004; **18**: 483-485
62 **Mawdsley JE**, Irving PM, Makins RJ, Rampton DS. Optimizing quality of outpatient care for patients with inflammatory bowel disease: the importance of specialist clinics. *Eur J Gastroenterol Hepatol* 2006; **18**: 249-253
63 **Casellas F**, Lopez-Vivancos J, Badia X, Vilaseca J, Malagelada JR. Influence of inflammatory bowel disease on different dimensions of quality of life. *Eur J Gastroenterol Hepatol* 2001; **13**: 567-572
64 **Tan WC**, Allan RN. Diffuse jejunoileitis of Crohn's disease. *Gut* 1993; **34**: 1374-1378
65 **Norman K**, Kirchner H, Lochs H, Pirlich M. Malnutrition affects quality of life in gastroenterology patients. *World J Gastroenterol* 2006; **12**: 3380-3385
66 **Mitchell A**, Guyatt G, Singer J, Irvine EJ, Goodacre R, Tompkins C, Williams N, Wagner F. Quality of life in patients with inflammatory bowel disease. *J Clin Gastroenterol* 1988; **10**: 306-310

S- Editor Xiao LL **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5540

World J Gastroenterol 2008 September 28; 14(36): 5540-5548
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Javier P Gisbert, Professor; Fernando Gomollón, Dr., MD, PhD, *Series Editors*

Postoperative Crohn's disease recurrence: A practical approach

Pilar Nos, Eugeni Domènech

Pilar Nos, Eugeni Domènech, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (Ciberehd), Spain
Pilar Nos, Servicio de Medicina Digestiva. Hospital Universitario La Fe, Valencia, Spain
Eugeni Domènech, Servicio de Aparato Digestivo, Hospital Universitari Germans Trias y Pujol, Badalona (Catalonia), Spain
Author contributions: Nos P and Domènech E contributed equally to this work.
Correspondence to: Pilar Nos, MD, PhD, Gastroenterology Unit, Hospital Universitario La Fe. Avenida Campanar 21. 46009 Valencia, Spain. pnosm@meditex.es
Telephone: +34-96-3862700 Fax: +34-96-1973065
Received: August 18, 2008 Revised: August 26, 2008
Accepted: September 3, 2008
Published online: September 28, 2008

Abstract

Crohn's disease is a chronic inflammatory condition that may involve any segment of the gastrointestinal tract. Although several drugs have proven efficacy in inducing and maintaining disease in remission, resectional surgery remains as a cornerstone in the management of the disease, mainly for the treatment of its stenosing and penetrating complications. However, the occurrence of new mucosal (endoscopic) lesions in the neoterminal ileum early after surgery is almost constant, it is followed in the mid-term by clinical symptoms and, in a proportion of patients, repeated intestinal resections are required. Pathogenesis of postoperative recurrence (POR) is not fully understood, but luminal factors (commensal microbes, dietary antigens) seem to play an important role, and environmental and genetic factors may also have a relevant influence. Many studies tried to identify clinical predictors for POR with heterogeneous results, and only smoking has repeatedly been associated with a higher risk of POR. Ileocolonoscopy remains as the gold standard for the assessment of appearance and severity of POR, although the real usefulness of the available endoscopic score needs to be revisited and alternative techniques are emerging. Several drugs have been evaluated to prevent POR with limited success. Smoking cessation seems to be one of the more beneficial therapeutic measures. Aminosalicylates have only proved to be of marginal benefit, and they are only used in low-risk patients. Nitroimidazolic antibiotics, although efficient, are associated with a high rate of intolerance and might induce irreversible side effects when used for a long-term. Thiopurines are not widely used after ileocecal resection, maybe because some concerns in giving immunomodulators in asymptomatic patients still remain. In the era of biological agents and genetic testing, a well-established preventive strategy for POR is still lacking, and larger studies to identify good clinical, serological, and genetic predictors of early POR as well as more effective drugs (or drug combinations) are needed.

Key words: Crohn's disease; Postoperative recurrence; Smoking; Aminosalicylates; Nitroimidazolic antibiotics; Thiopurines

Nos P, Domènech E. Postoperative Crohn's disease recurrence: A practical approach. *World J Gastroenterol* 2008; 14(36): 5540-5548 Available from: URL: http://www.wjgnet.com/1007-9327/14/5540.asp DOI: http://dx.doi.org/10.3748/wjg.14.5540

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory intestinal disease that frequently requires surgical bowel resection due to refractory activity in spite of medical treatment or complications such stenosis or fistulae. Approximately, 80% of patients need intestinal resection during their lifetime[1]. Nevertheless, resectional surgery is not definitively curative and disease frequently reappears at the site of the anastomosis and/or proximally. The term recurrence defines the presence of new objective intestinal lesions of CD after a radical or curative resection. Radical resection is considered as a complete excision with a sufficient length (5-15 cm) of macroscopic normal margins from both ends of the diseased bowel segment, together with local lymph nodes, to remove the whole involved gut[2]. Although some alternative surgical techniques such as stricturoplasty are emerging, according to the European consensus on definitions and diagnosis of CD, *recurrence* is best used to define the reappearance of lesions after surgical resection while *relapse* refers to

the reappearance of symptoms[3]. Early studies by the Leuven group showed a symptomatic recurrence rate of 20%-30% one year after ileal or ileocolonic resection, with a 10% increase in each subsequent year[4]. Moreover, the same group reported an endoscopic and histological recurrence rate of 72% within one year of the operation[5]. These key studies marked the basis of knowledge about postoperative recurrence (POR) in CD.

DEFINITION OF POSTOPERATIVE RECURRENCE

POR rates vary according to the criteria used for its definition: clinical, endoscopic, morphologic, or surgical (Figure 1). *Morphological* POR refers to the occurrence of new lesions assessed by imaging techniques; since radiological explorations are less accurate in detecting small or superficial mucosal lesions, morphological POR has been replaced by *endoscopic* POR. The occurrence of new mucosal (endoscopic) lesions in the neoterminal ileum early after surgery is almost constant, and it is followed in the mid-term by clinical symptoms and, in a proportion of patients repeated intestinal resections are required. As recently reviewed, *clinical* POR rates, assessed by conventional clinical activity indexes, varies between 17%-55% at 5 years, 32%-76% at 10 years and 72%-73% at 20 years[6], whereas *surgical* POR rates (patients requiring re-operation) are of 11%-32% at 5 years, 20%-44% at 10 years and 46%-55% at 20 years[6-8].

Although the real relevance of endoscopic POR is yet to be known, the emergence of mucosal healing as one of the most important therapeutic targets in CD increases the interest for endoscopic POR prevention as the main goal after resectional surgery, at least in the setting of clinical trials.

NATURAL HISTORY OF POSTOPERATIVE RECURRENCE

The physiopathology of POR still remains unknown and its course is unpredictable. Reasonably, pathogenic mechanisms of recurrence should be the same of CD itself, but even a more relevant role of luminal microorganisms is suspected. It is known that if ileocolonic anastomosis is protected by a proximal ileostomy and no faecal stream passes through the anastomosis, mucosal lesions rarely appear[9]. The infusion of intestinal luminal contents into the excluded ileum results in inflammatory mucosal changes, as shown in a classical interesting study by the Leuven group in 3 CD patients who were studied by histopathology and electron microscopy[10]. The authors demonstrated that, as soon as 8 d after the reinfusion of intestinal fluids, focal infiltration of mononuclear cells, eosinophils, and polymorphonuclear cells in the lamina propria, small vessels, and epithelium appeared in the previously normal excluded neoterminal ileum. Furthermore and considering this hypothesis, the same group showed some years later that nitroimidazolic antibiotics are efficient in preventing POR, as will be later discussed[11,12].

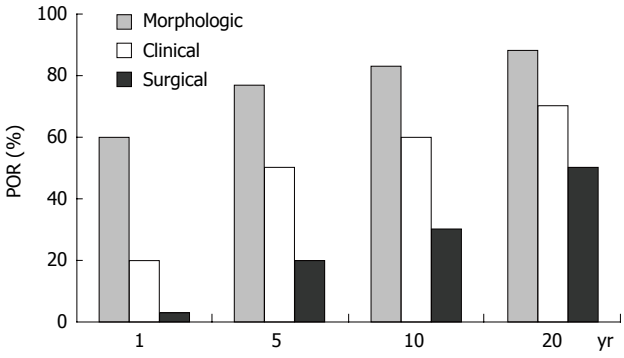


Figure 1 Mean rates of postoperative recurrence of Crohn's disease assessed by clinical relapse, imaging techniques (morphologic), and re-operation (surgical).

The onset of disease recurrence begins by the appearance of new mucosal lesions (morphologic recurrence) that can be assessed by means of radiological or endoscopic techniques and the evolution of these lesions mimics the previous phenotypic pattern in every particular patient, as shown when the indication for reoperation is evaluated[13].

Ileocolonoscopy is a very useful diagnostic tool for examination of neoileum after ileocolonic resection and it determine the presence or absence of new lesions. There is a sequence of endoscopic lesions in the anastomotic and preanastomotic regions followed by the development of clinical symptoms. The earliest lesions detected are aphthoid ulcers and it is possible to detect a disruption of capillary basement membrane with haemorrhaging and rails of fibrinogen in the surrounding lamina propria empting into the bowel lumen[14]. The presence of extensive lesions in the neoileum by endoscopic examination within some months after surgery predicts a rapid evolution to recurrent symptoms and eventual complications, and there is evidence that these early lesions do not originate from microscopic inflammation prior to surgery[15].

RISK FACTOR FOR POSTOPERATIVE RECURRENCE

Even though the determinants for disease recurrence remain as speculative as for the initial CD lesions, several risk factors for POR have been reported. The identification of such factors is very important in order to select those patients who may benefit the most from active preventive measures. Clinical parameters that influenced outcome are listed in Table 1, and they can be grouped in those related to the patient's and disease's characteristics, and those that depend on surgical questions. However, it has to be pointed out that most studies evaluating risk factors for POR are retrospective, and that prospective RCTs designed to evaluate different preventive treatment for POR (mostly with a smaller sample size than retrospective studies) have not confirmed these results.

Table 1 Clinical parameters that may influence the development of postoperative recurrence

Age (at onset of disease)
Gender
Family history of CD
Smoking
Duration of disease before surgery
Location
Genetic factors
Involvement of disease at the resection margin
Indication for surgery
Length of resected bowel
Anastomotic technique
Histological findings

Patient and disease characteristics

Smoking: It is known that smoking habits play an important role in the pathogenesis of inflammatory bowel diseases and it is maybe the only environmental factor extensively evaluated in both CD and ulcerative colitis. The effect of smoking and its cessation on disease location, behaviour, and disease progression in CD has been recently analyzed through a systematic *Medline* research[16]. The conclusions were that smoking duplicates the risk of recurrence after surgery and smoking cessation should be encouraged as one of the most useful measures for the management of the disease. Cigarette smoking may not be only a risk factor for development of CD but also may influence disease activity following surgery[17]. The risk of surgery as well as the risk for further resections during disease course is increased among smokers in most studies. The first study that analyzed the effect of cigarette smoking on POR (defined by the need for repeated surgery) in a historical cohort of 174 CD patients who had been operated on, found that the rate of reoperation after 5 and 10 years was significantly different for smokers (36% and 70%, respectively) that for nonsmokers (20% and 41%, respectively). When patients were stratified by gender, this increased risk was more apparent in women[18]. In another study, significant differences in surgical recurrence rates between smokers and non-smokers and for both male and female were also found. Five and 10-year recurrence rates were 43% and 64%, respectively, for smokers, with corresponding figures for non-smokers of 26% and 33%[19]. In addition, it has been shown that macroscopic lesions at the neoileum are present in 70% of smokers one year after surgery, for only 35% in non-smokers and 27% in ex-smokers. Finally, an Italian study correlated the risk of recurrence to the severity of smoking habits, with a more marked increase in the risk of symptomatic POR among heavy smokers than among mild smokers[20]. No interventional study evaluating the impact of smoking cessation has been performed and it is unlikely to occur.

Genetic factors: *NOD2/CARD15* gene variants play an important role in the susceptibility to CD. Studies of genotype-phenotype relationship suggest that these variants are associated with ileal location and the development of intestinal strictures. In a study evaluating the influence of NOD2/CARD15 variants on CD outcomes including 170 patients (70 of who had been operated on), Alvarez-Lobos and co-workers found that those patients carrying gene variants had a higher risk of surgical POR[21]. Recently, in another Mediterranean area study including a total of 110 patients, although these findings were not completely reproduced, those patients the L1007fs mutation also had higher rates of reoperation[22].

Disease phenotype-related factors: Early onset of CD might be associated to a higher recurrence rate. However, results were controversial in the largest series in which this item was evaluated, and age at diagnosis should not be considered as a definitive predictive factor. Similar results have been found regarding family history of CD[23].

Clinical parameters that have been associated to postoperative outcomes are preoperative disease activity[14] and location of disease at diagnosis[24]. When recurrence was defined by the need for reoperation, patients with disease affecting only large bowel had a higher risk when compared to those with isolated small bowel involvement or ileal and colonic disease[24]. The presence of jejunal CD is associated with a higher rate of early disease recurrence as compared to ileocecal disease, but long-term recurrence rates do not differ significantly[25]. In a European study, phenotype at diagnosis had a strong predictive value for clinical relapse or recurrence, upper gastrointestinal disease being the most important positive predictor; although the study did not focus on POR, it seems that these results agree with those obtained in the Japanese study[13].

Perforating disease seems to be associated with a more rapid or aggressive POR. In a recent meta-analysis, a trend for earlier reoperation in patients who were firstly operated on for perforating complications as compared to patients operated on for non-perforating indications was found[26]. However, there was a significant heterogeneity among the included studies, limiting the value of the results.

Histopathological factors: Although microscopic involvement of the resection margins was not associated to a higher risk of surgical POR in a RCT in which patients were randomized to have a limited or an extended resection from the macroscopically affected margin[27], recent data suggest that the presence of myenteric plexitis in the resection margins may be a relevant predictor of POR. Ferrante and colleagues studied the surgical specimen and the clinical and endoscopic outcome of 59 patients who were operated on and included in the placebo arm of two RCTs for POR prevention. Those patients with myenteric plexitis in the surgical specimen had significant higher rates of endoscopic POR at both 3 and 12 mo after surgery[28].

Surgery-related factors: Several prognostic factors related to surgical aspects have been investigated. The length of resected bowel does not appear to be a predis-

posing factor to POR[29,30]. As long as recurrence rate is unaffected by the presence of microscopic active disease at the surgical margins, the current surgical approach is that only macroscopically affected tissue must be removed in order to minimize the risk of a further short bowel syndrome.

Based on data suggesting that anastomotic stapling techniques result in a localized ischemic injury that might increase disease recurrence, the studies evaluating this topic show different results[31]. Firm conclusions are difficult to achieve because of the retrospective nature and heterogeneity of studies. Recently, a meta-analysis evaluated the incidence of recurrence and indication for reoperation between patients undergoing end-to-end anastomosis and patients with another anastomotic configuration after intestinal resection[32]. Eight studies (only two were prospective and randomized) reported data from 661 patients who underwent 712 anastomosis. Almost half the patients had end-to-end anastomosis and recurrence rate was significantly reduced in those patients who had other anastomotic configurations.

Laparoscopic resection is more adequate in CD since patients are usually young, with cosmetic and working concerns. This technique can be performed safely and effectively and it shows a faster resumption of oral feeds, lesser postoperative pain, and shorter hospital stay when compared to open surgery. No differences were found in the recurrence rate or the disease-free interval when laparoscopic technique was compared with open surgery in a retrospective analysis of 61 consecutive patients performed in a tertiary Canadian centre[33]. Similar results were obtained in a retrospective study involving a larger cohort of 113 patients[34]. Recently, long-term results of laparoscopic-assisted or open ileocolic resection for Crohn's disease have been compared in terms of surgical recurrence, quality of life, body image, and cosmetics[35]. Although surgical recurrence and quality of life were comparable, a better body image and cosmetic appearance favored laparoscopy.

ASSESSMENT OF POSTOPERATIVE RECURRENCE

Clinical monitoring should be the easiest way to assess the occurrence of POR (clinical recurrence). However, ileocecal resection (especially in cases of extensive ileal resection) may be associated with the development of abdominal symptoms such as diarrhoea or abdominal cramps secondary to bacterial overgrowth, bile salts malabsorption, or short bowel syndrome, and these might be misinterpreted as a disease recurrence. Unfortunately, no clinical activity index has been validated for patients with previous ileocolonic resection, and the value of the changes in the CDAI or the Harvey-Bradshaw indices in this clinical setting is still to be established.

Histology (histological recurrence) could also be used to diagnose POR, but microscopic inflammatory mucosal changes have been described to occur early after surgery[10] and the relationship with the timing or severity of clinical recurrence has not been adequately evaluated.

As mentioned before, Rutgeerts and colleagues showed that the severity of mucosal lesions correlated with the likeliness to develop clinical recurrence. Although these lesions should be detected radiologically or endoscopically, the higher accuracy of endoscopy to detect small lesions makes ileocolonoscopy the gold standard for POR assessment. Moreover, these authors proposed an endoscopic index to specifically describe and grade POR[15]. Since then, the so called Rutgeerts' endoscopic index has been used to define the primary endpoint in most RCTs evaluating preventive strategies for POR performed in the last decade, as a surrogate for clinical recurrence. This index scores recurrent ileal lesions as i0 (no lesions), i1 (less than 5 aphthous lesions), i2 (more than 5 aphthous lesions with normal mucosa between the lesions, or skip areas of larger lesions or lesions confined to < 1 cm from the ileocolonic anastomosis), i3 (diffuse aphthous ileitis with diffusely inflamed mucosa), and i4 (diffuse inflammation with larger ulcers, nodules, and/or narrowing). Although POR was initially defined as a Rutgeerts score $\geqslant$ i1, most RCTs use i2 as the cut-off to define endoscopic recurrence and i3 for defining "significant" or "severe" endoscopic recurrence, suggesting that i1 and i2 are of low value to predict clinical recurrence in the short-term.

The meaning of those endoscopic lesions confined to the anastomosis is still under debate. These lesions, although included in the Rutgeerts index as i2, have been questioned by some authors who claimed that they could be related to staples and/or ischemia[36]. In a recent prospective study, when considered apart, these lesions did not become symptomatic and showed a low probability to progress to more severe endoscopic lesions in the mid-term (22% and 42% after 3 and 5 years of surgery, respectively)[37]. Certainly, some authors already had not considered these lesions as disease recurrence in the setting of clinical trials[38]. Moreover, a careful reading of the initial paper by Rutgeerts *et al* in which the index was first defined, shows that whereas the clinical outcome was markedly different in patients with an endoscopic index greater or lesser than i2, patients with i2 had an unpredictable outcome. Therefore, the possibility that these lesions may be a confounding factor rather than a 'true' disease recurrence should be kept in mind, and their clinical relevance should be revisited.

Finally, although POR usually occurs at the neoterminal ileum after ileocecal resection, some patients (mostly after segmentary colonic resections) may develop mucosal lesions in the colon. This is not considered in the Rutgeerts score, and in a recently published RCT a modified version of this index including recurrent lesions in the colon has been used[39]. For all these reasons, and despite a Rutgeerts' index of $\geqslant$ 3 has been strongly associated with a poor prognosis, many authors do not recommend its use neither in clinical practice nor in the setting of RCTs since data supporting its clinical relevance are still lacking[36].

Table 2 Characteristics of available preventive measures of post-operative recurrence

Preventive measure	Pros	Cons
Smoking cessation	Many colateral beneficial effects Synergistic effect with thiopurines?	Lack of specifically designed prospective studies and RCTs (data coming from retrospective studies) Low adherence
Enteral nutrition	Excellent safety profile	Preliminary results Adherence?
5ASA compounds	Safety profile	Marginal benefit (high number needed to treat –NNT-)
Nitroimidazolic antibiotics	Proven efficacy in RCTs	High rate of intolerance/side effects (not applicable in a long-term basis)
Thiopurines	Strong positive results in open and retrospective studies Proven efficacy in RCT when combined with metronidazole Particularly efficient after second resection? Synergistic effect with smoking cessation?	Safety profile/intolerance Efficacy in monotherapy not conclusive (RCTs)

Alternative non-invasive imaging techniques to assess POR have been evaluated in recent years. Wireless capsule endoscopy (WCE) has potential advantages over ileocolonoscopy: it is more comfortable and better tolerated by patients, it does not need sedation, and it is less influenced by technical limitations (impossibility to access the neoterminal ileum). In small-sized controlled trials, WCE demonstrated a similar efficacy to ileocolonoscopy to detect mucosal lesions in the neoterminal ileum[38,40,41]. Furthermore, WCE is able to detect mucosal lesions one year after surgery in 60%-70% of patients in upper segments of the gastrointestinal tract that are outside the scope of ileocolonoscopy. The significance of these lesions is uncertain; whereas some authors defend that they must be considered as disease recurrence, the lack of a careful assessment of the upper gastrointestinal tract prior to surgery makes impossible to know if they were already present before surgery. In the early nineties, Lescut *et al* performed a perioperative retrograde endoscopy through the enterotomy and up to the angle of Treitz in 20 CD patients undergoing ileal and/or colonic resections, and found that 65% of patients had mucosal lesions[42]. Furthermore, a prospective endoscopic evaluation of these patients did not find any relationship between endoscopic recurrence 12 wk after surgery and the presence of small bowel lesions left in place after a "curative" surgery[43]. The spreading use of WCE for POR assessment may be additionally restricted by two major concerns: first, there are no studies evaluating the correlation between those mucosal lesions seen at WCE and the clinical outcome and, second, WCE interpretation is associated to a significant interobserver variability[44].

Abdominal ultrasonography is an attractive alternative to assess POR. It has the advantage over ileocolonoscopy to be non-invasive and well-tolerated, and it is cheaper than WCE. However, conventional transabdominal ultrasonography has the inherent technical limitations of the virtual lumen and the presence of gas in the intestinal loops for assessing intestinal lesions. To overcome these limitations, the administration of polyethylene glycol as an anechoic luminal contrast (the so called small intestine contrast ultrasonography -SICUS-) has proven to be able to dissociate intestinal loops, thus allowing the measurement of the bowel wall thickness and lumen diameter. In a recently published study in which SICUS was compared to ileocolonoscopy and WCE, even though it was found to be useful in assessing POR, it had a low diagnostic specificity and there was a poor correlation between wall thickness evaluated by SICUS and the Rutgeerts index[38]. In addition, it has to be kept in mind that, even when using improved modifications of abdominal ultrasonography, an experienced sonographist is always required to detect small intestine lesions.

Granulocyte-labelled scintigraphy has also been evaluated in a small study to assess POR suggesting that, although with a lower specificity, this technique might be useful[45]; nevertheless, it is time-consuming and it is not available in all centres.

The usefulness of inflammatory faecal markers for early and non-invasive evaluation of POR is still to be established. Recently, faecal markers have shown to be superior to CDAI and CRP in predicting endoscopic disease activity as measured by the CDEIS[46]. However, although promising results in pilot studies are available[47,48], the role of inflammatory faecal markers has not been appropriately evaluated in the post-operative setting.

PREVENTION OF POSTOPERATIVE RECURRENCE

Several different approaches have been unsuccessfully evaluated in the prevention of POR (Table 2). The preventive use of conventional drugs such as budesonide has not proven efficacy[49], although one single study suggested that this drug might be beneficial in those patients operated on for refractory inflammatory activity[50].

Despite they may be more appealing from a physiopathogenic point of view, neither the administration of anti-inflammatory cytokines such as interleukin-10[51], nor the use of probiotics[39,52,53] or a cocktail of probiotics and prebiotics[54], have demonstrated to be superior to placebo in preventing POR in RCTs.

As in many other clinical scenarios in CD, the role of mesalazine in the prevention of POR remains controversial. Many RCTs have been published, some of them with a large sample size, but with heterogeneous results.

Repeated meta-analyses suggest that 5-ASA compounds offer just a marginal benefit in this setting[55,56] and their use is only advised in patients with low-risk of POR or in non-treated patients with grade i1-i2 of endoscopical recurrence[2,57].

Enteral nutrition, although with a lesser effect than conventional steroids, is able to induce remission in up to 50% of patients with active CD with almost no associated serious adverse effects[58]. In fact, enteral nutrition is considered as the treatment of choice at the onset of paediatric CD. Some studies suggest that supplementation with enteral nutrition may also be beneficial for maintaining clinical remission[59]; however this strategy has been poorly explored after surgically-induced remission. Yamamoto and colleagues found in a prospective, controlled, open, pilot study that long-term nocturnal enteral nutrition administered by nasogastric self-intubation resulted in a significant reduction in the 12-mo rates of clinical and endoscopic POR[60].

Similarly, the efficacy of biological agents in this setting has not been assessed yet. Sorrentino and colleagues reported the results of a prospective, parallel, non-randomized study in which 7 patients were treated with a conventional dosing of infliximab plus low-dose oral methotrexate and compared to 16 patients treated with 2.4 g/d of mesalazine, to prevent POR. None of the infliximab-treated patients had evidence of disease recurrence by means of endoscopy, MRI and small-bowel enteroclysis 2 years after surgery, whereas 75% of the patients in the mesalazine group had endoscopic recurrence, 25% of them with concurrent clinical recurrence[61].

Even though it is well established that smokers have an increased risk of POR in comparison to non-smokers, the impact of smoking cessation on the risk of developing POR has only been evaluated in retrospective studies in which patients who quit smoking had a reduced risk for both clinical[62] and surgical[63] recurrence. Prospective, controlled studies evaluating the usefulness of smoking cessation to prevent POR will never be performed but, taking into account that the impact of this therapeutical measure has been compared to the start of immunomodulator therapy[64], it should be the first line therapy for POR prevention.

Nitroimidazolic antibiotics are the only drugs with clearly proven efficacy at preventing endoscopic and clinical POR. However, side effects are a major drawback for their long-term administration. In the two RCTs that evaluated metronidazole (for 3 mo) and ornidazole (for 12 mo) in the prevention of POR, almost one half of the patients developed unequivocal drug-related side effects, leading to treatment discontinuation in 13% and 21% of patients, respectively[11,12]. Therefore, nitroimidazolic antibiotics are not appropriate as monotherapy for maintaining postoperative remission.

Many arguments suggest that thiopurines may work in preventing CD recurrence. First, thiopurines (azathioprine and 6-mercaptopurine) are, together with infliximab and adalimumab, the only drugs that have proven efficacy for maintaining CD in remission[65-67]. Second, Rutgeerts *et al*, in the ornidazole trial, already found that discontinuation of immunomodulators at the time of operation was the only clinical parameter predicting clinical recurrence, irrespective of the treatment during the trial. This led the authors to suggest that patients receiving thiopurines before surgery should continue on these drugs after intestinal resection[12]. Finally, azathioprine may lead to healing or improvement of endoscopic lesions in a great proportion of patients with severe postoperative recurrence, as described by D'Haens *et al*[68]. However, thiopurines have not been appropriately evaluated in this clinical setting. Several retrospective studies reported good results with AZA to prevent POR[69-72]. The largest RCT published up to date compared thiopurines to mesalazine and placebo, and found that 6-mercaptopurine was more effective than placebo for preventing both endoscopic and clinical POR over 2 years[73]. Nevertheless, the study was strongly criticized because of a lower than expected efficacy of 6-mercaptopurine (maybe related to the low dose used) and the high rate of dropouts from the study. A previous small-sized RCT did not demonstrate any superiority of low-dose azathioprine over mesalazine 3 g/d[74]. In addition, the use of thiopurines seems to merely delay but not avoid the development of mucosal lesions in the neoterminal ileum, as stated in two recently published prospective, open studies[38,72], suggesting that the efficacy of these drugs should be improved. Despite that, thiopurines are the best drugs to prevent and treat POR and they are still considered the first line treatment in patients with severe endoscopic lesions or at high risk.

Given the limited efficacy and/or applicability of the available preventive measures, alternative or combined strategies must be investigated. Recently, D'Haens and colleagues reported positive results with azathioprine when associated to metronidazole for the first 3 mo after surgery in patients at high risk for POR. Such an association resulted to be better than metronidazole alone, with 12-mo endoscopic recurrence rates of 44% and 69%, respectively[75]. Smoking cessation might have a synergistic effect with thiopurines on the prevention of POR. In a retrospective study, Papay and co-workers found that treatment with thiopurines and non-smoking were the only factors associated with a lower risk of surgical recurrence in a large cohort of CD patients who had been operated on, although non-smoking did not reach statistical significance in the multivariate analysis (OR 1.6, 95% CI 0.99-2.7)[76].

A PRACTICAL APPROACH: WHICH PATIENTS MUST START PREVENTION TREATMENT AFTER SURGERY?

Whether POR must be prevented in all patients after intestinal resection and how, is still under debate. Even though everyone agrees in treating those patients at "high risk", it is difficult (if not impossible) in clinical practice to classify patients as being at "low" or "high" risk; therefore, this is not applicable in most instances.

As long as smoking is harmful not only for Crohn's evolution (and, of course, for the risk of developing POR) but also for developing cardiovascular and neoplastic diseases, smokers have to be earnestly encouraged to give up smoking once surgery have been planned. Those patients who continue smoking despite medical advice should be considered as being at high risk and, consequently, treated with thiopurines. It also seems clear that patients with previous intestinal resections must follow active prevention; in this setting, azathioprine has shown to be superior to no-treatment[71] or high-dose of mesalazine[77] at preventing clinical recurrence. There are no other clinical or epidemiological factors clearly associated with a high risk of POR; thus, studies in large cohorts of patients evaluating not only clinical and epidemiological but also genetic and serological factors are warranted. In the meantime, some authors are prone to endoscopically monitor or even prescribe mesalazine to those patients with no risk factors (mainly smoking, second resection, and/or penetrating disease pattern). Our opinion is that all patients must be given thiopurines. Crohn's disease is a chronic condition with a relapsing course and most patients will develop penetrating or stenosing complications within the first 5 to 10 years from diagnosis. This has led to the thought that maintenance treatment must be started right from the diagnosis of CD, and it has been already shown that early introduction of thiopurines is beneficial in both paediatric[78] and adult[79] CD patients. From this point of view, it makes no sense to leave untreated those patients who already had a complication of the disease. Even if this policy is chosen, many questions remain unanswered: must thiopurines be started together with a 3-mo course of metronidazole? Which are the alternatives in those patients intolerant to thiopurines or with a previous failure of these drugs to prevent POR? Can we stop thiopurines in smokers some months after smoking cessation?

ACKNOWLEDGMENTS

CIBEREHD is funded by the Instituto de Salud Carlos Ⅲ, from the Spanish Ministry of Health.

REFERENCES

1 **Caprilli R**, Gassull MA, Escher JC, Moser G, Munkholm P, Forbes A, Hommes DW, Lochs H, Angelucci E, Cocco A, Vucelic B, Hildebrand H, Kolacek S, Riis L, Lukas M, de Franchis R, Hamilton M, Jantschek G, Michetti P, O'Morain C, Anwar MM, Freitas JL, Mouzas IA, Baert F, Mitchell R, Hawkey CJ. European evidence based consensus on the diagnosis and management of Crohn's disease: special situations. *Gut* 2006; **55** Suppl 1: i36-i58

2 **Rutgeerts P**. Review article: recurrence of Crohn's disease after surgery - the need for treatment of new lesions. *Aliment Pharmacol Ther* 2006; **24** Suppl 3: 29-32

3 **Stange EF**, Travis SP, Vermeire S, Beglinger C, Kupcinkas L, Geboes K, Barakauskiene A, Villanacci V, Von Herbay A, Warren BF, Gasche C, Tilg H, Schreiber SW, Scholmerich J, Reinisch W. European evidence based consensus on the diagnosis and management of Crohn's disease: definitions and diagnosis. *Gut* 2006; **55** Suppl 1: i1-i15

4 **Rutgeerts P**. Protagonist: Crohn's disease recurrence can be prevented after ileal resection. *Gut* 2002; **51**: 152-153

5 **Rutgeerts P**, Geboes K, Vantrappen G, Kerremans R, Coenegrachts JL, Coremans G. Natural history of recurrent Crohn's disease at the ileocolonic anastomosis after curative surgery. *Gut* 1984; **25**: 665-672

6 **Yamamoto T**. Factors affecting recurrence after surgery for Crohn's disease. *World J Gastroenterol* 2005; **11**: 3971-3979

7 **Binder V**, Hendriksen C, Kreiner S. Prognosis in Crohn's disease--based on results from a regional patient group from the county of Copenhagen. *Gut* 1985; **26**: 146-150

8 **Goldberg PA**, Wright JP, Gerber M, Claassen R. Incidence of surgical resection for Crohn's disease. *Dis Colon Rectum* 1993; **36**: 736-739

9 **Rutgeerts P**, Goboes K, Peeters M, Hiele M, Penninckx F, Aerts R, Kerremans R, Vantrappen G. Effect of faecal stream diversion on recurrence of Crohn's disease in the neoterminal ileum. *Lancet* 1991; **338**: 771-774

10 **D'Haens GR**, Geboes K, Peeters M, Baert F, Penninckx F, Rutgeerts P. Early lesions of recurrent Crohn's disease caused by infusion of intestinal contents in excluded ileum. *Gastroenterology* 1998; **114**: 262-267

11 **Rutgeerts P**, Hiele M, Geboes K, Peeters M, Penninckx F, Aerts R, Kerremans R. Controlled trial of metronidazole treatment for prevention of Crohn's recurrence after ileal resection. *Gastroenterology* 1995; **108**: 1617-1621

12 **Rutgeerts P**, Van Assche G, Vermeire S, D'Haens G, Baert F, Noman M, Aerden I, De Hertogh G, Geboes K, Hiele M, D'Hoore A, Penninckx F. Ornidazole for prophylaxis of postoperative Crohn's disease recurrence: a randomized, double-blind, placebo-controlled trial. *Gastroenterology* 2005; **128**: 856-861

13 **Wolters FL**, Russel MG, Sijbrandij J, Ambergen T, Odes S, Riis L, Langholz E, Politi P, Qasim A, Koutroubakis I, Tsianos E, Vermeire S, Freitas J, van Zeijl G, Hoie O, Bernklev T, Beltrami M, Rodriguez D, Stockbrugger RW, Moum B. Phenotype at diagnosis predicts recurrence rates in Crohn's disease. *Gut* 2006; **55**: 1124-1130

14 **Sankey EA**, Dhillon AP, Anthony A, Wakefield AJ, Sim R, More L, Hudson M, Sawyerr AM, Pounder RE. Early mucosal changes in Crohn's disease. *Gut* 1993; **34**: 375-381

15 **Rutgeerts P**, Geboes K, Vantrappen G, Beyls J, Kerremans R, Hiele M. Predictability of the postoperative course of Crohn's disease. *Gastroenterology* 1990; **99**: 956-963

16 **Lakatos PL**, Szamosi T, Lakatos L. Smoking in inflammatory bowel diseases: good, bad or ugly? *World J Gastroenterol* 2007; **13**: 6134-6139

17 **Lindberg E**, Jarnerot G, Huitfeldt B. Smoking in Crohn's disease: effect on localisation and clinical course. *Gut* 1992; **33**: 779-782

18 **Sutherland LR**, Ramcharan S, Bryant H, Fick G. Effect of cigarette smoking on recurrence of Crohn's disease. *Gastroenterology* 1990; **98**: 1123-1128

19 **Breuer-Katschinski BD**, Hollander N, Goebell H. Effect of cigarette smoking on the course of Crohn's disease. *Eur J Gastroenterol Hepatol* 1996; **8**: 225-228

20 **Cottone M**, Rosselli M, Orlando A, Oliva L, Puleo A, Cappello M, Traina M, Tonelli F, Pagliaro L. Smoking habits and recurrence in Crohn's disease. *Gastroenterology* 1994; **106**: 643-648

21 **Alvarez-Lobos M**, Arostegui JI, Sans M, Tassies D, Plaza S, Delgado S, Lacy AM, Pique JM, Yague J, Panes J. Crohn's disease patients carrying Nod2/CARD15 gene variants have an increased and early need for first surgery due to stricturing disease and higher rate of surgical recurrence. *Ann Surg* 2005; **242**: 693-700

22 **Renda MC**, Orlando A, Civitavecchia G, Criscuoli V, Maggio A, Mocciaro F, Rossi F, Scimeca D, Modesto I, Oliva L, Cottone M. The role of CARD15 mutations and smoking in the course of Crohn's disease in a Mediterranean area. *Am J Gastroenterol* 2008; **103**: 649-655

23 **Ryan WR**, Allan RN, Yamamoto T, Keighley MR. Crohn's disease patients who quit smoking have a reduced risk of

reoperation for recurrence. *Am J Surg* 2004; **187**: 219-225
24 **Chardavoyne R**, Flint GW, Pollack S, Wise L. Factors affecting recurrence following resection for Crohn's disease. *Dis Colon Rectum* 1986; **29**: 495-502
25 **Keh C**, Shatari T, Yamamoto T, Menon A, Clark MA, Keighley MR. Jejunal Crohn's disease is associated with a higher postoperative recurrence rate than ileocaecal Crohn's disease. *Colorectal Dis* 2005; **7**: 366-368
26 **Simillis C**, Yamamoto T, Reese GE, Umegae S, Matsumoto K, Darzi AW, Tekkis PP. A meta-analysis comparing incidence of recurrence and indication for reoperation after surgery for perforating versus nonperforating Crohn's disease. *Am J Gastroenterol* 2008; **103**: 196-205
27 **Fazio VW**, Marchetti F, Church M, Goldblum JR, Lavery C, Hull TL, Milsom JW, Strong SA, Oakley JR, Secic M. Effect of resection margins on the recurrence of Crohn's disease in the small bowel. A randomized controlled trial. *Ann Surg* 1996; **224**: 563-571; discussion 571-573
28 **Ferrante M**, de Hertogh G, Hlavaty T, D'Haens G, Penninckx F, D'Hoore A, Vermeire S, Rutgeerts P, Geboes K, van Assche G. The value of myenteric plexitis to predict early postoperative Crohn's disease recurrence. *Gastroenterology* 2006; **130**: 1595-1606
29 **Speranza V**, Simi M, Leardi S, Del Papa M. Recurrence of Crohn's disease after resection. Are there any risk factors? *J Clin Gastroenterol* 1986; **8**: 640-646
30 **Pennington L**, Hamilton SR, Bayless TM, Cameron JL. Surgical management of Crohn's disease. Influence of disease at margin of resection. *Ann Surg* 1980; **192**: 311-318
31 **Penner RM**, Madsen KL, Fedorak RN. Postoperative Crohn's disease. *Inflamm Bowel Dis* 2005; **11**: 765-777
32 **Simillis C**, Purkayastha S, Yamamoto T, Strong SA, Darzi AW, Tekkis PP. A meta-analysis comparing conventional end-to-end anastomosis vs. other anastomotic configurations after resection in Crohn's disease. *Dis Colon Rectum* 2007; **50**: 1674-1687
33 **Tabet J**, Hong D, Kim CW, Wong J, Goodacre R, Anvari M. Laparoscopic versus open bowel resection for Crohn's disease. *Can J Gastroenterol* 2001; **15**: 237-242
34 **Lowney JK**, Dietz DW, Birnbaum EH, Kodner IJ, Mutch MG, Fleshman JW. Is there any difference in recurrence rates in laparoscopic ileocolic resection for Crohn's disease compared with conventional surgery? A long-term, follow-up study. *Dis Colon Rectum* 2006; **49**: 58-63
35 **Eshuis EJ**, Polle SW, Slors JF, Hommes DW, Sprangers MA, Gouma DJ, Bemelman WA. Long-term surgical recurrence, morbidity, quality of life, and body image of laparoscopic-assisted vs. open ileocolic resection for Crohn's disease: a comparative study. *Dis Colon Rectum* 2008; **51**: 858-867
36 **Sandborn WJ**, Feagan BG, Hanauer SB, Lochs H, Lofberg R, Modigliani R, Present DH, Rutgeerts P, Scholmerich J, Stange EF, Sutherland LR. A review of activity indices and efficacy endpoints for clinical trials of medical therapy in adults with Crohn's disease. *Gastroenterology* 2002; **122**: 512-530
37 **Domenech E**, Manosa M, Bernal I, Garcia-Planella E, Cabre E, Pinol M, Lorenzo-Zuniga V, Boix J, Gassull MA. Impact of azathioprine on the prevention of postoperative Crohn's disease recurrence: results of a prospective, observational, long-term follow-up study. *Inflamm Bowel Dis* 2008; **14**: 508-513
38 **Biancone L**, Calabrese E, Petruzziello C, Onali S, Caruso A, Palmieri G, Sica GS, Pallone F. Wireless capsule endoscopy and small intestine contrast ultrasonography in recurrence of Crohn's disease. *Inflamm Bowel Dis* 2007; **13**: 1256-1265
39 **Marteau P**, Lemann M, Seksik P, Laharie D, Colombel JF, Bouhnik Y, Cadiot G, Soule JC, Bourreille A, Metman E, Lerebours E, Carbonnel F, Dupas JL, Veyrac M, Coffin B, Moreau J, Abitbol V, Blum-Sperisen S, Mary JY. Ineffectiveness of Lactobacillus johnsonii LA1 for prophylaxis of postoperative recurrence in Crohn's disease: a randomised, double blind, placebo controlled GETAID trial. *Gut* 2006; **55**: 842-847
40 **Bourreille A**, Jarry M, D'Halluin PN, Ben-Soussan E, Maunoury V, Bulois P, Sacher-Huvelin S, Vahedy K, Lerebours E, Heresbach D, Bretagne JF, Colombel JF, Galmiche JP. Wireless capsule endoscopy versus ileocolonoscopy for the diagnosis of postoperative recurrence of Crohn's disease: a prospective study. *Gut* 2006; **55**: 978-983
41 **Pons Beltran V**, Nos P, Bastida G, Beltran B, Arguello L, Aguas M, Rubin A, Pertejo V, Sala T. Evaluation of postsurgical recurrence in Crohn's disease: a new indication for capsule endoscopy? *Gastrointest Endosc* 2007; **66**: 533-540
42 **Lescut D**, Vanco D, Bonniere P, Lecomte-Houcke M, Quandalle P, Wurtz A, Colombel JF, Delmotte JS, Paris JC, Cortot A. Perioperative endoscopy of the whole small bowel in Crohn's disease. *Gut* 1993; **34**: 647-649
43 **Klein O**, Colombel JF, Lescut D, Gambiez L, Desreumaux P, Quandalle P, Cortot A. Remaining small bowel endoscopic lesions at surgery have no influence on early anastomotic recurrences in Crohn's disease. *Am J Gastroenterol* 1995; **90**: 1949-1952
44 **Lai LH**, Wong GL, Chow DK, Lau JY, Sung JJ, Leung WK. Inter-observer variations on interpretation of capsule endoscopies. *Eur J Gastroenterol Hepatol* 2006; **18**: 283-286
45 **Biancone L**, Scopinaro F, Ierardi M, Paoluzi P, Marcheggiano A, Di Paolo MC, Porowska B, Colella AC, Pallone F. 99mTc-HMPAO granulocyte scintigraphy in the early detection of postoperative asymptomatic recurrence in Crohn's disease. *Dig Dis Sci* 1997; **42**: 1549-1556
46 **Sipponen T**, Savilahti E, Kolho KL, Nuutinen H, Turunen U, Farkkila M. Crohn's disease activity assessed by fecal calprotectin and lactoferrin: correlation with Crohn's disease activity index and endoscopic findings. *Inflamm Bowel Dis* 2008; **14**: 40-46
47 **Orlando A**, Modesto I, Castiglione F, Scala L, Scimeca D, Rispo A, Teresi S, Mocciaro F, Criscuoli V, Marrone C, Platania P, De Falco T, Maisano S, Nicoli N, Cottone M. The role of calprotectin in predicting endoscopic post-surgical recurrence in asymptomatic Crohn's disease: a comparison with ultrasound. *Eur Rev Med Pharmacol Sci* 2006; **10**: 17-22
48 **Boirivant M**, Pallone F, Ciaco A, Leoni M, Fais S, Torsoli A. Usefulness of fecal alpha 1-antitrypsin clearance and fecal concentration as early indicator of postoperative asymptomatic recurrence in Crohn's disease. *Dig Dis Sci* 1991; **36**: 347-352
49 **Papi C**, Luchetti R, Gili L, Montanti S, Koch M, Capurso L. Budesonide in the treatment of Crohn's disease: a meta-analysis. *Aliment Pharmacol Ther* 2000; **14**: 1419-1428
50 **Hellers G**, Cortot A, Jewell D, Leijonmarck CE, Lofberg R, Malchow H, Nilsson LG, Pallone F, Pena S, Persson T, Prantera C, Rutgeerts P. Oral budesonide for prevention of postsurgical recurrence in Crohn's disease. The IOIBD Budesonide Study Group. *Gastroenterology* 1999; **116**: 294-300
51 **Colombel JF**, Rutgeerts P, Malchow H, Jacyna M, Nielsen OH, Rask-Madsen J, Van Deventer S, Ferguson A, Desreumaux P, Forbes A, Geboes K, Melani L, Cohard M. Interleukin 10 (Tenovil) in the prevention of postoperative recurrence of Crohn's disease. *Gut* 2001; **49**: 42-46
52 **Prantera C**, Scribano ML, Falasco G, Andreoli A, Luzi C. Ineffectiveness of probiotics in preventing recurrence after curative resection for Crohn's disease: a randomised controlled trial with Lactobacillus GG. *Gut* 2002; **51**: 405-409
53 **Van Gossum A**, Dewit O, Louis E, de Hertogh G, Baert F, Fontaine F, DeVos M, Enslen M, Paintin M, Franchimont D. Multicenter randomized-controlled clinical trial of probiotics (Lactobacillus johnsonii, LA1) on early endoscopic recurrence of Crohn's disease after lleo-caecal resection. *Inflamm Bowel Dis* 2007; **13**: 135-142
54 **Chermesh I**, Tamir A, Reshef R, Chowers Y, Suissa A, Katz D, Gelber M, Halpern Z, Bengmark S, Eliakim R. Failure of Synbiotic 2000 to prevent postoperative recurrence of Crohn's disease. *Dig Dis Sci* 2007; **52**: 385-389
55 **Cottone M**, Camma C. Mesalamine and relapse prevention

in Crohn's disease. *Gastroenterology* 2000; **119**: 597
56 **Sutherland LR**. Mesalamine for the prevention of postoperative recurrence: is nearly there the same as being there? *Gastroenterology* 2000; **118**: 436-438
57 **Lemann M**. Review article: can post-operative recurrence in Crohn's disease be prevented? *Aliment Pharmacol Ther* 2006; **24** Suppl 3: 22-28
58 **Zachos M**, Tondeur M, Griffiths AM. Enteral nutritional therapy for induction of remission in Crohn's disease. *Cochrane Database Syst Rev* 2007; **1**: CD000542
59 **Akobeng AK**. Review article: the evidence base for interventions used to maintain remission in Crohn's disease. *Aliment Pharmacol Ther* 2008; **27**: 11-18
60 **Yamamoto T**, Nakahigashi M, Umegae S, Kitagawa T, Matsumoto K. Impact of long-term enteral nutrition on clinical and endoscopic recurrence after resection for Crohn's disease: A prospective, non-randomized, parallel, controlled study. *Aliment Pharmacol Ther* 2007; **25**: 67-72
61 **Sorrentino D**, Terrosu G, Avellini C, Maiero S. Infliximab with low-dose methotrexate for prevention of postsurgical recurrence of ileocolonic Crohn disease. *Arch Intern Med* 2007; **167**: 1804-1807
62 **Timmer A**, Sutherland LR, Martin F. Oral contraceptive use and smoking are risk factors for relapse in Crohn's disease. The Canadian Mesalamine for Remission of Crohn's Disease Study Group. *Gastroenterology* 1998; **114**: 1143-1150
63 **Ryan WR**, Allan RN, Yamamoto T, Keighley MR. Crohn's disease patients who quit smoking have a reduced risk of reoperation for recurrence. *Am J Surg* 2004; **187**: 219-225
64 **Johnson GJ**, Cosnes J, Mansfield JC. Review article: smoking cessation as primary therapy to modify the course of Crohn's disease. *Aliment Pharmacol Ther* 2005; **21**: 921-931
65 **Pearson DC**, May GR, Fick G, Sutherland LR. Azathioprine for maintenance of remission in Crohn's disease. *Cochrane Database Syst Rev* 2000; **2**: CD000067
66 **Hanauer SB**, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, Rachmilewitz D, Wolf DC, Olson A, Bao W, Rutgeerts P. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet* 2002; **359**: 1541-1549
67 **Sandborn WJ**, Hanauer SB, Rutgeerts P, Fedorak RN, Lukas M, MacIntosh DG, Panaccione R, Wolf D, Kent JD, Bittle B, Li J, Pollack PF. Adalimumab for maintenance treatment of Crohn's disease: results of the CLASSIC II trial. *Gut* 2007; **56**: 1232-1239
68 **D'Haens G**, Geboes K, Ponette E, Penninckx F, Rutgeerts P. Healing of severe recurrent ileitis with azathioprine therapy in patients with Crohn's disease. *Gastroenterology* 1997; **112**: 1475-1481
69 **Cuillerier E**, Lemann M, Bouhnik Y, Allez M, Rambaud JC, Modigliani R. Azathioprine for prevention of postoperative recurrence in Crohn's disease: a retrospective study. *Eur J Gastroenterol Hepatol* 2001; **13**: 1291-1296
70 **Domènech E**, Scala L, Bernal I, Garcia-Planella E, Casalots A, Piñol M, Esteve-Comas E, Cabré E, Boix J, Gassull MA. Azathioprine and mesalazine in the prevention of postoperative recurrence of Crohn's disease: a retrospective study. *Gastroenterol Hepatol* 2004; **27**: 63-67
71 **Alves A**, Panis Y, Joly F, Pocard M, Lavergne-Slove A, Bouhnik Y, Valleur P. Could immunosuppressive drugs reduce recurrence rate after second resection for Crohn disease? *Inflamm Bowel Dis* 2004; **10**: 491-495
72 **Myrelid P**, Svarm S, Andersson P, Almer S, Bodemar G, Olaison G. Azathioprine as a postoperative prophylaxis reduces symptoms in aggressive Crohn's disease. *Scand J Gastroenterol* 2006; **41**: 1190-1195
73 **Hanauer SB**, Korelitz BI, Rutgeerts P, Peppercorn MA, Thisted RA, Cohen RD, Present DH. Postoperative maintenance of Crohn's disease remission with 6-mercaptopurine, mesalamine, or placebo: a 2-year trial. *Gastroenterology* 2004; **127**: 723-729
74 **Nos P**, Hinojosa J, Aguilera V, Moles JR, Pastor M, Ponce J, Berenguer J. [Azathioprine and 5-ASA in the prevention of postoperative recurrence of Crohn's disease] *Gastroenterol Hepatol* 2000; **23**: 374-378
75 **D'Haens G**, Noman M, Van Assche, Van Olmen G, Aerden I, Vermeire S, Rutgeerts P. Severe postoperative recurrence of Crohn's disease is significantly reduced with combination therapy metronidazole + azathioprine: A double-blind controlled randomised trial. *J Crohn Colitis* (Suppl) 2007; **1**: 4
76 **Papay P**, Reinisch W, Miehsler W, Lissner D, Gratzer C, Dejaco C, Vogelsang H, Novacek G. Maintenance therapy with azathioprine/6-mercaptopurine in Crohn's disease reduces the risk of re-operation particularly in smokers. *J Crohn Colitis* (Suppl) 2008; **2**: 3
77 **Ardizzone S**, Maconi G, Sampietro GM, Russo A, Radice E, Colombo E, Imbesi V, Molteni M, Danelli PG, Taschieri AM, Bianchi Porro G. Azathioprine and mesalamine for prevention of relapse after conservative surgery for Crohn's disease. *Gastroenterology* 2004; **127**: 730-740
78 **Markowitz J**, Grancher K, Kohn N, Lesser M, Daum F. A multicenter trial of 6-mercaptopurine and prednisone in children with newly diagnosed Crohn's disease. *Gastroenterology* 2000; **119**: 895-902
79 **D'Haens G**, Baert F, van Assche G, Caenepeel P, Vergauwe P, Tuynman H, De Vos M, van Deventer S, Stitt L, Donner A, Vermeire S, Van de Mierop FJ, Coche JC, van der Woude J, Ochsenkuhn T, van Bodegraven AA, Van Hootegem PP, Lambrecht GL, Mana F, Rutgeerts P, Feagan BG, Hommes D. Early combined immunosuppression or conventional management in patients with newly diagnosed Crohn's disease: an open randomised trial. *Lancet* 2008; **371**: 660-667

S- Editor Xiao LL **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5549

World J Gastroenterol 2008 September 28; 14(36): 5549-5556
World Journal of Gastroenterology ISSN 1007-9327

Relationship between nm23H1 genetic instability and clinical pathological characteristics in Chinese digestive system cancer patients

Yue-Qin Yang, Liang Wu, Jin-Xing Chen, Jian-Zhong Sun, Meng Li, Dong-Mei Li, Hai-Ying Lu, Zhi-Hong Su, Xin-Qiu Lin, Ji-Cheng Li

Yue-Qin Yang, Meng Li, Dong-Mei Li, Hai-Ying Lu, Zhi-Hong Su, Xin-Qiu Lin, Ji-Cheng Li, Institute of Cell Biology, Zhejiang University, Hangzhou 310058, Zhejiang Province, China
Liang Wu, Jian-Zhong Sun, Zhejiang Qingtian People's Hospital, Qingtian 323900, Zhejiang Province, China
Jin-Xing Chen, Zhejiang Tonglu People's Hospital, Tonglu 311500, Zhejiang Province, China
Author contributions: Li JC designed the research; Yang YQ, Wu L, Chen JX, Sun JZ, Li M, Li DM, Lu HY, Su ZH and Lin XQ performed the experiments; Wu L, Chen JX and Sun JZ contributed to the new reagents/ analytic tools; Yang YQ and Li JC analyzed the data; Yang YQ, Li M, Li DM and Li JC wrote the paper.
Supported by National Fund for Fostering Talents of Basic Sciences, No. J0730856; Scientific Research Foundation of Public Health Bureau of Zhejiang Province, No.2007A179, No. 2007B209, Foundation for Analyzing and Testing of Zhejiang Province, No. 03149
Correspondence to: Ji-Cheng Li, MD, Professor, Institute of Cell Biology, Zhejiang University, Hangzhou 310058, Zhejiang Province, China. lijichen@zju.edu.cn
Telephone: +86-571-88208088 Fax: +86-571-88208094
Received: May 12, 2008 Revised: June 23, 2008
Accepted: June 30, 2008
Published online: September 28, 2008

Abstract

AIM: To study the relationship between nm23H1 gene genetic instability and its clinical pathological characteristics in Chinese digestive system cancer patients.

METHODS: Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) was used to analyze the microsatellite instability (MSI) and loss of heterozygosity (LOH). Immunohistochemistry was employed to detect the expression of nm23H1.

RESULTS: The MSI was higher in TNM stage Ⅰ + Ⅱ than in stage Ⅲ + Ⅳ of gastric, colonic and gallbladder carcinomas. The LOH was higher in TNM stage Ⅲ + Ⅳ than in stage Ⅰ + Ⅱ of gastric, colonic and hepatocellular carcinomas. Lymphatic metastasis was also observed. The expression of nm23H1 protein was lower in TNM stage Ⅲ + Ⅳ than in stage Ⅰ + Ⅱ of these tumors and in patients with lymphatic metastasis.The nm23H1 protein expression was higher in the LOH negative group than in the LOH positive group.

CONCLUSION: MSI and LOH may independently control the biological behaviors of digestive system cancers. MSI could serve as an early biological marker of digestive system cancers. Enhanced expression of nm23H1 protein could efficiently inhibit cancer metastasis and improve its prognosis. LOH mostly appears in late digestive system cancer.

Key words: nm23H1; Gastric cancer; Colonic cancer; Hepatocellular carcinoma; Gallbladder carcinoma

Peer reviewer: Marc Basson, MD, PhD, MBA, Chief of Surgery, John D. Dingell VA Medical Center, 4646 John R. Street, Detroit, MI 48301, United States

Yang YQ, Wu L, Chen JX, Sun JZ, Li M, Li DM, Lu HY, Su ZH, Lin XQ, Li JC. Relationship between nm23H1 genetic instability and clinical pathological characteristics in Chinese digestive system cancer patients. *World J Gastroenterol* 2008; 14(36): 5549-5556 Available from: URL: http://www.wjgnet.com/1007-9327/14/5549.asp DOI: http://dx.doi.org/10.3748/wjg.14.5549

INTRODUCTION

Digestive malignant tumor remains one of the commonly seen tumors in clinical practice, accounting for 70% of all digestive tumors. The mortality rate of gastric, colonic, liver and gallbladder tumors are the highest. Malignant tumors increase with the changing eating habit and environment changes. Since early diagnosis is always a challenge, the disease threatens the life of more and more patients. Therefore, great attention should be paid to finding new early markers and effective treatment methods for this disease.

Growing evidence suggests that accumulation of multiple alterations, such as activation of proto-oncogenes and inactivation of tumor suppressor genes, is responsible for the development and progression of digestive system cancer. Genetic instability of oncogenes, such as microsatellite instability (MSI) and loss of heterozygosity (LOH), is probably associated with mutations of genes

responsible for tumor-genesis, which play an important role in tumor pathology[1-5]. Studies on MSI and LOH of digestive system cancer have been focused on genetic instability of P53[6], P16 and FHIT[7]. However, few studies are available on gene nm23H1[8-11]. nm23H1 is a metastasis-associated gene of various tumors and its coding protein has the NDPK function, which determines the biological behaviors of cell proliferation, differentiation and migrations. Inhibited expression of nm23H1 shows malignant behaviors of melanoma[12], gastric[13], colon[14] and breast carcinomas[15]. The present study was to investigate the MSI and LOH of gene nm23H1 at locus D17S396 in Chinese patients with colon, gastric, hepatocellular and gallbladder cancers, and their influence on nm23H1 protein expression.

MATERIALS AND METHODS

Tissue sample

Tissue samples were obtained from 40 gastric, 30 colon, 48 HCC and 47 gallbladder carcinoma patients. The final pathological diagnosis was based on the result of histological examination. No patient received radioactive therapy or chemotherapy prior to operation. Fresh surgical tissue samples were fixed immediately in formaldehyde solution for 12-24 h and paraffin-embedded for polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and immunohistochemistry study.

DNA extraction and PCR amplification

DNA was extracted with a Qiagen's kit according its manufacturer's instructions. Designed primers were designed as previously described[16] and synthesized by Shanghai Shengyou Biology Company. The primer sequences are sense: 5'-TTGACCGGGGTAGAGA-ACTC-3', antisense: 5'-TCTCAGTACTTCCCGT-GACC-3'. The PCR mixture contained 200 ng of template-DNA while the PCR reaction buffer contained 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.4), 1.5 mmol/L $MgCl_2$, 0.5 μmol/L each of two fragment-specific primers, 100 μmol/L each of dATP, dGTP, dTTP and dCTP, and 2 units of Taq DNA polymerase (Shanghai Shengyou Biology Company) for a reaction volume of 50 μL. The conditions for PCR amplification were as follows: a pre-denaturation at 94℃ for 5 min, then 35 cycles at 94℃ for 45 s, at 62℃ for 45 s, at 72℃ for 45 s, and a final extension at 72℃ for 10 min. The amplified fragments were run on 1% agarose gel.

SSCP analysis

SSCP analysis of fragments was performed on a mini electrophoresis unit (Bio-Rad Company, USA). Ten microlitres of the PCR product was diluted with 10 μL of sample buffer containing 90% formamide, 0.05% bromophenol blue dye and 0.05% xylene cyanol. The samples were heated at 100℃ for 8 min, transferred into an ice-cold water bath for 3 min, and analyzed by 10% polyacrylamide gel electrophoresis (PAGE) in 45 mmol/L-Tris-borate (pH 8.0) of 1 mmol/L-EDTA (TBE) buffer under 13 v/cm at 10℃.

DNA silver staining

Gels were stained with silver as follows. In brief, gels were fixed in 100 mL/L alcohol for 10 min, and then oxidized in 100 mL/L nitric acid for 3 min. After washed for 1 min with double distilled water, they were stained in 2 g/L silver nitric acid for 5 min and washed for 1 min with double distilled water. Gels showed an appropriate color in 15 g/L anhydrous sodium carbonate and 4 mL/L formalin. The reaction was terminated by 7.5 mL/L glacial acetic acid, and finally, the gels were washed with double distilled water.

Immunohistochemical assay

Immunohistochemical study was performed using the Envision method. Briefly, 5 μm thick tissue sections were deparaffinized and dehydrated. Endogenous peroxide activity was blocked with 3% hydrogen peroxide for 20 min. After three times of rinsing with 0.01 mol/L phosphate-buffered saline (PBS, pH = 7.4), the sections were incubated with 10% normal goat serum at room temperature for 10 min to block the nonspecific reaction followed by 2 h with anti-nm23H1 antibody. After rinsed in PBS for 5 min, the sections were incubated with Envision complex for 2 h at room temperature, and stained with 3,3-diaminobenzidine (DAB) after washed in PBS.

Imaging analysis and data collection

After immunohistochemistry staining, the sections were analyzed with Leica-Qwin computer imaging techniques. We selected 20 continuous high microscopical views that did not overlap. Then we tested the gray-value of background and named it as GA. The gray-value of nm23H1 positive granules was named as Ga and area density of BRCA1 positive cells as A_{Aa}. We used Excel function to compute the value of positive unit (PU) which represents the expression intensity of nm23H1 protein in gastric cancer cells. The highest gray value was 255 in Leica-Qwin system.

Statistical analysis

Statistical analysis was performed using analysis of variance (AVONA). $P < 0.05$ was considered statistically significant.

RESULTS

Relationship between clinical pathological parameters and nm23H1 genetic instability in gastric cancer

The positive rate of D17S396 MSI (Figure 1A), LOH (Figure 1B) and nm23H1 protein (Figure 1C) was 20.00%, 17.50% and 55.00%, respectively, in 40 cases of gastric cancer (Table 1).

MSI and LOH were independent of the histological type of gastric cancer, the degree of differentiation and serosa infiltration. MSI was related to the clinical

Table 1 Relationship between clinical pathological parameters and nm23H1 genetic instability in gastric cancer

Clinical pathological factors	Cases (*n*)	MSI+ (%)	LOH+ (%)	nm23H1+ (%)	nm23H1 expression intensity (mean ± SD)
Histological type	40	8 (20.00)	7 (17.50)	22 (55.00)	40.63 ± 2.95
Tubular adenocarcinoma	33	7 (22.21)	6 (18.18)	21 (63.64)	41.56 ± 2.78
High differentiation	10	3 (30.00)	1 (10.00)	10 (100.00)	41.83 ± 1.52
Middle differentiation	15	3 (20.00)	2 (13.33)	9 (60.00)	40.69 ± 2.35
Low differentiation	8	1 (12.50)	3 (37.50)	2 (25.00)[1]	41.43 ± 1.89
Mucoid adenocarcinoma	7	1 (14.29)	1 (14.29)	1 (14.29)[2]	39.87 ± 2.31
Serosa infiltration					
Positive	24	3 (12.50)	6 (25.00)	10 (41.67)	39.76 ± 2.64
Negative	16	5 (31.25)	1 (6.25)	12 (75.00)[3]	41.45 ± 2.23
Lymph node metastasis					
Positive	20	1 (5.00)	6 (30.00)	6 (30.00)	39.14 ± 2.34
Negative	20	7 (35.00)[4]	1 (5.00)[5]	16 (80.00)[6]	41.75 ± 1.65
TNM stage					
Ⅰ + Ⅱ	22	7 (31.82)	1 (4.55)	17 (77.27)	41.22 ± 1.87
Ⅲ + Ⅳ	18	1 (5.56)[7]	6 (33.33)[8]	5 (17.86)[9]	40.13 ± 2.35

[1]P < 0.0001 *vs* differentiation degree of tubular adenocarcinoma; [2]P = 0.017 *vs* tubular adenocarcinoma; [3]P = 0.039 *vs* positive serosa infiltration; [4]P = 0.017, [5]P = 0.038, [6]P = 0.001 *vs* negative lymph node metastasis; [7]P = 0.04, [8]P = 0.017, [9]P = 0.001 *vs* TNM stage Ⅰ + Ⅱ.

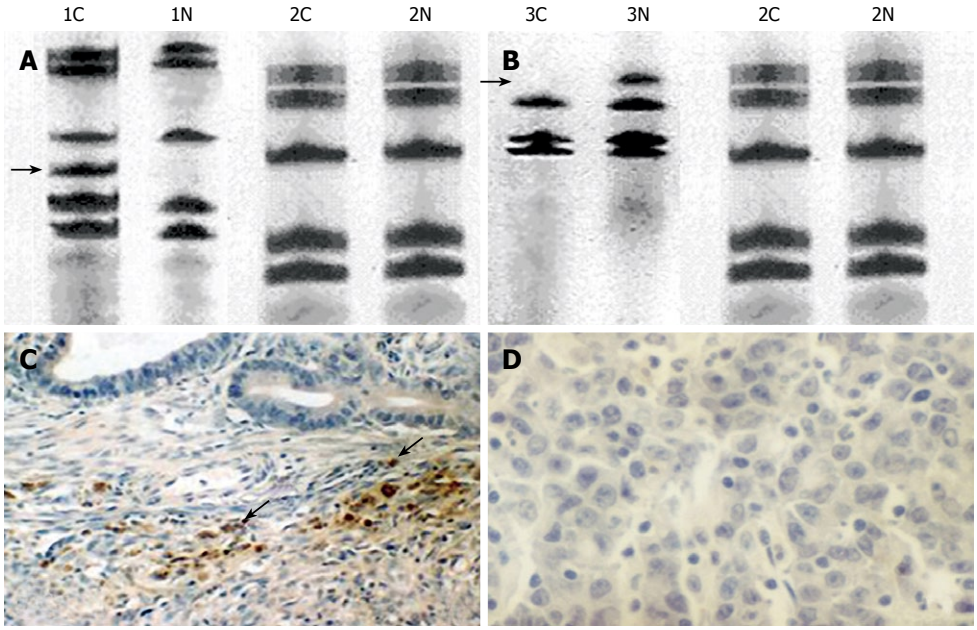


Figure 1 PAGE of D17S396 locus and immunohistochemistry of nm23H1 protein in gastric cancer. **A**: No difference in tumor tissue (2C) and normal tissue (2N). MSI (arrow) was positive when an allele band was added (1C) as compared with normal tissue (1N); **B**: No difference in tumor tissue (2C) and normal tissue (2N). LOH was positive when an allele band was added (arrow) in tumor tissue (3C) as compared with normal tissue (3N); **C**: Brown-yellow granules of nm23H1 protein mostly located in cytoplasm. The stained nucleolus and membrane of cells were observed (arrow, x 200); **D**: Control group, PBS replaced anti-nm23H1 protein as the first antibody (x 400).

TNM stage. The frequency of MSI was higher in stage Ⅰ + Ⅱ than in stages Ⅲ + Ⅳ of gastric cancer (31.82% *vs* 5.56%, P = 0.04). In contrast, LOH was higher in stage Ⅲ + Ⅳ than in stage Ⅰ + Ⅱ of gastric cancer (33.33% *vs* 4.55%, P = 0.017). In addition, MSI tended to decrease with lymph node metastasis (5.00% *vs* 35.00%, P = 0.017), while LOH did not (30.00% *vs* 5.00%, P = 0.038).

The positive rate of nm23H1 protein was closely correlated with the histological type, differentiation degree and clinical stage of gastric cancer. The expression of nm23H1 protein was significantly higher in tubular adenocarcinoma than in mucous adenocarcinoma (63.64% *vs* 14.29%, P = 0.017), and tended to increase with the differentiation degree of tubular adenocarcinoma (P < 0.0001). The positive rate of nm23H1 expression was higher in stage Ⅰ + Ⅱ than in stage Ⅲ + Ⅳ of gastric cancer (77.27% *vs* 17.86%, P = 0.001), and so was in negative than in positive lymph node metastasis. The same phenomenon was observed in patients with positive or negative MSI (87.50% *vs* 46.88%, P = 0.04, Table 2). On the contrary, the expression of nm23H1 protein was lower in LOH positive patients than in LOH negative patients (14.29% *vs* 63.64%, P = 0.017, Table 2). Computer imaging analysis showed that there was no statistical difference in nm23H1 protein expression between the two groups of patients (Table 3).

Relationship between clinical pathological parameters and nm23H1 genetic instability in colon cancer

Microsatellite fragments of D17S396 were amplified. The positive rate of D17S396 MSI (Figure 2A), LOH (Figure 2B) and nm23H1 protein (Figure 2C) was 26.67%, 20.00% and 53.33%, respectively, in 30 cases of colon cancer (Table 4).

MSI and LOH were independent of the histological type, the degree of differentiation and serosa infiltration

Table 2 Relationship between MSI, LOH and nm23H1 protein expression in different cancers of digestive system

Group	Positive frequency of nm23H1 protein % (*n*/*n*)			
	Gastric cancer	Colon cancer	HCC	Gallbladder cancer
MSI positive	87.50 (7/8)	75.00 (6/8)	83.33 (5/6)	63.64 (7/11)
MSI negative	46.88 (15/32)[1]	45.45 (10/22)	52.38 (22/42)	41.67 (15/36)
LOH positive	14.29 (1/7)	33.33 (2/6)	27.27 (3/11)	11.11 (1/9)
LOH negative	63.64 (21/33)[2]	58.33 (14/24)	64.86 (24/37)[3]	55.26 (21/38)[4]

[1]$P = 0.04$ *vs* MSI positive group; [2]$P = 0.017$, [3]$P = 0.027$, [4]$P = 0.016$ *vs* LOH positive group.

Table 3 Relationship between MSI, LOH and nm23H1 protein expression in different cancers of digestive system (mean ± SD)

Group	nm23H1 protein expression intensity ($n = 10$)			
	Gastric cancer	Colon cancer	HCC	Gallbladder cancer
MSI positive	26.34 ± 2.17	29.34 ± 2.14	29.34 ± 2.14	26.34 ± 2.17
MSI negative	25.78 ± 2.34	24.78 ± 2.06	24.78 ± 2.06	25.78 ± 2.34
LOH positive	27.64 ± 2.38	22.64 ± 2.38	22.64 ± 2.38	27.64 ± 2.38
LOH negative	25.88 ± 2.52	26.88 ± 2.52	26.88 ± 2.52	25.88 ± 2.52

Table 4 Relationship between clinical pathological parameters and nm23H1 genetic instability in colon cancer

Clinical pathological factors	Cases	MSI+ (%)	LOH+ (%)	nm23H1+ (%)	nm23H1 expression intensity (mean ± SD)
Histological type	30	8 (26.67)	6 (20.00)	16 (53.33)	40.21 ± 3.29
Tubular adenocarcinoma	25	7 (28.00)	5 (20.00)	15 (60.00)	40.76 ± 2.74
High differentiation	8	2 (25.00)	1 (12.50)	8 (100.00)	41.49 ± 2.01
Middle differentiation	13	5 (38.46)	2 (15.38)	6 (46.15)	40.41 ± 1.98
Low differentiation	4	0 (00.00)	2 (50.00)	1 (25.00)[1]	40.18 ± 2.17
Mucoid adenocarcinoma	5	1 (20.00)	1 (20.00)	1 (20.00)	39.53 ± 2.61
Serosa infiltration					
Positive	20	7 (35.00)	3 (15.00)	12 (48.00)	41.02 ± 2.14
Negative	10	1 (10.00)	3 (30.00)	4 (40.00)	41.45 ± 2.23
Lymph node metastasis					
Positive	11	1 (9.09)	5 (45.45)	3 (27.27)	39.14 ± 2.34
Negative	19	7 (36.84)	1 (5.26)[2]	13 (68.42)[3]	41.75 ± 1.65
TNM stage					
Ⅰ + Ⅱ	16	7 (43.75)	1 (11.76)	13 (81.25)	41.49 ± 2.01
Ⅲ + Ⅳ	14	1 (7.14)[4]	5 (35.71)[5]	3 (21.43)[6]	39.53 ± 2.61

[1]$P = 0.004$ *vs* differentiation degree of tubular adenocarcinoma; [2]$P = 0.003$, [3]$P = 0.074$ *vs* positive lymph node metastasis; [4]$P = 0.023$, [5]$P = 0.046$, [6]$P < 0.0001$ *vs* TNM stage Ⅰ + Ⅱ.

of colon cancer. MSI was related to the clinical TNM stage. In TNM staging, the frequency of MSI was higher in stage Ⅰ + Ⅱ than in stages Ⅲ + Ⅳ of colon cancer (43.75% *vs* 7.14%, $P = 0.023$). In contrast, LOH was higher in stage Ⅲ + Ⅳ than in stage Ⅰ + Ⅱ of colon cancer (35.71% *vs* 11.76%, $P = 0.046$). In addition, LOH tended to decrease with lymph node metastasis (5.26% *vs* 45.45%, $P = 0.003$).

The positive rate of nm23H1 protein was closely correlated with the biological behaviors, differentiation degree and clinical stage of colon cancer. The expression of nm23H1 protein trended to increase with the differentiation degree of tubular adenocarcinoma ($P = 0.004$). The positive rate of nm23H1 was higher in stage Ⅰ + Ⅱ than in stage Ⅲ + Ⅳ of colon cancer (81.25% *vs* 21.43%, $P < 0.0001$), and higher in negative than in positive lymph node metastasis patients (68.42% *vs* 27.27%, $P = 0.074$). Computer imaging analysis showed that there was no difference in nm23H1 protein expression level between the two groups of patients (Table 3).

Relationship between clinical pathological parameters and nm23H1 genetic instability in HCC

The positive rate of D17S396 MSI (Figure 3A), LOH (Figure 3B) and nm23H1 protein (Figure 3C) was 12.50%, 22.92% and 56.25%, respectively, in 48 cases of HCC (Table 5).

MSI and LOH were independent of the differentiation degree, liver infiltration and lymph node metastasis of HCC. The frequency of MSI showed no correlation to the differentiation degree, liver infiltration and lymph node metastasis of HCC. However, LOH was higher in stage Ⅲ + Ⅳ than in stage Ⅰ + Ⅱ of HCC (52.63% *vs* 3.45%, $P < 0.0001$).

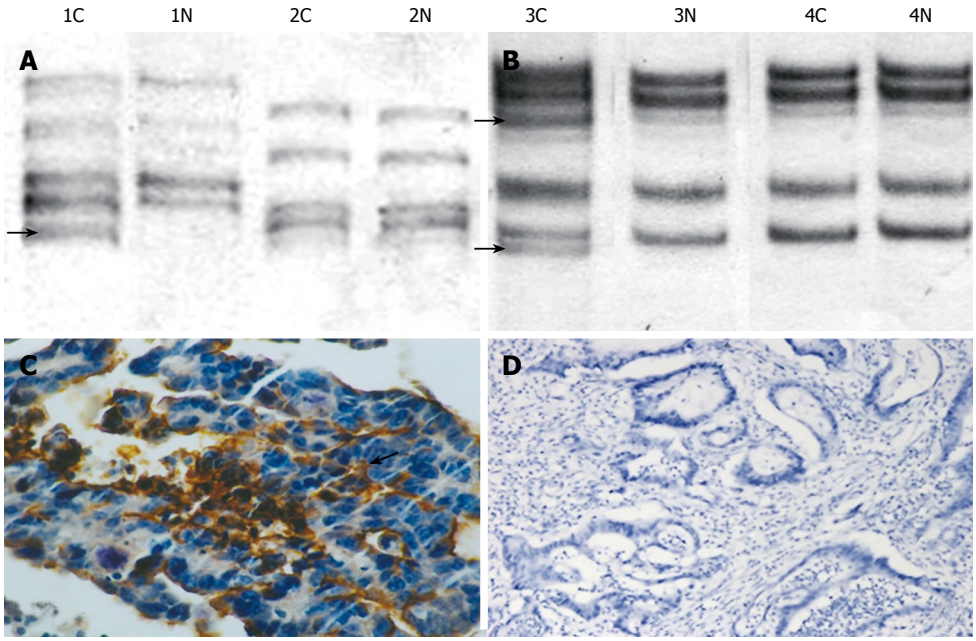


Figure 2 PAGE of D17S396 locus and immunohistochemistry of nm23H1 protein in colon cancer. **A**: No difference in tumor tissue (2C) and normal tissue (2N). MSI was positive when an allele band was added (arrow) in tumor tissue (1C) as compared with normal tissue (1N); **B**: No difference in tumor tissue (4C) and normal tissue (4N). LOH was positive when an allele band was added (arrow) in tumor tissue (3C) as compared with normal tissue (3N); **C**: Brown-yellow granules of nm23H1 protein mostly located in membrane. The stained nucleolus and cytoplasm of cells were observed (arrow, x 400); **D**: Control group, PBS replaced anti-nm23H1 protein as the first antibody (x 200).

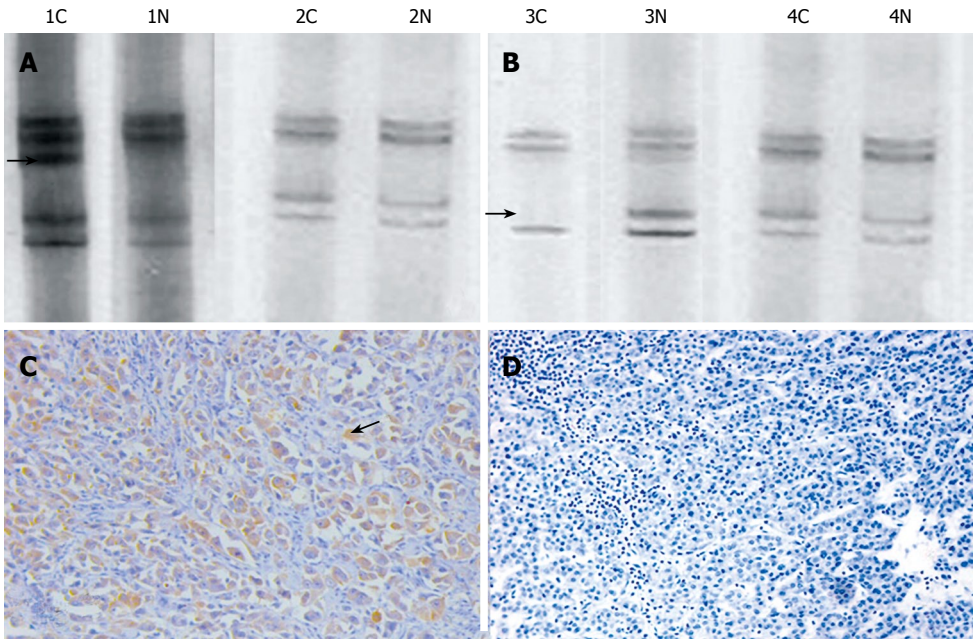


Figure 3 PAGE of D17S396 locus and immunohistochemistry of nm23H1 protein in HCC. **A**: No difference in tumor tissue (2C) and normal tissue (2N). MSI was positive when an allele band was added (arrow) in tumor tissue (1C) as compared with normal tissue (1N); **B**: No difference in tumor tissue (4C) and normal tissue (4N). LOH was positive in the absence of an allele band (arrow) in tumor tissue (3C) as compared with normal tissue (3N); **C**: Brown-yellow granules of nm23H1 protein mostly located in cytoplasm. The stained nucleolus and membrane of cells were observed (arrow, x 400); **D**: Control group, PBS replaced anti-nm23H1 protein as the first antibody (x 200).

The positive rate of nm23H1 protein was related with lymph node metastasis and clinical stage of HCC. The expression of nm23H1 protein was higher in stage Ⅰ + Ⅱ than in stage Ⅲ + Ⅳ of HCC (75.86% *vs* 26.32%, $P < 0.0001$), and in negative lymph node metastasis patients than in positive lymph node metastasis patients (82.14% *vs* 20.00%, $P < 0.0001$). The same phenomenon occurred in negative and positive LOH patients (64.86% *vs* 27.27%, $P = 0.027$, Table 2). However, MSI had no effect on the expression of nm23H1 protein (Table 2). Computer imaging analysis showed that there was no difference in the nm23H1 protein expression level between the two groups of patients (Table 3).

Relationship between clinical pathological parameters and nm23H1 genetic instability in gallbladder carcinoma

In gallbladder carcinoma, the positive rate of D17S396 MSI (Figure 4A), LOH (Figure 4B) and nm23H1 protein (Figure 4C) was 22.40%, 19.14% and 46.81%, respectively, in 47 cases of gallbladder carcinoma (Table 6).

MSI and LOH were correlated to the differentiation degree, liver infiltration, lymph node metastasis and TNM staging of gallbladder carcinoma. MSI trended to increase with the differentiation degree of gallbladder carcinoma ($P < 0.0001$). Furthermore, MSI was higher in negative than in positive liver infiltration patients (34.48% *vs* 5.56%, $P = 0.023$), and in negative than in positive lymph node metastasis patients (37.04% *vs* 5.00%, $P < 0.0001$). The frequency of MSI was higher in stage Ⅰ + Ⅱ than in stage Ⅲ + Ⅳ of gallbladder carcinoma (43.48% *vs* 4.17%, $P = 0.001$). On the other hand, the frequency LOH was lower than that of MSI (6.90% *vs* 38.89%, $P = 0.006$).

The positive rate of nm23H1 protein was higher in

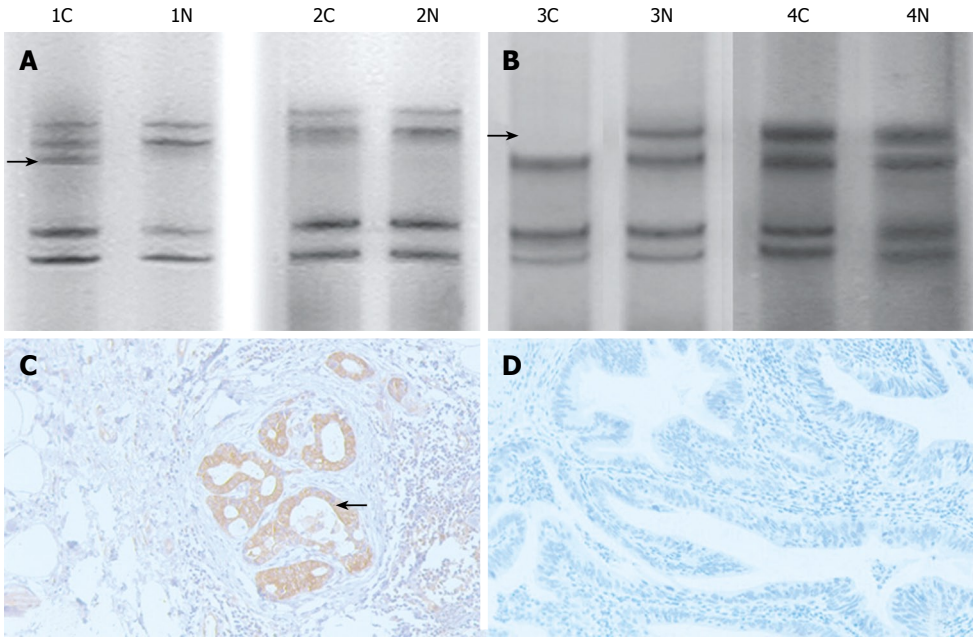


Figure 4 PAGE of D17S396 locus and immunohistochemistry of nm23H1 protein in gallbladder carcinoma. **A**: No difference in tumor tissue (2C) and normal tissue (2N). MSI was positive when an allele band was added (arrow) in tumor tissue (1C) as compared with normal tissue (1N); **B**: No difference in tumor tissue (4C) and normal tissue (4N). LOH was positive in the absence of an allele band (arrow) in tumor tissue (3C) as compared with normal tissue (3N); **C**: Brown-yellow granules of nm23H1 protein mostly located in cytoplasm, and part of the stained nucleolus and membrane of cells were observed (arrow, × 200); **D**: Control group, PBS replaced anti-nm23H1 protein as the first antibody (× 200).

Table 5 Relationship between clinical pathological parameters and nm23H1 genetic instability in HCC

Clinical pathological factors	Cases	MSI+ (%)	LOH+ (%)	nm23H1+ (%)	nm23H1 expression intensity (mean ± SD)
Differentiation degree	48	6 (12.50)	11 (22.92)	27 (56.25)	25.96 ± 2.53
High differentiation	15	3 (20.00)	2 (13.33)	10 (66.67)	27.87 ± 2.83
Middle differentiation	20	2 (10.00)	5 (25.00)	12 (60.00)	22.37 ± 2.35
Low differentiation	13	1 (7.70)	4 (30.77)	5 (38.46)	22.58 ± 2.56
Liver infiltration					
Positive	15	0 (00.00)	8 (53.33)	6 (40.00)	23.84 ± 2.43
Negative	33	6 (18.18)	3 (9.09)	21 (63.64)	25.63 ± 2.63
Lymph node metastasis					
Positive	20	1 (5.00)	9 (45.00)	4 (20.00)	21.67 ± 2.56
Negative	28	5 (17.86)	2 (7.14)	23 (82.14)[1]	28.56 ± 2.01
TNM stage					
Ⅰ + Ⅱ	29	5 (17.24)	1 (3.45)	22 (75.86)	25.58 ± 2.33
Ⅲ + Ⅳ	19	1 (5.26)	10 (52.63)[2]	5 (26.32)[2]	24.27 ± 2.27

[1]$P < 0.0001$ *vs* positive lymph node metastasis; [2]$P < 0.0001$ *vs* TNM stage Ⅰ + Ⅱ.

Table 6 Relationship between clinical pathological parameters and nm23H1 genetic instability in gallbladder cancer

Clinical pathological factors	Cases	MSI + (%)	LOH + (%)	nm23H1 + (%)	nm23H1 expression intensity (mean ± SD)
Differentiation degree	47	11 (22.40)	9 (19.14)	22 (46.81)	25.96 ± 2.73
High differentiation	17	9 (52.94)	0 (00.00)	10 (58.82)	24.15 ± 2.49
Middle differentiation	13	2 (15.38)	3 (23.08)	7 (53.85)	27.07 ± 2.53
Low differentiation	17	0 (00.00)[1]	6 (35.29)[2]	5 (29.41)	28.03 ± 2.63
Liver infiltration					
Positive	18	1 (5.56)	7 (38.89)	6 (33.33)	24.87 ± 2.83
Negative	29	10 (34.48)[3]	2 (6.90)[4]	16 (55.17)	26.37 ± 2.45
lymph node metastasis					
Positive	20	1 (5.00)	8 (40.00)	5 (25.00)	23.56 ± 2.19
Negative	27	10 (37.04)[5]	1 (3.70)[6]	17 (62.96)[7]	26.67 ± 2.33
TNM stage					
Ⅰ + Ⅱ	23	10 (43.48)	0 (00.00)	15 (65.22)	24.15 ± 2.78
Ⅲ + Ⅳ	24	1 (4.17)[8]	9 (37.50)[9]	7 (29.17)[10]	29.84 ± 2.53

[1]$P < 0.0001$; [2]$P = 0.008$ *vs* differentiation degree; [3]$P = 0.023$; [4]$P = 0.006$ *vs* positive liver infiltration; [5]$P < 0.0001$, [6]$P = 0.001$, [7]$P = 0.009$ *vs* positive lymph node metastasis; [8]$P = 0.001$; [9]$P < 0.0001$; [10]$P = 0.013$ *vs* TNM stage Ⅰ + Ⅱ.

stage Ⅰ + Ⅱ than in stage Ⅲ + Ⅳ gallbladder carcinoma (65.22% *vs* 29.17%, $P = 0.013$), and in negative than in positive lymph node metastasis patients (62.96% *vs* 25.00%, $P = 0.009$). The same phenomenon occurred

in the negative and positive LOH patients (55.26% *vs* 11.11%, $P = 0.016$, Table 2). However, MSI had no effect on the expression of nm23H1 protein. Computer imaging analysis showed that there was no difference in nm23H1 protein expression level between the two groups of patients (Table 3).

DISCUSSION

Microsatellites are short tandem repeat sequences with unknown functions scattered throughout the human genome. LOH and MSI are the phenotypes with genetic instability caused by abnormalities of tumor suppressor and DNA mismatch repair (MMR) genes. MSI is associated with slippage of DNA polymerase during DNA synthesis resulting in changing units of repetitive sequences, while LOH is allelic loss in a certain region of chromosome. Genetic instability of oncogenes, such as MSI and LOH, is probably associated with mutations of genes responsible for tumor-genesis, and plays an important role in tumor pathology[17].

MSI was first found in hereditary non-polyposis colorectal cancer (HNPCC), and then in some kinds of sporadic tumors, such as colon cancer[18], gastric cancer[19], uterus cancer[20], breast cancer[21], prostate cancer[22] and pancreatic cancer[23]. Alexander[24] examined the utility of histopathology for the identification of MSI-H cancers by evaluating the features of 323 sporadic carcinomas using specified criteria and comparing the results to MSI-H status, showing that MSI is more often found in patients with early stage cancers. In our experiment, the MSI frequency was significantly correlated with the clinical TNM staging and lymph node metastasis of gastric, colonic and gallbladder cancers. The frequency of MSI was higher in stages Ⅰ and Ⅱ than in stages Ⅲ and Ⅳ of these cancers. Furthermore, the frequency tended to decrease with lymph node metastasis. Our data indicate that MSI of nm23H1 may be a molecule marker of early digestive system cancer.

Berney[25] investigated the relationship between LOH and MSI in liver metastasis and nm23 protein expression, showing that the increasing proportion of LOH in nm23H1 is positively associated with liver metastasis. Candusso[26] found that LOH often occurs in later stage cancer with lymph metastasis. The frequency of LOH in 4 kinds of digestive system cancer in our study was significantly higher in stage TNM Ⅲ + Ⅳ than in stage TNM Ⅰ + Ⅱ. Moreover, the frequency of LOH was significantly higher in patients with lymph node metastasis than in those without lymph node metastasis, suggesting that LOH of nm23H1 gene may occur in later stage digestive system cancer and accelerate lymphatic metastasis, and thus can be used as an estimate marker for malignant degree, lymphatic metastasis and prognosis. In the present study, the difference in pathology of MSI and LOH had a strong influence on the four digestive system cancers, indicating that MSI and LOH can control the carcinogenesis and metastasis through different pathways.

Lower expression of nm23H1 is closely associated with high metastasis potential and poor prognosis of human mammary cancer, gastric cancer, lung cancer, melanoma, and ovary cancer. The lower the nm23H1 expression is, the poorer the prognosis is. Steeg[27] found that nm23H1 can prevent tumor metastasis by inhibiting the ability of cancer cells to clone. In our study, TNM stage, lymphatic metastasis, and local expression of nm23H1 protein were significantly reduced in gastric cancer, colonic cancer, HCC and gallbladder cancer. Also, the expression of m23H1 protein was higher in gastric and colon cancer, which was associated with the degree of differentiation. These findings suggest that nm23H1 is actively involved in cancer metastasis as a prohibitive gene, and restrains the digestive system cancer, and can serve as a valuable biological marker in the evaluation of tumor development and prognosis.

In our study, the frequency of nm23H1 protein was higher in negative than in positive LOH patients, suggesting that LOH on D17S396 can induce loss or aberrance of anti-oncogenes, and decrease the expression of nm23H1 protein, which endows cancer with a high invasiveness and a poor prognosis. In contrast, the frequency of nm23H1 protein was higher in positive than in negative MSI patients, suggesting that MSI can accelerate the expression of nm23H1 protein. However, its molecular mechanism is not clear, and remains to be elucidated.

COMMENTS

Background

nm23H1 has been regarded as a metastasis-associated gene in various tumors. Genetic instabilities of oncogenes, such as microsatellite instability (MSI) and loss of heterozygosity (LOH), are probably associated with mutations of genes responsible for tumor-genesis, and play an important role in tumor pathology. Studies on MSI and LOH of digestive system cancer have been focused on the genetic instability of P53, P16 and FHIT, but few studies are available on gene nm23H1.

Research frontiers

The present study investigated the MSI and LOH of gene nm23H1 at locus D17S396 in Chinese patients with digestive system cancers and their influence on the nm23H1 protein expression, to reveal their clinical pathological characteristics.

Innovations and breakthroughs

The results of this study indicate that MSI and LOH may independently control the biological behaviors of digestive system cancers. Enhanced expression of nm23H1 protein could efficiently inhibit cancer metastasis and improve the prognosis.

Applications

MSI could serve as an early biological marker of digestive system cancers. The data strongly suggest that LOH usually appears in the late digestive system cancer, indicating a higher malignancy and a poor prognosis.

Peer review

This is an interesting study, which showed a certain significant correlation between nm23H1 genetic instability and clinical pathological characteristics of different kinds of digestive system cancer, which may serve as a marker for the diagnosis of such cancers.

REFERENCES

1 **Cai YC**, So CK, Nie AY, Song Y, Yang GY, Wang LD, Zhao X, Kinzy TG, Yang CS. Characterization of genetic alteration patterns in human esophageal squamous cell carcinoma using selected microsatellite markers spanning multiple

loci. *Int J Oncol* 2007; **30**: 1059-1067
2 **Chakrabarti S**, Sengupta S, Sengupta A, Basak SN, Roy A, Panda C, Roychoudhury S. Genomic instabilities in squamous cell carcinoma of head and neck from the Indian population. *Mol Carcinog* 2006; **45**: 270-277
3 **Inoue Y**, Miki C, Watanabe H, Ojima E, Kusunoki M. Genomic instability and tissue expression of angiogenic growth factors in sporadic colorectal cancer. *Surgery* 2006; **139**: 305-311
4 **Nowacka-Zawisza M**, Brys M, Hanna RM, Zadrozny M, Kulig A, Krajewska WM. Loss of heterozygosity and microsatellite instability at RAD52 and RAD54 loci in breast cancer. *Pol J Pathol* 2006; **57**: 83-89
5 **Yoshida K**, Miki Y. Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription, and cell cycle in response to DNA damage. *Cancer Sci* 2004; **95**: 866-871
6 **Juvan R**, Hudler P, Gazvoda B, Repse S, Bracko M, Komel R. Significance of genetic abnormalities of p53 protein in Slovenian patients with gastric carcinoma. *Croat Med J* 2007; **48**: 207-217
7 **Xiao YP**, Wu DY, Xu L, Xin Y. Loss of heterozygosity and microsatellite instabilities of fragile histidine triad gene in gastric carcinoma. *World J Gastroenterol* 2006; **12**: 3766-3769
8 **Horak CE**, Lee JH, Elkahloun AG, Boissan M, Dumont S, Maga TK, Arnaud-Dabernat S, Palmieri D, Stetler-Stevenson WG, Lacombe ML, Meltzer PS, Steeg PS. Nm23-H1 suppresses tumor cell motility by down-regulating the lysophosphatidic acid receptor EDG2. *Cancer Res* 2007; **67**: 7238-7246
9 **Hsu NY**, Chen CY, Hsu CP, Lin TY, Chou MC, Chiou SH, Chow KC. Prognostic significance of expression of nm23-H1 and focal adhesion kinase in non-small cell lung cancer. *Oncol Rep* 2007; **18**: 81-85
10 **Korabiowska M**, Honig JF, Jawien J, Knapik J, Stachura J, Cordon-Cardo C, Fischer G. Relationship of nm23 expression to proliferation and prognosis in malignant melanomas of the oral cavity. *In Vivo* 2005; **19**: 1093-1096
11 **Ouatas T**, Salerno M, Palmieri D, Steeg PS. Basic and translational advances in cancer metastasis: Nm23. *J Bioenerg Biomembr* 2003; **35**: 73-79
12 **Ferrari D**, Lombardi M, Ricci R, Michiara M, Santini M, De Panfilis G. Dermatopathological indicators of poor melanoma prognosis are significantly inversely correlated with the expression of NM23 protein in primary cutaneous melanoma. *J Cutan Pathol* 2007; **34**: 705-712
13 **Guan-Zhen Y**, Ying C, Can-Rong N, Guo-Dong W, Jian-Xin Q, Jie-Jun W. Reduced protein expression of metastasis-related genes (nm23, KISS1, KAI1 and p53) in lymph node and liver metastases of gastric cancer. *Int J Exp Pathol* 2007; **88**: 175-183
14 **Kapitanovic S**, Cacev T, Berkovic M, Popovic-Hadzija M, Radosevic S, Seiwerth S, Spaventi S, Pavelic K, Spaventi R. nm23-H1 expression and loss of heterozygosity in colon adenocarcinoma. *J Clin Pathol* 2004; **57**: 1312-1318
15 **Sgouros J**, Galani E, Gonos E, Moutsatsou P, Belechri M, Skarlos D, Dionyssiou-Asteriou A. Correlation of nm23-H1 gene expression with clinical outcome in patients with advanced breast cancer. *In Vivo* 2007; **21**: 519-522
16 **Jackson AL**, Loeb LA. Microsatellite instability induced by hydrogen peroxide in Escherichia coli. *Mutat Res* 2000; **447**: 187-198
17 **Storchova Z**, Pellman D. From polyploidy to aneuploidy, genome instability and cancer. *Nat Rev Mol Cell Biol* 2004; **5**: 45-54
18 **Svrcek M**, El-Bchiri J, Chalastanis A, Capel E, Dumont S, Buhard O, Oliveira C, Seruca R, Bossard C, Mosnier JF, Berger F, Leteurtre E, Lavergne-Slove A, Chenard MP, Hamelin R, Cosnes J, Beaugerie L, Tiret E, Duval A, Flejou JF. Specific clinical and biological features characterize inflammatory bowel disease associated colorectal cancers showing microsatellite instability. *J Clin Oncol* 2007; **25**: 4231-4238
19 **Sakurai M**, Zhao Y, Oki E, Kakeji Y, Oda S, Maehara Y. High-resolution fluorescent analysis of microsatellite instability in gastric cancer. *Eur J Gastroenterol Hepatol* 2007; **19**: 701-709
20 **An HJ**, Kim KI, Kim JY, Shim JY, Kang H, Kim TH, Kim JK, Jeong JK, Lee SY, Kim SJ. Microsatellite instability in endometrioid type endometrial adenocarcinoma is associated with poor prognostic indicators. *Am J Surg Pathol* 2007; **31**: 846-853
21 **Pizzi C**, Di Maio M, Daniele S, Mastranzo P, Spagnoletti I, Limite G, Pettinato G, Monticelli A, Cocozza S, Contegiacomo A. Triplet repeat instability correlates with dinucleotide instability in primary breast cancer. *Oncol Rep* 2007; **17**: 193-199
22 **Burger M**, Denzinger S, Hammerschmied CG, Tannapfel A, Obermann EC, Wieland WF, Hartmann A, Stoehr R. Elevated microsatellite alterations at selected tetranucleotides (EMAST) and mismatch repair gene expression in prostate cancer. *J Mol Med* 2006; **84**: 833-841
23 **House MG**, Herman JG, Guo MZ, Hooker CM, Schulick RD, Cameron JL, Hruban RH, Maitra A, Yeo CJ. Prognostic value of hMLH1 methylation and microsatellite instability in pancreatic endocrine neoplasms. *Surgery* 2003; **134**: 902-908; discussion 909
24 **Alexander J**, Watanabe T, Wu TT, Rashid A, Li S, Hamilton SR. Histopathological identification of colon cancer with microsatellite instability. *Am J Pathol* 2001; **158**: 527-535
25 **Berney CR**, Fisher RJ, Yang J, Russell PJ, Crowe PJ. Genomic alterations (LOH, MI) on chromosome 17q21-23 and prognosis of sporadic colorectal cancer. *Int J Cancer* 2000; **89**: 1-7
26 **Candusso ME**, Luinetti O, Villani L, Alberizzi P, Klersy C, Fiocca R, Ranzani GN, Solcia E. Loss of heterozygosity at 18q21 region in gastric cancer involves a number of cancer-related genes and correlates with stage and histology, but lacks independent prognostic value. *J Pathol* 2002; **197**: 44-50
27 **Palmieri D**, Horak CE, Lee JH, Halverson DO, Steeg PS. Translational approaches using metastasis suppressor genes. *J Bioenerg Biomembr* 2006; **38**: 151-161

S- Editor Zhong XY **L- Editor** Wang XL **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5557

World J Gastroenterol 2008 September 28; 14(36): 5557-5563
World Journal of Gastroenterology ISSN 1007-9327

Gene expression and MR diffusion-weighted imaging after chemoembolization in rabbit liver VX-2 tumor model

You-Hong Yuan, En-Hua Xiao, Jian-Bin Liu, Zhong He, Ke Jin, Cong Ma, Jun Xiang, Jian-Hua Xiao, Wei-Jian Chen

You-Hong Yuan, Jian-Bin Liu, Department of Radiology, Hunan Province People's Hospital, Changsha 410005, Hunan Province, China
En-Hua Xiao, Zhong He, Cong Ma, Jun Xiang, Department of Radiology, the Second Xiang-Ya Hospital, Central South University, Changsha 410005, Hunan Province, China
Ke Jin, Wei-Jian Chen, Department of Pathology, Hunan Province Children's Hospital, Changsha 410005, Hunan Province, China
Jian-Hua Xiao, Department of Epidemiology, Center for Disease Control of Hunan Province, Changsha 410005, Hunan Province, China
Author contributions: Yuan YH, Xiao EH designed research; Yuan YH, Xiao EH, Liu JB, He Z, Jin K, Ma C, Xiang J, Xiao JH, Chen WJ performed research; Yuan YH analyzed data; and Yuan YH, Xiao EH wrote the paper.
Supported by The National Natural Science Foundation of China, No. 30070235 and 30470508; The Natural Science Foundation of Hunan Province, No. 08JJ5043; The Science and Technology Foundation of Hunan Province, No. 06FJ3120 and 2007SK3072; and The Medical Science and Technology Foundation of Hunan Province, No. B2006-159
Correspondence to: Dr. You-Hong Yuan, Department of Radiology, Hunan Province People's Hospital, Changsha 410005, Hunan Province,
China. heyuanyouhong@yahoo.com.cn
Telephone: +86-731-2278047 Fax: +86-731-2278047
Received: Mary 3, 2008 Revised: August 26, 2008
Accepted: September 2, 2008
Published online: September 28, 2008

Abstract

AIM: To investigate the dynamic characteristics and the correlation between PCNA, Bax, nm23, E-cadherin expression and apparent diffusion coefficient (ADC) on MR diffusion-weighted imaging (DWI) after chemoembolization in rabbit liver VX-2 tumor model.

METHODS: Forty New Zealand rabbit liver VX-2 tumor models were included in the study. DWI was carried out periodically after chemoembolization. All VX-2 tumor samples in each group were examined by histopathology and Strept Avidin-Biotin Complex (SABC) immunohistochemical staining.

RESULTS: The PCNA expression index in VX-2 tumors was higher than in the normal parenchyma around the tumor ($P < 0.001$). Nm23, Bax or E-caderin expression index in VX-2 tumors were lower than in the normal parenchyma around the tumor (all $P < 0.001$). PCNA and nm23 expression in the VX-2 tumor periphery first increased and then decreased ($P < 0.001$ and $P = 0.03$, respectively), while the expression of Bax and E-cadherin before and after chemoembolization was insignificant. When b-value was 100 s/mm^2, there was a linear correlation between PCNA expression and ADC in the area of VX-2 tumor periphery ($P < 0.001$), and PCNA expression in VX-2 tumor periphery influenced the ADC.

CONCLUSION: The potential of VX-2 tumor infiltrating and metastasizing decreases, while its ability to proliferate increases for a short time after chemoembolization. To some degree, the ADC value indirectly reflects the proliferation of VX-2 tumor cells.

Key words: Rabbit liver; VX-2 tumor; Chemoembolization; Diffusion-weighted imaging; Gene expression

Peer reviewer: Dr. Serdar Karakose, Professor, Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey

Yuan YH , Xiao EH , Liu JB, He Z, Jin K, Ma C, Xiang J, Xiao JH, Chen WJ. Gene expression and MR diffusion-weighted imaging after chemoembolization in rabbit liver VX-2 tumor model. *World J Gastroenterol* 2008; 14(36): 5557-5563 Available from: URL: http://www.wjgnet.com/1007-9327/14/5557.asp DOI: http://dx.doi.org/10.3748/wjg.14.5557

INTRODUCTION

Hepatocellular carcinoma is one of the most common malignant tumors worldwide. The genetic mutation and abnormal gene expression of hepatocellular carcinoma during its origin, progression and after transcatheter arterial chemoembolization (TACE) have been investigated in several clinical and experimental studies, with respect to PCNA, Bax, E-caderin (E-cad) and nm23 gene expression

The expression of Proliferating Cell Nuclear Antigen (PCNA) is high in the S, G_2 and M stages of cell division, and low in the G_0 and G_1 stages. The degree of PCNA expression reflects cellular reproductive activity, which correlates significantly and positively with

the pathological grade, the potential for recurrence, and survival time of malignant tumors. Therefore, it is important to measure the degree of PCNA expression in evaluating the malignant potential and prognosis of tumors[1-5]. Shi *et al*[4] observed that the degree of PCNA expression correlated with the size, invasive potential and degree of enhancement on CT scan of hepatocellular carcinoma. As a member of the Bcl-2 family, Bax gene is an important apoptotic controlling gene. Most investigations have demonstrated that Bax is a protein-inducing apoptosis, which promotes cell apoptosis and suppresses cell proliferation through multiple mechanism[6-9]. The nm23-H1 gene expresses nucleoside diphosphate kinase (ndpk), and has a significant impact on tumor metastasis. As reported in several studies, low expression of nm23 and the absence of alleles correlates with a high rate of infiltration and metastasis in many tumors, including hepatocellular carcinoma[4-10]. The coding product of E-cad gene is a transmembrane glycoprotein that is calcium depended and is able to mediate adhesion between cells. When it is low or absent, the adhesion between cells decreases, enabling the cancer cells to separate easily from the primary tumor, resulting in local infiltration and distant metastasis[10].

Diffusion-weighted imaging (DWI) is used much less frequently in the diagnosis of hepatic tumor and for the evaluation of tumor progression, because of its poor imaging quality[11-14]. With the development of MRI software and scanning technology, especially for echoplanar imaging (EPI) in recent years, many deficiencies, such as poor imaging quality and slow scanning speed, have been resolved[15-17]. As reported by several workers (Ichikawa *et al*[18,19], Yamashita *et al*[20], Taouli *et al*[21], Sun *et al*[22] and Yang *et al*[23,24]), the apparent diffusion coefficient (ADC) of hepatic cysts, hemangioma, hepatocellular carcinoma, metastatic lesions, normal parenchyma and liver cirrhosis decrease gradually on DWI. The ADC of hepatic cysts is the highest, while ADC of benign tumors is higher than that of malignant tumors. Colagrande *et al*[25] demonstrated that coagulation necrosis had low signal compared to viable tumor. Kamel *et al*[26] confirmed these findings in a study on 8 patients with hepatocellular carcinoma; ADC increased with the degree of tumor necrosis, and in 6 tumors the DWI values were higher compared to the normal parenchyma. Based on these observations, DWI, and especially ADC have potential value of differentiating benign tumor from malignant tumor.

However, few studies have been carried out to investigate the dynamic characteristics of hepatocellular carcinoma gene expression after chemoembolization. Moreover, the ADC of hepatocellular carcinoma on DWI has not been investigated by image-pathology-gene expression.

The purpose of the present study was to investigate the dynamic characteristics of PCNA, Bax, nm23 and E-cad expression after chemoembolization, and to evaluate the correlation between ADC on DWI and gene expression in rabbit VX-2 tumor model. However, the signal dynamic characteristics on DWI and the pathological mechanisms will be discussed in separate report.

MATERIALS AND METHODS

Animals and establishment of VX-2 tumor model

Animal studies were carried out under the supervision of a veterinarian according to the guidelines on the Use of Laboratory Animals of the Ministry of Public Health of China. All animals were provided by the Laboratory Animal Center of the Second Xiangya Hospital and all protocols were approved by the Animal Use and Care Committee of the Second Xiangya Hospital.

Forty New Zealand rabbits were included in the study. Twenty-two were male and eighteen were female rabbits. The weight ranged from 1.7 kg to 2.5 kg, and the ages from 5 mo to 6 mo. All New Zealand white rabbits were healthy. Forty-seven rabbit VX-2 tumor models were developed by implanting directly into the liver, after opening the abdominal cavity. The rabbit VX-2 tumor strain was provided by the Fourth Military Medical University.

Forty VX-2 tumor models were chosen randomly from forty-seven VX-2 tumor models and were divided into four groups, a control group (non-interventional group, group A) and three investigation groups (group A, 16 h after chemoembolization; group B, 32 h after chemoembolization; and group C, 48 h after chemoembolization).

Interventional protocol

After DWI was performed on the 21st day after implantation, trans-hepatic artery catheterization chemoembolization was carried out in the animal operating room of the Second Xiangya Hospital, in study groups B, C and D.

First, the rabbits were anesthetized by injecting 3% soluble pentobarbitone. The abdominal skin was then disinfected, and the abdomen was opened by incising through the skin and the musculi recti abdominis. The organs of hepatic hilar region were exposed and the different structures such as arteria coeliaca, arteria hepatica communis, arteria hepatica propria, arteria gastroduodenalis and portal vein were recognized. The distal end of arteria gastroduodenalis was occluded, and a drop of aethocaine was placed on it. The arteria gastroduodenalis was punctured and a plastic transfixion pin was introduced and advanced into arteria hepatica propria, followed by infusion of iodized oil (0.3 mL/kg) and pharmorubicin (2 mg/kg). Finally, after making certain that there was no bleeding from the liver and other structures, the abdominal membrane, musculature and skin were sutured layer by layer.

Magnetic resonance imaging protocol

After the animals were anesthetized with 3% soluble pentobarbitone at a dose of 1 mL/kg or with different

Table 1 Gene expression of different areas of VX-2 tumour and the normal parenchyma around the tumor (%)

	PCNA	nm23	Bax	E-cad
Normal parenchyma	8.28 ± 5.61	16.53 ± 14.66	40.00 ± 23.72	78.15 ± 18.35
Tumor outer layer	65.45 ± 3.43	1.74 ± 4.07	1.91 ± 2.66	6.18 ± 7.55
Tumor periphery area	72.73 ± 14.23	0.42 ± 0.64	1.05 ± 1.09	1.96 ± 1.79
Tumor center area	58.37 ± 15.25	6.74 ± 10.13	2.16 ± 4.59	1.55 ± 3.65

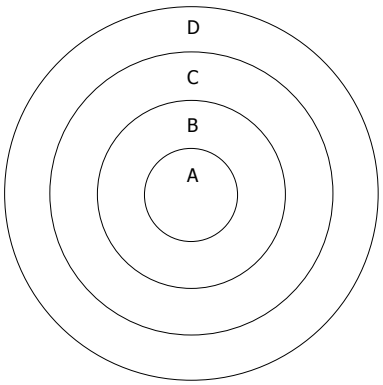


Figure 1 A-D represent VX-2 tumor center, VX-2 tumor periphery, VX-2 tumor outer layer and normal liver parenchyma around tumor respectively. The ADC levels and signal were measured on DWI, and the samples were investigated by histopathology.

doses based on the animal status, in order to make certain that the animals had stable breathing, DWI(axial) was performed on a 1.5-Tesla Signa Twinspeed MR scanner (General Electron Medical Systems, USA), using a small diameter cylindrical brain radiofrequency coil. The scans were done before chemoembolization, and again at 6 h, 16 h, 32 h and 48 h after chemoembolization. The DWI scanning parameters included spin echo echoplanar imaging (SE-EPI) series, *b*-value 100 and 300 s/mm^2, repetition time (TR) 6000 ms, echo time (TE) 45 ms, 20 cm × 15 cm field of view (FOV), 8 NEX, 2 mm thickness layer, and 0.5 mm Space, 128 × 128 matrix.

The ADC values and signal values were obtained in the VX-2 tumor periphery, VX-2 tumor center and normal liver parenchyma around the tumor (Figure 1) using Function software in GE workstation. Three different regions of interest (ROIs, 50 mm^2 each area) were chosen in the normal liver parenchyma (area D in Figure 1). The average reading was considered as the ADC value or the signal value of the normal liver parenchyma around the tumor. The thickness of the area A and B in Figure 1 was respectively two fifth of the diameter of the VX-2 tumor. Similarly, the average readings of three different ROIs ADC values or signal values in area B were considered as the ADC value or the signal value of VX-2 tumor periphery. The ADC value or signal value of area A was the center of the VX-2 tumor. All measurements were made by two senior attending physicians or associate professors.

Immunohistochemical staining protocol

DWI was performed before chemoembolization, and 16 h, 32 h and 48 h after chemoembolization. The rabbits were then sacrificed by injecting an overdose of 3% soluble pentobarbitone. Under aseptic conditions, we obtained layer by layer VX-2 samples (Figure 1). Each VX-2 tumor was divided into the outer layer area, the periphery area and center area (Figure 1). All tissue samples were fixed in formaldehyde for 24 h before embedding in mineral wax.

Immunohistochemical staining was performed, using the SP method. Monoclonal antibody against PCNA, Bax, nucleoside diphosphate kinase (ndpk, expression product of nm23 gene) and e-cadherin endogenous avidin and biotin block ade solution, biotin-marked SP were used for immunostaining. PBS was used as a negative control, and all the results were negative. A known positive specimen of mammary adenocarcinoma was used as a positive control for PCNA, Bax and ndpk instead of PHC tissue, while pancreatic carcinoma was used for E-cad; the results were all positive.

Expression index was used to estimate the degree of gene expression, which was based on the contrast of positive cells with the total number of cells, as seen under the microscope. When the cell nucleus appeared as brown-yellow with PCNA immunohistochemical staining, the PCNA expression was considered as positive. When cytolymph appeared as brown-yellow in Bax, nm23 or E-cad immunohistochemical staining, the test was considered as positive. Ten random locations were examined under a 400 × microscope in non-necrotic areas, and the number of PCNA, Bax, nm23 and E-cad expressing cells as well as the total number of cells were counted in a double blind fashion by two investigators who were not part of our study group. The average value was considered as the expression index. When the antibody was replaced by the balanced solution, all the gene expressions were negative.

Statistical analysis

Based on the apparent diffusion coefficient (ADC) value of ROIs and expression index of PCNA, Bax, nm23 and E-cad, the differences between the different areas, the different time groups and the correlation between ADC and gene expression was assessed. The statistical significance was calculated by analysis of variance (ANOVA), non-parameter and multiple linear regression, using the SPSS 12.0 software.

RESULTS

Gene expression information of VX-2 tumor

The results of PCNA, nm23, Bax and E-cad expression in VX-2 tumors and in the normal parenchyma around the tumor before chemoembolization are shown in Table 1 and Figure 2.

The differences in the PCNA, Bax, nm23 and

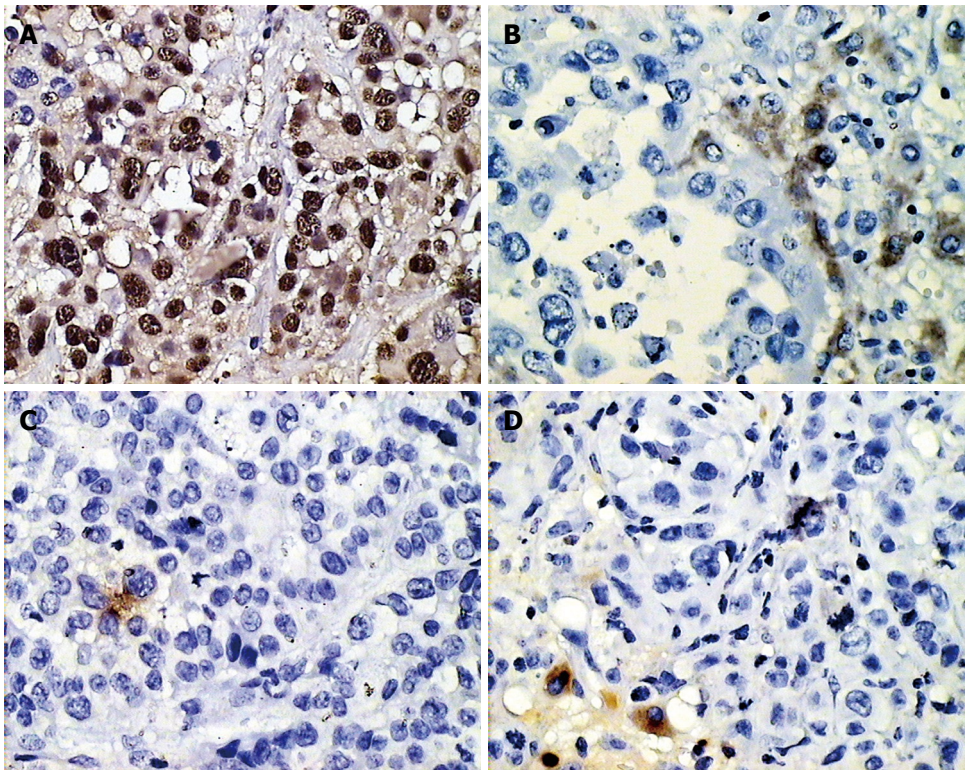


Figure 2 Positive gene expression in VX-2 tumor. **A**: PCNA; **B**: Bax; **C**: nm23; **D**: E-cad.

Table 2 Dynamic information of the gene expression in the peripheral area of VX-2 tumor and the normal parenchyma around the tumor (%)

Time	Area	PCNA	nm23	Bax	E-cad
A time	E	72.73 ± 14.23	0.42 ± 0.64	1.05 ± 1.09	1.96 ± 1.79
	F	8.28 ± 5.61	16.53 ± 14.66	40.00 ± 23.72	78.15 ± 18.35
B time	E	80.04 ± 22.84	2.29 ± 1.58	0.86 ± 1.44	2.87 ± 2.04
	F	4.04 ± 2.06	57.35 ± 28.36	81.22 ± 23.31	86.41 ± 12.65
C time	E	92.60 ± 4.76	7.56 ± 8.88	0.45 ± 0.57	0.93 ± 1.48
	F	21.87 ± 24.59	67.64 ± 30.54	85.63 ± 20.51	91.03 ± 9.65
D time	E	87.63 ± 7.12	4.14 ± 4.71	0.78 ± 1.05	1.07 ± 0.81
	F	19.38 ± 17.74	40.84 ± 34.90	87.08 ± 10.16	91.03 ± 5.11

A time is before chemoembolization or on 21th day after implantation. B time, C time, and D time represent samples obtained after 16 h, 32 h and 48 h after chemoembolization respectively. E indicates the peripheral area of VX-2 tumor and F normal parenchyma around VX-2 tumor.

E-cad expression index in the different areas before chemoembolization were statistically significant (χ^2 = 19.08, $P < 0.001$; χ^2 = 20.165, $P < 0.001$; χ^2 = 12.86, $P < 0.001$; χ^2 = 22.20, $P < 0.001$). The differences in the PCNA, Bax, nm23 and E-cad expression index between the tumor periphery and the normal parenchyma around the tumor were statistically significant (Z = -5.51, $P < 0.001$; Z = -5.39, $P < 0.001$; Z = -5.51, P = 0.003; Z = -5.511, $P < 0.001$).

The results of PCNA, nm23, Bax and E-cad expression in groups A, B, C, and D in the VX-2 tumor periphery and normal parenchyma around the tumor are shown in Table 2 and Figure 2.

The differences in the PCNA, nm23 and Bax expression in the normal parenchyma between groups A, B, C, and D were statistically significant (χ^2 = 10.92, P = 0.04; χ^2 = 14.83, $P < 0.001$; χ^2 = 17.28, P = 0.03), but E-cad expression was not significant (χ^2 = 3.410, $P > 0.05$). The differences in PCNA and nm23 expression in the tumor periphery between groups A, B, C, and D were significant (χ^2 = 14.37, $P < 0.001$; χ^2 = 8.94, P = 0.03) but that for Bax and E-cad were not significant (χ^2 = 1.98, $P > 0.05$; χ^2 = 3.88, $P > 0.05$).

Table 3 ADC values of the tumor and normal parenchyma (mean ± SD, × 10^{-3} mm^2/s)

	ADC in tumor periphery area		ADC in normal parenchyma	
	b = 100	b = 300	b = 100	b = 300
A time	1.71 ± 0.27	1.48 ± 0.23	2.71 ± 0.42	2.30 ± 0.40
B time	1.24 ± 0.22	1.12 ± 0.20	2.10 ± 0.54	1.65 ± 0.37
C time	1.48 ± 0.37	1.23 ± 0.16	2.10 ± 0.49	1.97 ± 0.29
D time	1.57 ± 0.23	1.40 ± 0.18	2.43 ± 0.33	2.06 ± 0.23

A time is before chemoembolization or on the 21th day after implantation. B time, C time, and D time represent samples obtained after 16 h, 32 h and 48 h after chemoembolization respectively.

Dynamic ADC values before and after chemoembolization (Table 3, Figures 3 and 4)

When the b-value was 100 s/mm^2, there was a significant correlation between the expression index of PCNA, nm23, Bax, E-cad and ADC values in the normal parenchyma around VX-2 tumor (r = -0.50, $P < 0.001$; r = -0.38, P = 0.08; r = -0.27, P = 0.04; r = -0.25; P = 0.04) . There was a linear correlation between PCNA and nm23 expression index and ADC value (F = 8.15, $P < 0.001$), and PCNA and nm23 expression influenced ADC. There was a linear correlation between PCNA expression index and ADC value in the VX-2 tumor periphery (F = 32.69, $P < 0.001$), while PCNA

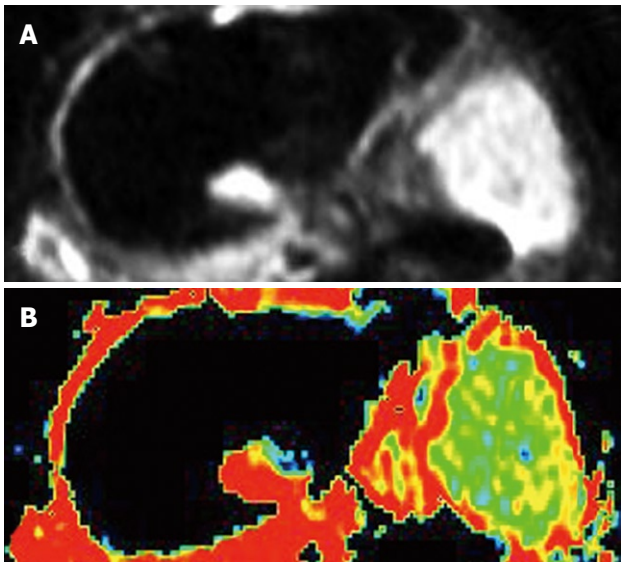


Figure 3 Image manifestations of hepatic VX-2 tumor on DWI and ADC map when b-value was 100 s/mm^2, 6 h after chemoembolization. **A**: High signal and distinct margins of VX-2 tumor on DWI; **B**: Low signal on the ADC map.

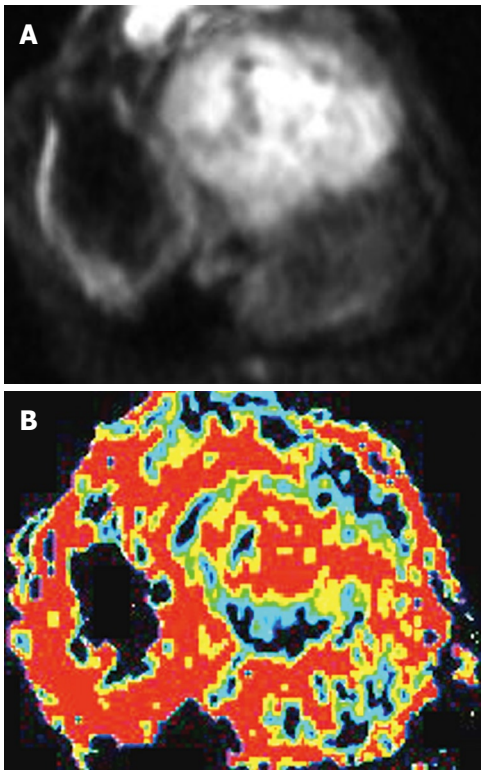


Figure 4 High uneven signal and distinct margins of hepatic VX-2 tumor on DWI and ADC map when b-value was 100 s/mm^2, 32 h after chemoembolization. **A**: High and uneven signal and distinct margins on DWI; **B**: Low or equal signal on ADC map.

expression influenced ADC. However, there was no correlation between nm23, Bax and E-cad expression index, and ADC value (r = -0.20, P = 0.11; r = 0.17, P = 0.15; r = -0.10, P = 0.28).

When the b-value was 300 s/mm^2, there was a correlation between PCNA, nm23, Bax and E-cad expression index, and ADC value in the normal parenchyma around VX-2 tumor (r = -0.37, P = 0.01; r = -0.37, P = 0.01; r = -0.28, P = 0.04; r = -0.31, P = 0.03), and there was a linear correlation between PCNA and nm23 expression index and ADC value (F = 5.00, P < 0.05), and PCNA and nm23 expression influenced ADC. There was a linear correlation between PCNA expression index and ADC value in the VX-2 tumor periphery (F = 29.08, P < 0.001), and PCNA expression influenced ADC, but there was no correlation between nm23, Bax and E-cad expression index and ADC value in the VX-2 tumor periphery (r = -0.15, P = 0.18; r = 0.14, P = 0.19; r = -0.04, P = 0.40).

DISCUSSION

Before and after chemoembolization, PCNA expression was significantly higher in VX-2 tumors compared with the normal parenchyma, whereas nm23, Bax and E-cad expression in the normal parenchyma was significantly higher. PCNA expression in the VX-2 tumor periphery was higher compared to the tumor outer layer and its center, while nm23 and Bax expression in the VX-2 tumor center were higher compared to the outer layer and the peripheral area of the tumor. The high level of PCNA expression indicated that the VX-2 tumor cells had powerful heteromorphism and reproductive activity. The considerable reduction in Bax expression resulted in a decrease in cell apoptosis such that the cell reproductive activity increased, while the reduction in nm23 and E-cad expression led to a marked increase in the ability of the tumor to infiltrate and metastasize. In general, the VX-2 tumor had a high malignant potential, with capacity for rapid reproduction, infiltration and metastasis.

At the same time, the ADC values of VX-2 tumor were significantly lower than the normal parenchyma, and ADC showed an inverse correlation with PCNA expression, but not with nm23, Bax and E-cad expression. These findings suggest that the ability of cell proliferation increases because gene expression increases relative to tumor proliferation such that water molecular diffusion is limited and ADC decreases. In other words, reduction in ADC indirectly increases the gene expression relative to the tumor proliferation. Similarly, Ding *et al*[27] reported that decrease in E-cad expression leads an increase in tumor reproductive activity and Luo *et al*[5] demonstrated that nm23 expression had an inverse correlation with PCNA. To a certain extent, tumor ADC can reflect its ability to infiltrate and metastasize.

After chemoembolization, PCNA expression in VX-2 tumors was slightly higher than before chemoembolization. PCNA expression increased gradually until 32 h, and then decreased slightly. PCNA expression in the normal parenchyma was significantly lower than that in VX-2 tumors, and its expression increased after chemoembolization. Soon after chemoembolization, the tumor reproductive activity may increase, but then decreases which may be related to the stimulation caused by chemoembolization. Therefore, the frequency of chemoembolization should be determined

carefully because viable tumor cells may have powerful reproductive activity. In the present study, the increase in the reproductive activity of normal parenchyma cells may be related to nonselective catheterization, indicating the importance of selective catheterization. After chemoembolization, nm23 expression in the VX-2 tumor or normal parenchyma was higher than before chemoembolization, and it continued to increase until 32 h, followed by decreased expression. These findings indicate that the risk of tumor metastasis may decrease after chemoembolization. The expression of E-cad and Bax in the tumors did not show a significant change after chemoembolization, while E-cad and Bax expression increased in the normal parenchyma. On the other hand, both with b-value 100 s/mm^2 and b-value 300 s/mm^2, the ADC levels in the VX-2 tumor periphery decreased gradually until 16 h after chemoembolization, and then increased gradually. The ADC level in the tumor was inversely related to PCNA expression. Besides water molecules "Brownian Motion", to a certain degree the ADC value indirectly impacts the reproductive activity of cells. With an increase in tumor cell reproduction, the diffusion motion of water cells becomes limited, such that the ADC value decreases. This may be one of the reasons why the ADC of malignant tumors is lower than that of benign tumors. Therefore, the ADC value has potential in the diagnosis and differentiation of tumors, as reported in previous studies by Ichikawa *et al*[18,19], Yamashita *et al*[20] and Kim *et al*[28].

ACKNOWLEDGMENTS

We thank the staff of the Radiology Department and the Laboratory Animal Center of the Second Xiangya Hospital for their help and support, especially Miss Ying-Si He.

COMMENTS

Background

Diffusion-weighted imaging (DWI) has not been extensively used in the diagnosis as well as in the evaluation of the progression and outcome of hepatic tumors, and few studies have been carried out to investigate the dynamic characteristics of hepatocellular carcinoma gene expression after chemoembolization. We believe that DWI has the potential value of differentiating benign tumors from malignant tumors after chemoembolization. In the present study, we investigated the characteristics of proliferating cell nuclear antigen (PCNA), Bax, nm23 and E-cadherin (E-cad) expression after chemoembolization, and evaluated the correlation between the apparent diffusion coefficient (ADC) on DWI and gene expression, in the rabbit VX-2 tumor model.

Research frontiers

There are very few studies on hepatic pathological changes on DWI. The ADC value of benign lesions, such as hepatic cysts and hemangiomas was higher than that of malignant lesions such as hepatocellular carcinoma and metastasis. Several studies have shown that signals from areas of tumor coagulation necrosis are lower compared to tumor viable areas. Several studies have reported on the PCNA, Bax, nm23 and E-cad expression after chemoembolization. However, few studies have investigated the dynamic characteristics of hepatocellular carcinoma gene expression after chemoembolization, using image-pathology-gene expression

Innovations and breakthroughs

The present study clearly demonstrates that DWI has the potential application of detecting and differentiating viable tumors from necrotic tumors after chemoembolization. PCNA expression in VX-2 tumors was slightly higher compared to the level before chemoembolization. The nm23 expression in VX-2 tumor was higher, such that the potential for VX-2 tumor to infiltrate and metastasize decreases, while the ability to proliferate increases for a short period after chemoembolization. To some extent, the ADC value is able to indirectly reflect the proliferation of VX-2 tumor cells.

Applications

Physicians can apply this knowledge to evaluate hepatic tumors that are clearly progressive in nature, and accurately differentiate areas of necrotic tumor from viable tumor after chemoembolization, as well as determine to some extent the proliferation of VX-2 tumor cells on ADC.

Peer review

This is an interesting, well designed and written study on the clinical significance of diffusion-weighted imaging. The manuscript contains important information on the manifestations of viable, necrotic and liquefied or cystic areas in liver tumors.

REFERENCES

1 **Nan KJ**, Ruan ZP, Jing Z, Qin HX, Wang HY, Guo H, Xu R. Expression of fragile histidine triad in primary hepatocellular carcinoma and its relation with cell proliferation and apoptosis. *World J Gastroenterol* 2005; **11**: 228-231

2 **Nan KJ**, Jing Z, Gong L. Expression and altered subcellular localization of the cyclin-dependent kinase inhibitor p27Kip1 in hepatocellular carcinoma. *World J Gastroenterol* 2004; **10**: 1425-1430

3 **Nanashima A**, Yano H, Yamaguchi H, Tanaka K, Shibasaki S, Sumida Y, Sawai T, Shindou H, Nakagoe T. Immunohistochemical analysis of tumor biological factors in hepatocellular carcinoma: relationship to clinicopathological factors and prognosis after hepatic resection. *J Gastroenterol* 2004; **39**: 148-154

4 **Shi YH**, Wang B, Zhu YL, Gao ZQ. Correlation of Spiral CT Signs with the Expression of p21~(WAF1), p16~(INK4) and PCNA in HCC. *Linchuang Fangshexue Zazhi* 2003; **22**: 847-851

5 **Luo HY**, Wang HC, Feng DY. [Expression of proliferating cell nucleus antigen and nm23 protein and their relation with metastasis in hepatocellular carcinoma] *Hunan Yikedaxue Xuebao* 2003; **28**: 17-19

6 **Simile MM**, Pagnan G, Pastorino F, Brignole C, De Miglio MR, Muroni MR, Asara G, Frau M, Seddaiu MA, Calvisi DF, Feo F, Ponzoni M, Pascale RM. Chemopreventive N-(4-hydroxyphenyl)retinamide (fenretinide) targets deregulated NF-{kappa}B and Mat1A genes in the early stages of rat liver carcinogenesis. *Carcinogenesis* 2005; **26**: 417-427

7 **Osada S**, Saji S, Kuno T. Clinical significance of combination study of apoptotic factors and proliferating cell nuclear antigen in estimating the prognosis of hepatocellular carcinoma. *J Surg Oncol* 2004; **85**: 48-54

8 **Xiao EH**, Liu DT, Shen SB, Zhou SK, Tan LH, Wang YH, Luo JG, Wu YZ, Tan CL, Liu H, Zhu H. Effect of preoperative transcatheter arterial chemoembolization on apoptosis of hepatocellular carcinoma cells. *Zhonghua Yixue Zazhi* 2003; **116**: 203-207

9 **Xiao EH**, Hu GD, Li JQ. Relationship between apoptosis and invasive and metastatic potential of hepatocellular carcinoma. *Hepatobiliary Pancreat Dis Int* 2002; **1**: 574-576

10 **Miyoshi A**, Kitajima Y, Sumi K, Sato K, Hagiwara A, Koga Y, Miyazaki K. Snail and SIP1 increase cancer invasion by upregulating MMP family in hepatocellular carcinoma cells. *Br J Cancer* 2004; **90**: 1265-1273

11 **Chan JH**, Tsui EY, Luk SH, Fung AS, Yuen MK, Szeto ML, Cheung YK, Wong KP. Diffusion-weighted MR imaging of the liver: distinguishing hepatic abscess from cystic or necrotic tumor. *Abdom Imaging* 2001; **26**: 161-165

12 **Moteki T**, Horikoshi H, Oya N, Aoki J, Endo K. Evaluation of hepatic lesions and hepatic parenchyma using diffusion-weighted reordered turboFLASH magnetic resonance

images. *J Magn Reson Imaging* 2002; **15**: 564-572
13 **Aube C**, Racineux PX, Lebigot J, Oberti F, Croquet V, Argaud C, Cales P, Caron C. [Diagnosis and quantification of hepatic fibrosis with diffusion weighted MR imaging: preliminary results] *J Radiol* 2004; **85**: 301-306
14 **Chow LC**, Bammer R, Moseley ME, Sommer FG. Single breath-hold diffusion-weighted imaging of the abdomen. *J Magn Reson Imaging* 2003; **18**: 377-382
15 **Boulanger Y**, Amara M, Lepanto L, Beaudoin G, Nguyen BN, Allaire G, Poliquin M, Nicolet V. Diffusion-weighted MR imaging of the liver of hepatitis C patients. *NMR Biomed* 2003; **16**: 132-136
16 **Hori M**, Ichikawa T, Sou H, Tsukamoto T, Kitamura T, Okubo T, Araki T, Amemiya Y, Okamoto E, Obara M. [Improving diffusion-weighted imaging of liver with SENSE technique: a preliminary study] *Nippon Igaku Hoshasen Gakkai Zasshi* 2003; **63**: 177-179
17 **Murtz P**, Flacke S, Traber F, van den Brink JS, Gieseke J, Schild HH. Abdomen: diffusion-weighted MR imaging with pulse-triggered single-shot sequences. *Radiology* 2002; **224**: 258-264
18 **Ichikawa T**, Haradome H, Hachiya J, Nitatori T, Araki T. Diffusion-weighted MR imaging with a single-shot echoplanar sequence: detection and characterization of focal hepatic lesions. *AJR Am J Roentgenol* 1998; **170**: 397-402
19 **Ichikawa T**, Haradome H, Hachiya J, Nitatori T, Araki T. Diffusion-weighted MR imaging with single-shot echo-planar imaging in the upper abdomen: preliminary clinical experience in 61 patients. *Abdom Imaging* 1999; **24**: 456-461
20 **Yamashita Y**, Tang Y, Takahashi M. Ultrafast MR imaging of the abdomen: echo planar imaging and diffusion-weighted imaging. *J Magn Reson Imaging* 1998; **8**: 367-374
21 **Taouli B**, Martin AJ, Qayyum A, Merriman RB, Vigneron D, Yeh BM, Coakley FV. Parallel imaging and diffusion tensor imaging for diffusion-weighted MRI of the liver: preliminary experience in healthy volunteers. *AJR Am J Roentgenol* 2004; **183**: 677-680
22 **Sun XJ**, Quan XY, Liang W, Wen ZB, Zeng S, Huang FH, Tang M. [Quantitative study of diffusion weighted imaging on magnetic resonance imaging in focal hepatic lesions less than 3 cm] *Zhonghua Zhongliu Zazhi* 2004; **26**: 165-167
23 **Yang ZH**, Xie JX, Hu BF, Zhang YW, Zhou C. Evaluation of Liver Cirrhosis with Diffusion-weighted MR Imaging: An Experimental Study. *Zhongguo Yixue Yingxiang Jishu* 2002; **18**: 849-851
24 **Yang ZH**, Xie JX. Clinical application of diffusion weighted MR imaging in the liver. *Zhonghua Fangshexue Zazhi* 1999; **10**: 684-688
25 **Colagrande S**, Politi LS, Messerini L, Mascalchi M, Villari N. Solitary necrotic nodule of the liver: imaging and correlation with pathologic features. *Abdom Imaging* 2003; **28**: 41-44
26 **Kamel IR**, Bluemke DA, Ramsey D, Abusedera M, Torbenson M, Eng J, Szarf G, Geschwind JF. Role of diffusion-weighted imaging in estimating tumor necrosis after chemoembolization of hepatocellular carcinoma. *AJR Am J Roentgenol* 2003; **181**: 708-710
27 **Ding YL**, Zhao RY, You CZ, Li ZT. Study of the relation ship between expression of E cadherin and PCNA in hepatocellular carcinoma. *Zhonghua Zhongliu Fangzhi Zazhi* 2002; **9**: 556-557
28 **Kim T**, Murakami T, Takahashi S, Hori M, Tsuda K, Nakamura H. Diffusion-weighted single-shot echoplanar MR imaging for liver disease. *AJR Am J Roentgenol* 1999; **173**: 393-398

S- Editor Zhong XY **L- Editor** Anand BS **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5564

World J Gastroenterol 2008 September 28; 14(36): 5564-5569
World Journal of Gastroenterology ISSN 1007-9327

LIVER CANCER

Interferon-alpha restrains growth and invasive potential of hepatocellular carcinoma induced by hepatitis B virus X protein

Jian-Qing Yang, Guang-Dong Pan, Guang-Ping Chu, Zhen Liu, Qiang Liu, Yi Xiao, Lin Yuan

Jian-Qing Yang, Guang-Dong Pan, Guang-Ping Chu, Zhen Liu, Qiang Liu, Yi Xiao, Lin Yuan, Department of Surgery, The People's Hospital of Liuzhou City, Liuzhou 545001, Guangxi Zhuang Autonomous Region, China
Author contributions: Yang JQ designed the research; Yang JQ, Pan GD, Chu GP and Liu Z performed experiments, analyzed data and wrote the paper; Liu Q, Xiao Y and Yuan L provided some technical support and conceptual advice.
Supported by The grant from Natural Science Foundation of Guangxi Zhuang Autonomous Region, No. 0542068
Correspondence to: Dr. Jian-Qing Yang, Department of Surgery, The People's Hospital of Liuzhou City, Liuzhou 545001, Guangxi Zhuang Autonomous Region, China. hawk1218@sina.com
Telephone: +86-772-2812433 Fax: +86-772-2823618
Received: May 7, 2008 Revised: July 28, 2008
Accepted: August 4, 2008
Published online: September 28, 2008

Abstract

AIM: To investigate the effects of interferon-alpha (IFN-α) to restrain the growth and invasive potential of hepatocellular carcinoma (HCC) induced by hepatitis B virus (HBV) X protein.

METHODS: The pcDNA3.1-HBx plasmid was transfected into Chang cells by Lipofectamine *in vitro*, and Chang/HBx was co-cultured with IFN-α. Cell survival growth curve and clonogenicity assay were used to test the growth potential of Chang/pcDNA3.1, Chang/HBx and IFN-α-Chang/HBx *in vitro*. Growth assay in nude mice was used to detect the growth potential of Chang/pcDNA3.1, Chang/HBx and IFN-α-Chang/HBx *in vivo*. Wound healing and transwell migration assays were used to detect the invasive ability of Chang/pcDNA3.1, Chang/HBx and IFN-α-Chang/HBx.

RESULTS: Compared with CCL13 cells transfected with pcDNA3.1, CCL13 with stable expression of hepatitis B virus X protein showed the characteristics of malignant cells with high capability of growth and invasion by detecting their growth curves, colony forming efficiency, wound healing , transwell migration assays and growth assays in nude mice. Its capability of growth and invasion could be controlled by IFN-α.

CONCLUSION: IFN-α can restrain the growth and invasive potential of HCC cells induced by HBx protein, which has provided an experimental basis for IFN-α therapy of HCC.

Key words: Hepatitis B virus X protein; Interferon-alpha; Hepatocellular carcinoma; Growth; Invasion

Peer reviewer: Yogesh K Chawla, PhD, Professor, Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

Yang JQ, Pan GD, Chu GP, Liu Z, Liu Q, Xiao Y, Yuan L. Interferon-alpha restrains growth and invasive potential of hepatocellular carcinoma induced by hepatitis B virus X protein. *World J Gastroenterol* 2008; 14(36): 5564-5569 Available from: URL: http://www.wjgnet.com/1007-9327/14/5564.asp DOI: http://dx.doi.org/10.3748/wjg.14.5564

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most prevalent malignant tumors in Asia, especially in China, where hepatitis B virus (HBV) is the major etiologic factor for it[1-3]. The HBV X protein (HBx), a small regulatory protein, that is required for the establishment of viral infection[4,5], is believed to contribute to the development of HCC[6-8].

Interferon-alpha (IFN-α) has been used clinically for the treatment of viral infections[9-11] and malignances[12-14], for it plays an important role in both antiviral and antitumor host defenses. IFN-α also delays or prevents HCC in patients with HBV-related cirrhosis[15]. Therefore, IFN-α may exhibit an antitumor activity in HBV-related HCC through inhibiting the HBx-mediated cellular responses.

In this study, we aimed to explore the strong effect of IFN-α to restrain the growth and invasive potential of HCC induced by HBx protein through transfection of the HBx gene expression to human hepatoma cells.

MATERIALS AND METHODS

Cell lines and interferon

pcDNA3.1-HBx Chang liver cells (Chang/HBx) and pcDNA3.1 Chang liver cells (Chang/pcDNA3.1) were kindly donated by Liver Cancer Laboratory, Department of Surgery, Xiangya Hospital, Central South University (Changsha, Hunan Province, China). The IFN-α was

purchased from Roche Pharmaceuticals Ltd. (Guangxi, China).

Cell culture and passage

The Chang/HBx and Chang/pcDNA3.1 were cultured and subcultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37℃ for further research. The IFN-α concentration in the nutritive medium of IFN-α-Chang/HBx was 1000 ku/L.

Growth curve

The growth curve was designed to compare the proliferative ability of Chang/HBx cells with that of Chang/HBx cells cocultured with IFN-α and Chang/pcDNBA3.1 cells. First, 2×10^4 cells per well were placed into 24-well plates. Next, the cells in 3 wells were counted daily during the 8 d of culture for evaluation of cell proliferation. Finally, the viability of the cells was determined by Trypan blue exclusion.

Clonogenic assay

Chang/HBx, IFN-α-Chang/HBx and Chang/pcDNA3.1 cells were plated onto 60-mm Petri dishes at a density of 2×10^3 cells per dish and cultured in conditioned medium for 2 wk. The formed colonies were stained with Giemsa to allow calculation of their average colony-forming efficiency.

Wound healing assays

Six-well plates (Costar) were coated overnight with 10 mg/mL fibronectin (Becton Dickinson) in phosphate-buffered saline (PBS) and blocked with 1% bovine serum albumin (Sigma) in DMEM. Chang/HBx, IFN-α-Chang/HBx and Chang/pcDNA3.1 cells were suspended with 0.05% trypsin and 5.3 mmol/L ethylene diaminetetraacetic acid (EDTA) (Gibco) and then counted. Next, 5×10^5 cells per well were plated in serum-free DMEM with applicable inhibitors or DMSO for 3 h. Monolayer cultures were wounded with a P-200 pipette tip, and the medium was replaced with DMEM, 5% fetal bovine serum, and 2 mg/mL G418. Cells were photographed at a magnification of × 20 adjacent to a reference line etched onto the bottom of the plate. Cells were allowed to grow and migrate for 24 h at 37℃ in 5% CO_2 before being photographed a second time. DMSO levels were adjusted to be equal in all wells within each experiment and were never higher than 0.5% DMSO. Each experiment was performed in triplicate and repeated three times.

Transwell migration assay

Matrigel-coated filter inserts with 8-μm pores that fit into 24-well invasion chambers were obtained from Becton Dickinson. Chang/HBx cells, IFN-α-Chang/HBx cells, and Chang/pcDNA3.1 cells were detached from the tissue culture plates, washed, resuspended in conditioned medium (5×10^4 cells/200 μL), and then added to the upper compartment of the invasion chamber with or without plasmin (1.8 μg). Conditioned medium (500 μL) was added to the lower compartment of the invasion chamber. The Matrigel invasion chambers were incubated at 37℃ for 24 h in 5% CO_2. After incubation, the filter inserts were removed from the wells, and the cells on the upper side of the filter were removed using cotton swabs. The filters were fixed, mounted, and stained according to the manufacturer's instructions. The cells that invaded through the Matrigel were counted on the underside of the filter. Three to five invasion chambers were used for each experimental condition. The values obtained were calculated by averaging the total number of cells from three filters.

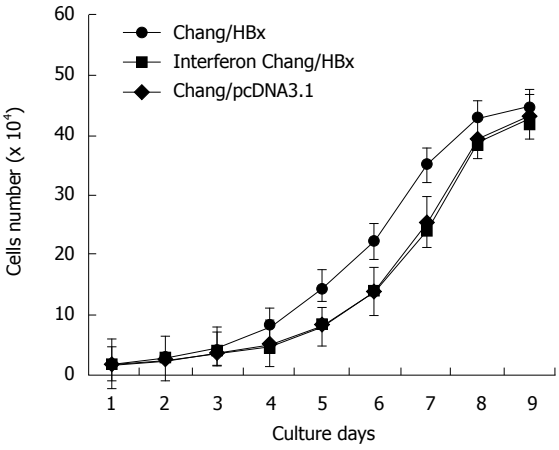


Figure 1 The growth curve of Chang/HBx cells shows a shift to the left from that of Chang/pcDNA3.1 and IFN-α-Chang/HBx cells.

Growth assays in nude mice

Male BALB/C-nu/nu athymic nude mice were obtained from Shanghai Laboratory Animal Center (China) at 4 wk of age. The Chang/HBx, IFN-α-Chang/HBx and Chang/pcDNA3.1 cells were collected by trypsinization and were washed with D-Hank's solution. To produce experimental metastasis, 1×10^7 cells from each culture were injected into the right forelimb armpit of the mice. IFN-α was administrated subcutaneously at dosage of 1.5 × 10 U/kg per day in the group of IFN-α-Chang/HBx cells. After 2 wk, 2 mice from each group were killed and analyzed per week. Neoplasms were removed, rinsed in ice-cold PBS, fixed, stained with hematoxylin and eosin, and photographed.

Statistical analysis

All the data were presented as the mean ± SE. The significance of differences from the control values was determined with Student's t test or the χ^2 test. $P < 0.05$ was considered statistically significant.

RESULTS

Cell survival growth curve and clonogenicity assay

The cell survival growth curve of Chang/HBx cells showed faster growth than that of Chang/pcDNA3.1 cells and IFN-α-Chang/HBx cells (Figure 1). Their average colony-forming efficiency was also significantly different (Figure 2). These findings showed that Chang/

Figure 2 Cell proliferative ability comparisons. The average colony-forming efficiency of Chang/HBx (**A**) was 29.3 ± 4.5, which was significantly different from that of Chang/pcDNA3.1 (**B**) (12.8 ± 2.6) and IFN-α-Chang/HBx (**C**) cells (13.5 ± 2.3) ($P < 0.05$).

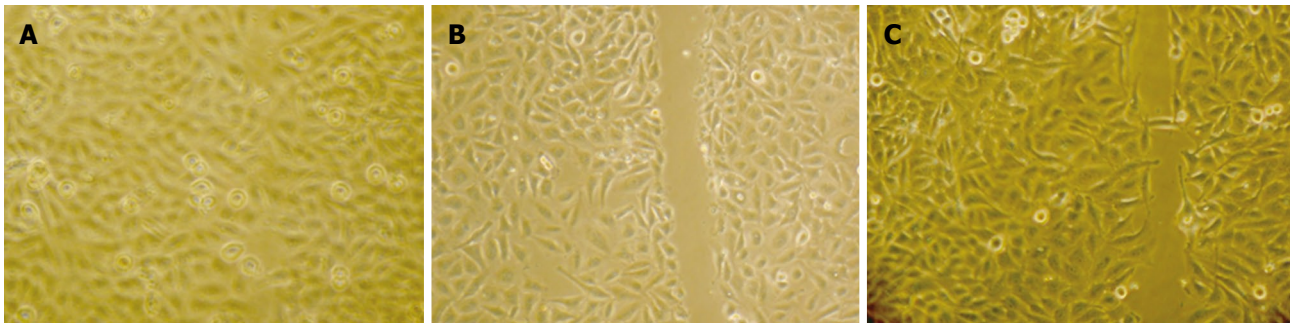


Figure 3 HBx promotes the invasive capacity of Chang liver cells *in vitro*. Wound-healing assays were performed at 0 and 24 h and observed under a phase-contrast microscope (x 100). **A**: Chang liver cells with pcDNA3.1-HBx were disrupted, and wound healing occurred after 24 h; **B**: IFN-α-Chang/HBx cells were disrupted, and the wound did not heal completely after 24 h; **C**: Chang cells with pcDNA3.1 were disrupted, and the wound did not heal completely after 24 h.

HBx was more proliferative than Chang/pcDNA3.1 and IFN-α-Chang/HBx cells.

HBx promotes invasion of chang cells in vitro

The wound-healing assay showed that Chang/HBx cells had a higher ability to heal themselves than the control cells and IFN-α-Chang/HBx cells (Figure 3).

In the transwell migration assay, edge collection phenomena was found on cells in the transwell chamber, with few cells in the center of the wells; therefore, we selected superior, inferior, left, right, and center sites so as to represent the migration assay correctly. The assay showed that the Chang/HBx cells also had greater migration ability than the control cells and IFN-α-Chang/HBx cells ($P < 0.05$) (Figure 4A-C). The average number of Chang/HBx cells that migrated through the Matrigel was 41.6 ± 3.1 cells/site compared with 7.4 ± 1.2 cells/site in the control cells and 9.2 ± 1.6 cells/site in IFN-α-Chang/HBx cells (Figure 4D).

IFN-α inhibits proliferation of chang/HBx cells in vivo

The growth assay in nude mice showed a 100% growth rate of neoplasms after inoculation of the HCC cells. The average latency period was 5 d, and the growth rate was 65.5 ± 7.6 mm^3/d. There was no distant metastasis from the transfectant. The sizes of the neoplasms from IFN-α-Chang/HBx were obviously smaller than the tumors of Chang/HBx injected mice ($P < 0.05$), and the neoplasms from Chang/HBx grew more slowly than those from Chang/pcDNA3.1 ($P < 0.05$), but there was no statistical difference between them in our test. Although Chang liver cells contained the Hela marker, the formed tumor indeed was hepatoma (Figure 5), which could progress to HCC.

DISCUSSION

Interferons are a family of cytokine that are produced by cells of the immune system. Three classes of interferons have been identified: α, β and γ. Each class has different effects though their activities overlap. The interferons direct the attack of the immune system on viruses, bacteria, tumors and other foreign substances that may invade the body[16]. IFN-α is known to have antiproliferative effects on human HCC cells, both *in vivo* and *in vitro*[17,18]. The effectiveness of IFN-α in treating HCC patients has been reported to be positive in a few previous clinical trials[19-21]. It was also shown that IFN-α delayed or prevented HCC in patients with HBV-related cirrhosis[15]. In addition, combination of IFN-α and 5-FU significantly prolonged the survival rate of patients with HCC[22]. These observations suggest that IFN-α has a capability of anticancer agents against HCC. But the precise mechanisms remain poorly understood. To investigate weather IFN-α could regulate the function of HBx protein and inhibit the growth and invasion potential of HBV-related HCC cells, we investigated Chang hepatocytes encoding *HBx* gene and observed that the transfected cells had acquired growth and invasive properties *in vitro*, but these properties could be

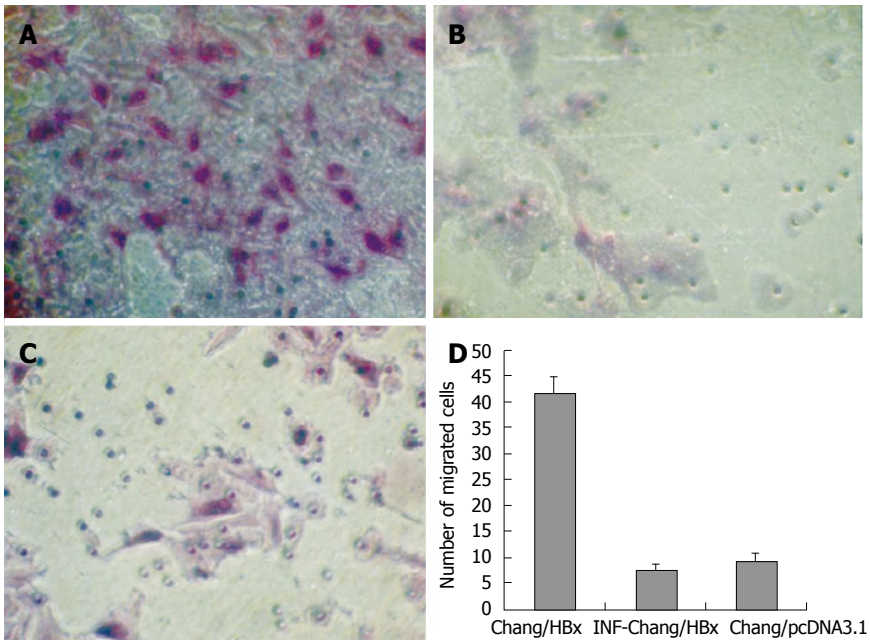


Figure 4 HBx promotes the invasive capacity of Chang liver cells *in vitro*. **A**: Chang cells transfected with pcDNA3.1-HBx invaded through the Matrigel were counted on the underside of the transwell filter and compared with Chang/HBx cells (**B**) and control cells Chang/pcDNA3.1 (**C**) (x 200). There was no difference between IFN-α-Chang/HBx cells and Chang/pcDNA3.1; **D**: The average number of migrated cells per site seen under a high-power microscope (x 400) was 41.6 ± 3.1 for the transfected Chang/HBx cells and 7.4 ± 1.2 for IFN-α-Chang/HBx cells and 9.2 ± 1.6 for the control Chang/pcDNA3.1 cells.

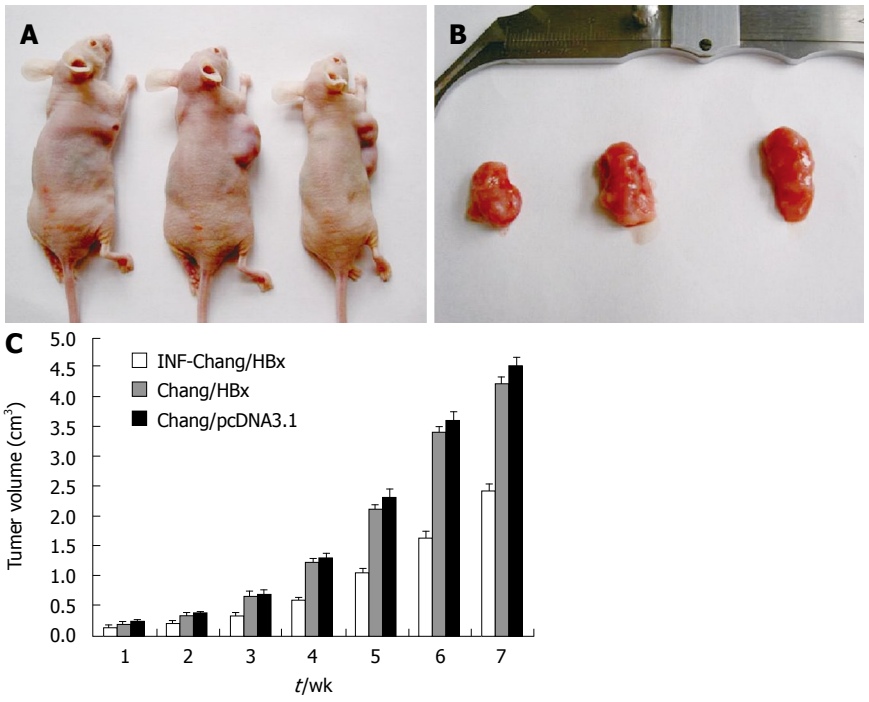


Figure 5 **A**: A nude mouse migration model with pcDNA3.1-HBx Chang cells, IFN-α-Chang/HBx cells and Chang/pcDNA3.1 cells were constructed on the 7th wk; **B**: The volume of neoplasms in the three groups were observed. The sizes of the hepatomas from Chang/pcDNA3.1 and IFN-α-Chang/HBx injected nude mice were obviously larger than the tumors of Chang/HBx injected mice; **C**: Neoplasm growth in the Chang/HBx-inoculated nude mice compared between IFN-α-Chang/HBx and the control Chang/pcDNA3.1-inoculated mice.

controlled by IFN-α effectively.

Through cell survival growth curve and clonogenic assays, we found that Chang cells encoding HBx had higher dynamics for growth than the Chang cells. These cells also showed multilaminar growth. Because they had lost contact inhibition completely, their colony-forming ability was greater than that of the parental cell line. However, the growth and colony-forming ability of Chang-HBx could be inhibited by IFN-α obviously.

Our study also showed that HBx-transfected Chang had higher wound healing ability than the control cells. The transwell migration assay showed that Chang/HBx had a more obviously invasive ability than the control cells. But IFN-α could inhibit these malignant biologic behavior of Chang/HBx induced by HBx protein.

Nude mice were used as the mimic system in our study for observing the effects of cell tumorigenicity of HBx protein in Chang liver cells *in vivo*. However, in the growth assays of nude mice, we found that Chang/HBx cells grew more slowly than Chang/pcDNA3.1 cells. At the same time, Chang/HBx cells treated with IFN-α were found growing more slowly than Chang/HBx cells without IFN-α treatment, which inhibited the tumorigenicity of Chang/HBx cells.

Some studies have shown that HBx can induce tumor proliferation and invasion through down-regulating the function of P53[23,24], up-regulating the expression of survivin[25], activating with several important cell signaling pathways, including PKB/Akt[26], RAS/RAF/MAPK[27], wnt/β-catenin[28] and NF-κB[29]. It has also proved that

IFN-α can induce p53-dependent apoptosis[30], down-regulate the expression of survivin[31] and inactive NF-κB[32] respectively. It is possible that IFN-α could control the growth and invasive potential of HBV-related HCC by inhibiting the function of HBx. The reason why Chang/HBx cells grew more slowly than Chang/pcDNA3.1 cells, is the growth of Chang/HBx cells in nude mice need more nutrient than Chang/pcDNA3.1 cells, however, as the early tumorigenesis of Chang/HBx cells formed, the nutrient was not sufficient and the cells might enter into rest phase as proliferation ceases.

In conclusion, IFN-α could inhibit the growth and invasive potential of Chang cells induced by HBx. IFN-α is a main drug for type B hepatitis and can delay and prevent HBV-related cirrhosis effectively. Postoperative IFN-α therapy can improve the survival of patients with HBV-related HCC. IFN-α therapy might be a very important means for preventing and curing HBV-related HCC. Our study provides a new evidence for this.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world, especially in China. About 53% of HCC cases in the world are related to hepatitis B virus (HBV) infection. Chronic HBV infection is a leading risk factor for HCC. It has been proved that IFN-α could control the infection of HBV, prevent HBV-related cirrhosis, lower tumor recurrence rate after surgical resection or ablation of HCC and prolong the survival rate of patients with HCC. IFN-α therapy has been applied to treat HBV-related HCC as an effective drug, but its precise mechanism remains poorly understood.

Research frontiers

Some studies have proved that IFN-α could inhibit the growth of HCC cells *in vivo* and *in vitro*. It could regulate expression of VEGF, bFGF and angiopoietins and inhibit tumor angiogenesis in human HCC cells. But there are few reports about the impact of IFN-α on the malignant characteristics of HCC cells induced by HBx protein.

Innovations and breakthroughs

Our results reveal that the liver cell lines CCL13 containing HBx gene expression have the characteristics of malignant cells, which was verified by means of detecting their growth curve, colony forming efficiency, wound healing, transwell migration assays and growth assay in nude mice. All of these could be controlled by IFN-α.

Applications

IFN-α therapy is a very important means for preventing and curing HBV-related HCC. This study provides some experimental evidence for it.

Terminology

Interferons are natural proteins produced by the cells of the immune system of most vertebrates in response to challenges by foreign agents such as viruses, parasites and tumor cells. Three classes of interferons have been identified: α, β and γ. The interferons direct the attack of immune system on viruses, bacteria, tumors and other foreign substances that may invade the body. The JAK-STAT signaling pathway is the most specific and commonly accepted IFN signaling pathway.

Peer review

This is an interesting article which investigated the effect of IFN-α on the malignant characters of human liver cell lines CCL13 containing HBx gene expression. The design of experiment is novel and the result is significant. It would be helpful to explore the mechanism of IFN-α therapy for HCC and direct the clinical therapy of HCC.

REFERENCES

1 **El-Serag HB**. Hepatocellular carcinoma: recent trends in the United States. *Gastroenterology* 2004; **127**: S27-S34

2 **Llovet JM**, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet* 2003; **362**: 1907-1917

3 **Shibuya K**, Mathers CD, Boschi-Pinto C, Lopez AD, Murray CJ. Global and regional estimates of cancer mortality and incidence by site: II. Results for the global burden of disease 2000. *BMC Cancer* 2002; **2**: 37

4 **Zoulim F**, Saputelli J, Seeger C. Woodchuck hepatitis virus X protein is required for viral infection in vivo. *J Virol* 1994; **68**: 2026-2030

5 **Melegari M**, Wolf SK, Schneider RJ. Hepatitis B virus DNA replication is coordinated by core protein serine phosphorylation and HBx expression. *J Virol* 2005; **79**: 9810-9820

6 **Chan HL**, Sung JJ. Hepatocellular carcinoma and hepatitis B virus. *Semin Liver Dis* 2006; **26**: 153-161

7 **Koike K**, Tsutsumi T, Fujie H, Shintani Y, Kyoji M. Molecular mechanism of viral hepatocarcinogenesis. *Oncology* 2002; **62** Suppl 1: 29-37

8 **Staib F**, Hussain SP, Hofseth LJ, Wang XW, Harris CC. TP53 and liver carcinogenesis. *Hum Mutat* 2003; **21**: 201-216

9 **Sene D**, Touitou V, Bodaghi B, Saadoun D, Perlemuter G, Cassoux N, Piette JC, Hoang PL, Cacoub P. Intraocular complications of IFN-alpha and ribavirin therapy in patients with chronic viral hepatitis C. *World J Gastroenterol* 2007; **13**: 3137-3140

10 **Yang YF**, Zhao W, Zhong YD, Yang YJ, Shen L, Zhang N, Huang P. Comparison of the efficacy of thymosin alpha-1 and interferon alpha in the treatment of chronic hepatitis B: a meta-analysis. *Antiviral Res* 2008; **77**: 136-141

11 **Ueno T**, Mitsuishi T, Kimura Y, Kato T, Hasegawa H, Katano H, Sata T, Kurane S, Kawana S. Immune reconstitution inflammatory syndrome associated with Kaposi's sarcoma: successful treatment with interferon-alpha. *Eur J Dermatol* 2007; **17**: 539-540

12 **Kujawski LA**, Talpaz M. The role of interferon-alpha in the treatment of chronic myeloid leukemia. *Cytokine Growth Factor Rev* 2007; **18**: 459-471

13 **Amato RJ**, Mohammad T. Interferon-alpha plus capecitabine and thalidomide in patients with metastatic renal cell cancer. *J Exp Ther Oncol* 2008; **7**: 41-47

14 **Nagano H**, Miyamoto A, Wada H, Ota H, Marubashi S, Takeda Y, Dono K, Umeshita K, Sakon M, Monden M. Interferon-alpha and 5-fluorouracil combination therapy after palliative hepatic resection in patients with advanced hepatocellular carcinoma, portal venous tumor thrombus in the major trunk, and multiple nodules. *Cancer* 2007; **110**: 2493-2501

15 **Ikeda K**, Saitoh S, Suzuki Y, Kobayashi M, Tsubota A, Fukuda M, Koida I, Arase Y, Chayama K, Murashima N, Kumada H. Interferon decreases hepatocellular carcinogenesis in patients with cirrhosis caused by the hepatitis B virus: a pilot study. *Cancer* 1998; **82**: 827-835

16 **Chelbi-Alix MK**, Wietzerbin J. Interferon, a growing cytokine family: 50 years of interferon research. *Biochimie* 2007; **89**: 713-718

17 **Kondo M**, Nagano H, Wada H, Damdinsuren B, Yamamoto H, Hiraoka N, Eguchi H, Miyamoto A, Yamamoto T, Ota H, Nakamura M, Marubashi S, Dono K, Umeshita K, Nakamori S, Sakon M, Monden M. Combination of IFN-alpha and 5-fluorouracil induces apoptosis through IFN-alpha/beta receptor in human hepatocellular carcinoma cells. *Clin Cancer Res* 2005; **11**: 1277-1286

18 **Hagiwara S**, Kudo M, Nakatani T, Sakaguchi Y, Nagashima M, Fukuta N, Kimura M, Hayakawa S, Munakata H. Combination therapy with PEG-IFN-alpha and 5-FU inhibits HepG2 tumour cell growth in nude mice by apoptosis of p53. *Br J Cancer* 2007; **97**: 1532-1537

19 **Fischer M**, Runkel L, Schaller H. HBx protein of hepatitis B virus interacts with the C-terminal portion of a novel human proteasome alpha-subunit. *Virus Genes* 1995; **10**: 99-102

20 **Sun HC**, Tang ZY, Wang L, Qin LX, Ma ZC, Ye QH, Zhang BH, Qian YB, Wu ZQ, Fan J, Zhou XD, Zhou J, Qiu SJ, Shen YF. Postoperative interferon alpha treatment postponed

recurrence and improved overall survival in patients after curative resection of HBV-related hepatocellular carcinoma: a randomized clinical trial. *J Cancer Res Clin Oncol* 2006; **132**: 458-465

21 **Nakamura M**, Nagano H, Marubashi S, Miyamoto A, Takeda Y, Kobayashi S, Wada H, Noda T, Dono K, Umeshita K, Monden M. Pilot study of combination chemotherapy of S-1, a novel oral DPD inhibitor, and interferon-alpha for advanced hepatocellular carcinoma with extrahepatic metastasis. *Cancer* 2008; **112**: 1765-1771

22 **Obi S**, Yoshida H, Toune R, Unuma T, Kanda M, Sato S, Tateishi R, Teratani T, Shiina S, Omata M. Combination therapy of intraarterial 5-fluorouracil and systemic interferon-alpha for advanced hepatocellular carcinoma with portal venous invasion. *Cancer* 2006; **106**: 1990-1997

23 **Chung TW**, Lee YC, Ko JH, Kim CH. Hepatitis B Virus X protein modulates the expression of PTEN by inhibiting the function of p53, a transcriptional activator in liver cells. *Cancer Res* 2003; **63**: 3453-3458

24 **Hsieh JL**, Wu CL, Lee CH, Shiau AL. Hepatitis B virus X protein sensitizes hepatocellular carcinoma cells to cytolysis induced by E1B-deleted adenovirus through the disruption of p53 function. *Clin Cancer Res* 2003; **9**: 338-345

25 **Zhang X**, Dong N, Yin L, Cai N, Ma H, You J, Zhang H, Wang H, He R, Ye L. Hepatitis B virus X protein upregulates survivin expression in hepatoma tissues. *J Med Virol* 2005; **77**: 374-381

26 **Shih WL**, Kuo ML, Chuang SE, Cheng AL, Doong SL. Hepatitis B virus X protein inhibits transforming growth factor-beta -induced apoptosis through the activation of phosphatidylinositol 3-kinase pathway. *J Biol Chem* 2000; **275**: 25858-25864

27 **Yun C**, Cho H, Kim SJ, Lee JH, Park SY, Chan GK, Cho H. Mitotic aberration coupled with centrosome amplification is induced by hepatitis B virus X oncoprotein via the Ras-mitogen-activated protein/extracellular signal-regulated kinase-mitogen-activated protein pathway. *Mol Cancer Res* 2004; **2**: 159-169

28 **Cha MY**, Kim CM, Park YM, Ryu WS. Hepatitis B virus X protein is essential for the activation of Wnt/beta-catenin signaling in hepatoma cells. *Hepatology* 2004; **39**: 1683-1693

29 **Wang T**, Wang Y, Wu MC, Guan XY, Yin ZF. Activating mechanism of transcriptor NF-kappaB regulated by hepatitis B virus X protein in hepatocellular carcinoma. *World J Gastroenterol* 2004; **10**: 356-360

30 **Yuan XW**, Zhu XF, Huang XF, Sheng PY, He AS, Yang ZB, Deng R, Feng GK, Liao WM. Interferon-alpha enhances sensitivity of human osteosarcoma U2OS cells to doxorubicin by p53-dependent apoptosis. *Acta Pharmacol Sin* 2007; **28**: 1835-1841

31 **Thyrell L**, Arulampalam V, Hjortsberg L, Farnebo M, Grander D, Pokrovskaja Tamm K. Interferon alpha induces cell death through interference with interleukin 6 signaling and inhibition of STAT3 activity. *Exp Cell Res* 2007; **313**: 4015-4024

32 **Shigeno M**, Nakao K, Ichikawa T, Suzuki K, Kawakami A, Abiru S, Miyazoe S, Nakagawa Y, Ishikawa H, Hamasaki K, Nakata K, Ishii N, Eguchi K. Interferon-alpha sensitizes human hepatoma cells to TRAIL-induced apoptosis through DR5 upregulation and NF-kappa B inactivation. *Oncogene* 2003; **22**: 1653-1662

S- Editor Li DL **L- Editor** Ma JY **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5570

World J Gastroenterol 2008 September 28; 14(36): 5570-5583
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Potentially probiotic bacteria induce efficient maturation but differential cytokine production in human monocyte-derived dendritic cells

Sinikka Latvala, Taija E Pietilä, Ville Veckman, Riina A Kekkonen, Soile Tynkkynen, Riitta Korpela, Ilkka Julkunen

Sinikka Latvala, Taija E Pietilä, Ville Veckman, Ilkka Julkunen, Department of Viral Diseases and Immunology, National Public Health Institute, Mannerheimintie 166, Helsinki 00300, Finland
Riina A Kekkonen, Riitta Korpela, Pharmacology, Institute of Biomedicine, University of Helsinki, PO Box 63, Helsinki 00014, Finland
Riina A Kekkonen, Soile Tynkkynen, Riitta Korpela, Research Center, Valio Ltd, Meijeritie 4, Helsinki 00370, Finland
Author contributions: Latvala S, Pietilä TE, Veckman V, Kekkonen RA, Tynkkynen S, Korpela R, and Julkunen I designed research and took part in data analysis and paper writing; Latvala S, Pietilä TE, and Kekkonen RA performed the experiments and carried out data analysis; Latvala S, Julkunen I wrote the paper.
Supported by The Medical Research Council of the Academy of Finland and the Sigrid Juselius Foundation
Correspondence to: Sinikka Latvala, Department of Viral Diseases and Immunology, National Public Health Institute, Mannerheimintie 166, Helsinki FIN-00300, Finland. sinikka.latvala@ktl.fi
Telephone: +358-9-47448943 Fax: +358-9-47448355
Received: April 23, 2008 Revised: August 29, 2008
Accepted: September 5, 2008
Published online: September 28, 2008

Abstract

AIM: To analyze the ability of nine different potentially probiotic bacteria to induce maturation and cytokine production in human monocyte-derived dendritic cells (moDCs).

METHODS: Cytokine production and maturation of moDCs in response to bacterial stimulation was analyzed with enzyme-linked immunosorbent assay (ELISA) and flow cytometric analysis (FACS), respectively. The kinetics of mRNA expression of cytokine genes was determined by Northern blotting. The involvement of different signaling pathways in cytokine gene expression was studied using specific pharmacological signaling inhibitors.

RESULTS: All studied bacteria induced the maturation of moDCs in a dose-dependent manner. More detailed analysis with *S. thermophilus* THS, *B. breve* Bb99, and *L. lactis* subsp. *cremoris* ARH74 indicated that these bacteria induced the expression of moDC maturation markers HLA class Ⅱ and CD86 as efficiently as pathogenic bacteria. However, these bacteria differed in their ability to induce moDC cytokine gene expression. *S. thermophilus* induced the expression of pro-inflammatory (TNF-α, IL-12, IL-6, and CCL20) and Th1 type (IL-12 and IFN-γ) cytokines, while *B. breve* and *L. lactis* were also potent inducers of anti-inflammatory IL-10. Mitogen-activated protein kinase (MAPK) p38, phosphatidylinositol 3 (PI3) kinase, and nuclear factor-kappa B (NF-κB) signaling pathways were shown to be involved in bacteria-induced cytokine production.

CONCLUSION: Our results indicate that potentially probiotic bacteria are able to induce moDC maturation, but their ability to induce cytokine gene expression varies significantly from one bacterial strain to another.

Key words: Probiotic bacteria; Immune response; Cytokine; Chemokine; Dendritic cell; Human; Gene expression; Signal transduction

Peer reviewer: Magali J Fontaine, PhD, Stanford University, 300 Pasteur drive H1402, M/C5626, Stanford CA/ 94305-5626, United States

Latvala S, Pietilä TE, Veckman V, Kekkonen RA, Tynkkynen S, Korpela R, Julkunen I. Potentially probiotic bacteria induce efficient maturation but differential cytokine production in human monocyte-derived dendritic cells. *World J Gastroenterol* 2008; 14(36): 5570-5583 Available from: URL: http://www.wjgnet.com/1007-9327/14/5570.asp DOI: http://dx.doi.org/10.3748/wjg.14.5570

INTRODUCTION

The importance of the intestinal microbiota, and the ways in which it can be modified, has been under intensive investigation in recent years. Evidence from randomized and placebo-controlled clinical trials and molecular studies has attracted significant interest to the use of probiotic bacteria as therapeutic substances. Probiotics are live microbial food supplements that have a beneficial effect on human health. They can

change the composition or metabolic activity of the intestinal microbiota and modulate the immune system of the host. This seems to be dependent on the probiotic bacterial species used and the overall health status of the host[1]. Probiotic bacteria have been used as an alternative form of therapy for various diseases and symptoms[2,3]. Probiotic supplement containing *L. rhamnosus* GG, *L. rhamnosus* LC705, *P. freudenreichii* JS, and *B. breve* Bb99 alleviated the symptoms of irritable bowel syndrome (IBS) and improved eradication of *H pylori*[4,5]. The best studied probiotic bacterium, *L. rhamnosus* GG has been found to be effective in the treatment of antibiotic-associated[6] and nosocomial diarrhea[7] in children. In addition, *L. rhamnosus* GG has been used to reduce the onset of atopy[8]. It has also been shown to have immunomodulatory effects on the host by decreasing the production of IL-6 and TNF-α in healthy individuals[9] and TNF-α production in allergic children[10]. All bacteria used in the present work are Gram-positive bacteria and some of them are widely used in the food industry. *S. thermophilus* THS, *L. mesenteroides* subsp. *cremoris* PIA2, and *L. lactis* subsp. *cremoris* ARH74 are used for the fermentation of milk products. *L. rhamnosus* GG and *B. animalis* subsp. *lactis* Bb12 are used as supplements in dairy products due to their proved probiotic effects. *B. animalis* subsp. *lactis* Bb12 is shown to be effective in reducing the risk of diarrhea and modulating the immune response[11,12]. Although many probiotic bacterial strains are used as food supplements, their mechanisms of action have remained poorly characterized.

The gut is rich in antigen-presenting cells such as dendritic cells (DCs), which reside underneath the epithelial cell layer in an immature state being constantly on alert for foreign antigens or invading pathogens[13,14]. DCs can reach their dendrites through the tight junctions of epithelial cells and in this manner sample the gut for different antigens[14,15]. Upon contact with microbes or foreign antigens, DCs undergo a maturation process, which is associated with the expression of cell surface costimulatory molecules CD80, CD83, and CD86 and migration from the peripheral tissues into local lymph nodes. In the lymph nodes mature DCs present antigens to naive T cells, and the development of adaptive immune responses is initiated[16-18]. Depending on the nature of the antigen, the magnitude of the activation of innate and adaptive immune responses can vary greatly. Cytokine production profile and the maturation status of DCs depend on the nature of the stimulus and determine whether T cells are polarized towards T helper (Th) 1, Th2, or T regulatory (Treg) type responses[19-21].

DCs have multiple receptor systems including scavenger receptors, lectin-binding molecules, Toll-like receptors (TLRs), and nucleotide-binding oligomerization domain (NOD) molecules that recognize different types of bacterial structural components or genetic material[22-24]. Activation of DCs *via* TLRs leads to the activation of intracellular signal transduction pathways and to the activation and nuclear translocation of NF-κB, interferon regulatory factor (IRF), or mitogen-activated protein kinase (MAPK)-regulated transcription factors, which ultimately regulate the activation status of DCs and enhance the expression of cytokine genes[25-27]. However, it still remains elusive how DCs can differentiate between commensal and pathogenic bacteria.

In the present study we have analyzed the ability of nine different, potentially probiotic bacteria to induce maturation and cytokine gene expression in human moDCs. By analyzing the cytokine production profiles in moDCs induced by these bacteria, we hope to gain insight into different immunomodulatory effects of probiotic bacteria. This information is of value for selecting new probiotics for *in vivo* trials.

MATERIALS AND METHODS

Bacterial strains

Two well-known probiotic strains *L. rhamnosus* GG (ATCC 53103)[7,10] and *B. animalis* subsp. *lactis* Bb12 (DSM15954)[12], and seven potentially probiotic strains *L. rhamnosus* LC705 (DSM 7061)[4,5], *L. helveticus* 1129 (DSM 13137)[28], *B. longum* 1/10, *B. breve* Bb99 (DSM 13692)[4,5], *S. thermophilus* THS[29,30], *L. lactis* subsp. *cremoris* ARH74 (DSM 18891)[31], and *L. mesenteroides* subsp. *cremoris* PIA2 (DSM 18892)[29] were obtained from Valio Research Centre (Helsinki, Finland). Pathogenic *S. pyogenes* serotype T1M1 (IH32030)[32], isolated from a child with bacteremia, was from the collection of National Public Health Institute (Helsinki, Finland), and was used as a positive control[33,34]. Bacteria were stored in skimmed milk at -70℃ and grown to the end of logarithmic growth phase before they were used in experiments. *S. pyogenes* and *L. rhamnosus* GG were grown as previously described[32,34]. All strains were passaged three times, except for the Bifidobacterium strains which were passaged four times, before they were used in stimulation experiments. The number of bacteria was determined by counting them in a Petroff-Hausser chamber.

Frozen stocks of *L. rhamnosus* GG, *L. rhamnosus* LC705, and *L. mesenteroides* subsp. *cremoris* PIA2 strains were inoculated in deMan, Rogosa, and Sharpe (MRS) medium (Lab M, Topley House, Lancashire, UK) and grown under aerobic conditions at 37℃. *L. helveticus* 1129 was grown at 42℃ under aerobic conditions in MRS medium (Lab M, Topley House). Bifidobacterium strains were grown anaerobically in MRS (Lab M, Topley House) supplemented with 5 g/L L-cysteine (Merck, Darmstadt, Germany) at 37℃. *S. thermophilus* was grown aerobically at 37℃ on M17 agar (Lab M, Topley House) supplemented with 20 g/L D (+) lactose monohydrate (J.T. Baker B.V., Deventer, Holland), and transferred to M17 broth (Difco, Beckton Dickinson, MD, USA) containing 20 g/L lactose (J.T. Baker B.V.). *L. lactis* was grown on calcium citrate agar (Valio Ltd.) and M17 broth (Difco) containing 20 g/L lactose (J.T. Baker B.V.) at 22℃ under aerobic conditions. For stimulation experiments bacteria were grown to a late logarithmic growth phase, and the indicated amount of bacteria was collected by centrifugation. After this cells were

suspended to RPMI-1640 medium (Sigma, St. Louis, MO, USA) and added to moDC cultures.

DC purification and differentiation

Monocytes were purified from freshly collected, leukocyte-rich buffy coats obtained from healthy blood donors (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) as described previously[33]. Human peripheral blood mononuclear cells were isolated by density gradient centrifugation over a Ficoll-Pague gradient (Amersham Biotech, Uppsala, Sweden) followed by purification of monocytes in Percoll gradient (Amersham Biotech) centrifugation. Monocytes were collected from the top layer of the gradient, and T and B cells were depleted using anti-CD3 and anti-CD19 magnetic beads (Dynal, Oslo, Norway). Purified monocytes were allowed to adhere to six-well plates 2.5×10^6 cells/well (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) for 1 h at 37℃ in RPMI-1640 supplemented with 0.6 μg/mL penicillin (Gibco, BRL, Paisley, Scotland), 60 μg/mL streptomycin (Gibco), 2 mmol/L L-glutamine (Sigma), and 20 mmol/L HEPES. For inhibitor assays, 24-well plates were used (1.25×10^6 cells/well). To differentiate monocytes into immature DCs, they were grown in RPMI-1640 medium (Sigma) supplemented as above, plus 100 mL/L fetal calf serum (FCS) (Integro, Zaandam, Holland), 10 ng/mL recombinant human granulocyte macrophage-colony stimulating factor, GM-CSF (Biosource, Camarillo, CA, USA), and 20 ng/mL recombinant human interleukin (IL)-4 (R&D Systems, Abingdon, UK). Fresh medium was added every 2 d, and the cells were used in experiments after 6 to 7 d of cultivation. Cultured cells were $CD1a^+$, $CD14^-$, $CD80^+$, $CD83^-$, and $CD86^+$ as analyzed by flow cytometry, and they showed a typical DC morphology (data not shown).

Stimulation experiments

All stimulation experiments were carried out with cells obtained from three to four blood donors and conducted in RPMI-1640 medium containing 100 mL/L FCS. MoDCs were stimulated with a bacteria:host cell ratio of 2:1, 10:1, or 40:1. Cells were collected either for flow cytometric analysis (FACS) or for isolation of total cellular RNA. Supernatants were collected and stored at -20℃.

In cytokine priming experiments moDCs were pre-treated with IL-6 (Biosource), TNF-α (Biosource), or IFN-γ (Finnish Red Cross Blood Transfusion Center) for 16 h prior to bacterial stimulation. IL-6 and TNF-α were used at a concentration of 10 ng/mL, and IFN-γ at 100 IU/mL. Supernatants were collected at 24 h after bacterial stimulation for cytokine measurements.

In inhibitor experiments moDCs were treated with different signaling inhibitors for 30 min prior to bacterial stimulation. Five different inhibitors were used at two concentrations: PD98059 (10 μmol/L or 2 μmol/L) and LY294002 (50 μmol/L or 10 μmol/L) both from Calbiochem (San Diego, CA, USA), and cyclosporin A (CsA) (1 μg/mL or 0.2 μg/mL), SB202190 (10 μmol/L or 2 μmol/L), and pyrrolidine dithiocarbamate (PDTC) (100 μmol/L or 20 μmol/L) all from Alexis Biochemicals (Lausen, Switzerland). Supernatants were collected at 24 h after stimulation, and cytokine levels were determined.

Cytokine-specific enzyme-linked immunosorbent assays (ELISAs)

Cytokine and chemokine levels from cell culture supernatants were analyzed by a sandwich ELISA method as previously described[34]. TNF-α, IL-6, IL-10, and CXCL10 levels were determined by using antibody pairs and standards obtained from BD Pharmingen (San Diego, CA, USA). IL-12p70 and IFN-γ levels were determined with Eli-pair kits (Biosite, Täby, Sweden), and CCL19 and CCL20 levels with Duoset kits (R&D Systems) as instructed by the manufacturer. Cytokine levels in the bacterial dose response experiments were measured using FlowCytomix human Th1/Th2 10plex kit (Bender Medsystems, Vienna, Austria) according to the manufacturer's instructions.

FACS analysis

MoDCs were stimulated with different bacteria for 24 h. Cells were collected, washed once with PBS, and fixed with 10 mL/L paraformaldehyde for 15 min in PBS. After fixation, cells were washed twice with PBS and suspended in PBS + 20 mL/L FCS. For staining of cell surface marker proteins FITC- and PE-conjugated monoclonal antibodies against CD86, HLA class Ⅱ, and isotype matched control antibodies (Caltag Laboratories) were used. Paraformaldehyde-fixed moDCs were stained with mAbs for 30 min at 4℃. The cells were washed twice with PBS + 20 mL/L FCS, suspended in the same solution and analyzed with FACScan flow cytometric device using CellQuest software (Becton Dickinson).

In inhibitor experiments the viability of the cells was confirmed by dead cell discriminator (DCD) (Caltag Laboratories, Burlingame, CA, USA), containing propidium iodide (PI). PI integrates with DNA in non-viable cells with compromised membranes and can be detected by flow cytometry. DCD was added to a suspension of non-fixed cells, 5 μL of DCD per 10^6 cells, immediately prior to FACS, performed as previously described[35].

RNA isolation and Northern blot analysis

For RNA analysis, cells were collected, washed once with PBS, and total cellular RNA was isolated with Rneasy Midi kit (Qiagen). RNA was quantified spectrophotometrically (A_{260nm}) and samples containing equal amounts of RNA (10 μg) were separated by size on 1% formaldehyde-agarose gels and transferred to hybond-N-membranes (Amersham Pharmacia Biotech). To control equal RNA loading, ethidium bromide staining was used. The following human cytokine cDNA probes were used for hybridization: CCL20[36], TNF-α (American Type Culture Collection, Manassas, VA), IL-12p35[33], IFN-γ[37], and IL-10 (DNAX, Palo Alto, CA, USA).

Table 1 Bacteria used in the study

Bacterial species/subspecies	Strain abbreviation	ATCC/DSM number	Ref.	Use in dairy products
Streptococcus				
Streptococcus pyogenes	GAS	NA	[32]	NA
Streptococcus thermophilus	THS	NA	[29,30]	Yoghurt
Lactobacillus				
Lactobacillus rhamnosus GG	LGG	ATCC 53103	[10,7]	Probiotic supplement
Lactobacillus rhamnosus LC705	LC705	DSM 7061	[4,5]	Cheese
Lactobacillus helveticus	1129	DSM 13137	[28]	Cheese, fermented milk
Lactococcus				
Lactococcus lactis subsp. *cremoris*	ARH74	DSM 18891	[31]	Sour milk production
Leuconostoc				
Leuconostoc mesenteroides subsp. *cremoris*	PIA2	DSM 18892	[29]	Sour milk production
Bifidobacterium				
Bifidobacterium animalis subsp. *lactis*	Bb12	DSM 15954	[12]	Probiotic supplement
Bifidobacterium breve	Bb99	DSM 13692	[4,5]	NA
Bifidobacterium longum	1/10	NA	NA	NA

NA: Not applicable.

The probes were labelled with [α-^{32}P] deoxy-adenosine 5'-triphosphate (3000 Ci/mmol, Amersham Pharmacia Biotech) using a random-primed DNA labelling kit (Fermentas, Burlington, Ontario, Canada). Membranes were hybridized o/n in Ultrahyb buffer (Ambion, Austin, TX, USA) at 42℃. Membranes were washed three times with 1 g/L SDS in 1 × saline sodium citrate at 42℃ for 30 min and once at 65℃ for 30 min. Membranes were exposed to Kodak X-Omat AR films (Eastman Kodak, Rochester, NY, USA) at -70℃ with intensifying screens.

RESULTS

Bacteria-induced cytokine production in human moDCs

MoDCs were stimulated with different doses of probiotic bacteria to determine the optimal bacterial dose for further stimulation experiments (Figure 1). The bacteria used in the present study are summarized in Table 1. *S. thermophilus* THS efficiently induced TNF-α, IL-6, and IL-12 production. The two Bifidobacterium strains, *B. animalis* subsp. *lactis* Bb12 and *B. breve* Bb99, were potent inducers of all measured cytokines TNF-α, IL-1β, IL-6, IL-10, IL-12, and IFN-γ. In contrast, *B. longum* strain 1/10 was not as efficient as *B. animalis* subsp. *lactis* Bb12 and *B. breve* Bb99 in inducing cytokine, production. *L. lactis* subsp. *cremoris* ARH74 and *L. helveticus* 1129 were as good as bifidobacteria at inducing cytokine production in moDCs. *L. rhamnosus* strains GG and LC705 as well as *L. mesenteroides* subsp. *cremoris* PIA2 were poor inducers of cytokine production and stimulation of moDCs with these bacteria did not result in increased cytokine production compared to unstimulated cells. There was a direct correlation between bacterial dose and cytokine response. Bacteria: host cell ratio of 40:1 showed the highest cytokine production levels (Figure 1).

All studied bacteria induced CCL20 production in a dose-dependent manner (Figure 2), while none of the bacteria were able to induce CCL19 production. *S. thermophilus* was the most efficient probiotic bacterium in inducing CXCL10 production in moDCs. The dose-dependent responses demonstrated that the bacteria: host cell ratio of 40:1 was also most effective in inducing moDC chemokine production.

The effects of bacteria on moDC maturation

Since different probiotic bacteria are able to induce variable cytokine and chemokine responses in human moDCs (Figures 1 and 2), we analyzed whether these bacteria would also differ in their ability to induce moDC maturation. Maturation of DCs is characterized by up-regulation of cell surface maturation marker proteins CD80, CD86, and HLA class Ⅱ[38]. For these studies we chose *B. breve* Bb99, *S. thermophilus* THS, and *L. lactis* subsp. *cremoris* ARH74 that represent different genera and were able to stimulate a variety of moDC cytokine responses (Figure 1). MoDCs were stimulated with selected bacteria at bacteria:host cell ratio of 2:1, 10:1, or 40:1. After stimulation the expression of CD86 and HLA class Ⅱ was analyzed by flow cytometry. Cells from different donors were pooled before the analysis. In *S. thermophilus* THS and *B. breve* Bb99 stimulated moDCs highest up-regulation of HLA class Ⅱ was observed with bacteria:host cell ratio of 10:1, whereas with *L. lactis* subsp. *cremoris* ARH74 a ratio of 40:1 was required for maximal HLA class Ⅱ induction (Figure 3A). The highest CD86 expression was seen at bacteria:host cell ratio of 40:1 (Figure 3B) with all studied bacteria. It is noteworthy that even the lowest bacterial dose (2:1) increased the expression of HLA Ⅱ and CD86. MoDCs stimulated with probiotic bacteria matured equally well as cells stimulated with pathogenic *S. pyogenes*, a known inducer of moDC maturation[33]. The majority of the bacteria-stimulated cells were HLA class Ⅱ/CD86 double positive (Figure 3C). Maturation of moDCs was also observed with all other studied bacteria (data not shown).

Kinetics of bacteria-induced cytokine and chemokine mRNA expression

To further compare the cytokine gene expression profiles induced by *S. thermophilus* THS, *B. breve* Bb99,

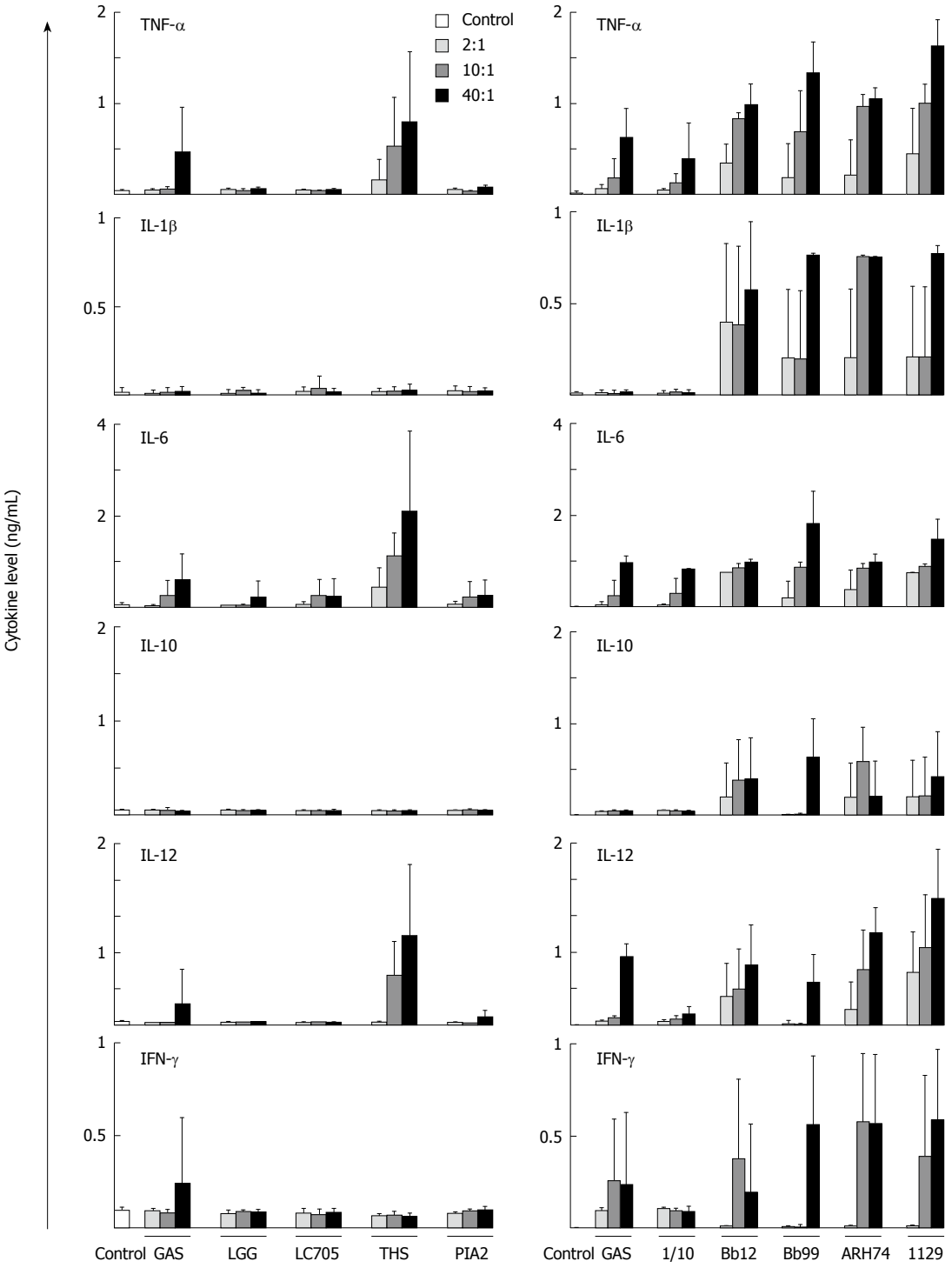


Figure 1 Probiotic bacteria induce cytokine production in human moDCs in a dose-dependent manner. MoDCs were stimulated with bacteria:host cell ratio of 2:1, 10:1, or 40:1. At 24 h after stimulation cell culture supernatants were collected and cytokine levels were determined by ELISA. The experiment was carried out with cells obtained from four different blood donors, and the columns represent means and error bars indicate the standard deviations. The data is from a representative experiment out of two. GAS: *S. pyogenes*; LGG: *L. rhamnosus* GG; LC705: *L. rhamnosus* LC705; THS: *S. thermophilus* THS; PIA2: *L. mesenteroides* subsp. *cremoris* PIA2; 1/10: *B. longum* 1/10; Bb12: *B. animalis* subsp. *lactis* Bb12; Bb99: *B. breve* Bb99; ARH74: *L. lactis* subsp. *cremoris* ARH74; 1129: *L. helveticus* 1129.

and *L. lactis* subsp. *cremoris* ARH74, moDCs were stimulated with these bacteria at a 40:1 bacteria:host cell ratio for 8, 24, or 48 h. Cells from different donors were collected and pooled. Total cellular RNA was isolated for Northern blot analysis (Figure 4A). The mRNA expression of CCL20 and TNF-α was enhanced 8 h after stimulation with *S. thermophilus* THS, *B. breve* Bb99, and *L. lactis* subsp. *cremoris* ARH74. The expression of IL-12p35 or IFN-γ mRNA was not enhanced by any of the potentially probiotic bacteria. CCL20 mRNA level was highest in *B. breve* Bb99 stimulated cells at 8 h after stimulation. Low levels of CCL20 mRNA were detected in *S. pyogenes* and *S. thermophilus* THS stimulated cells at 8 h after stimulation. CCL20 protein level was highest at the 48 h time point in *B. breve* Bb99 stimulated cells (Figure 4B). TNF-α mRNA expression was strongest in

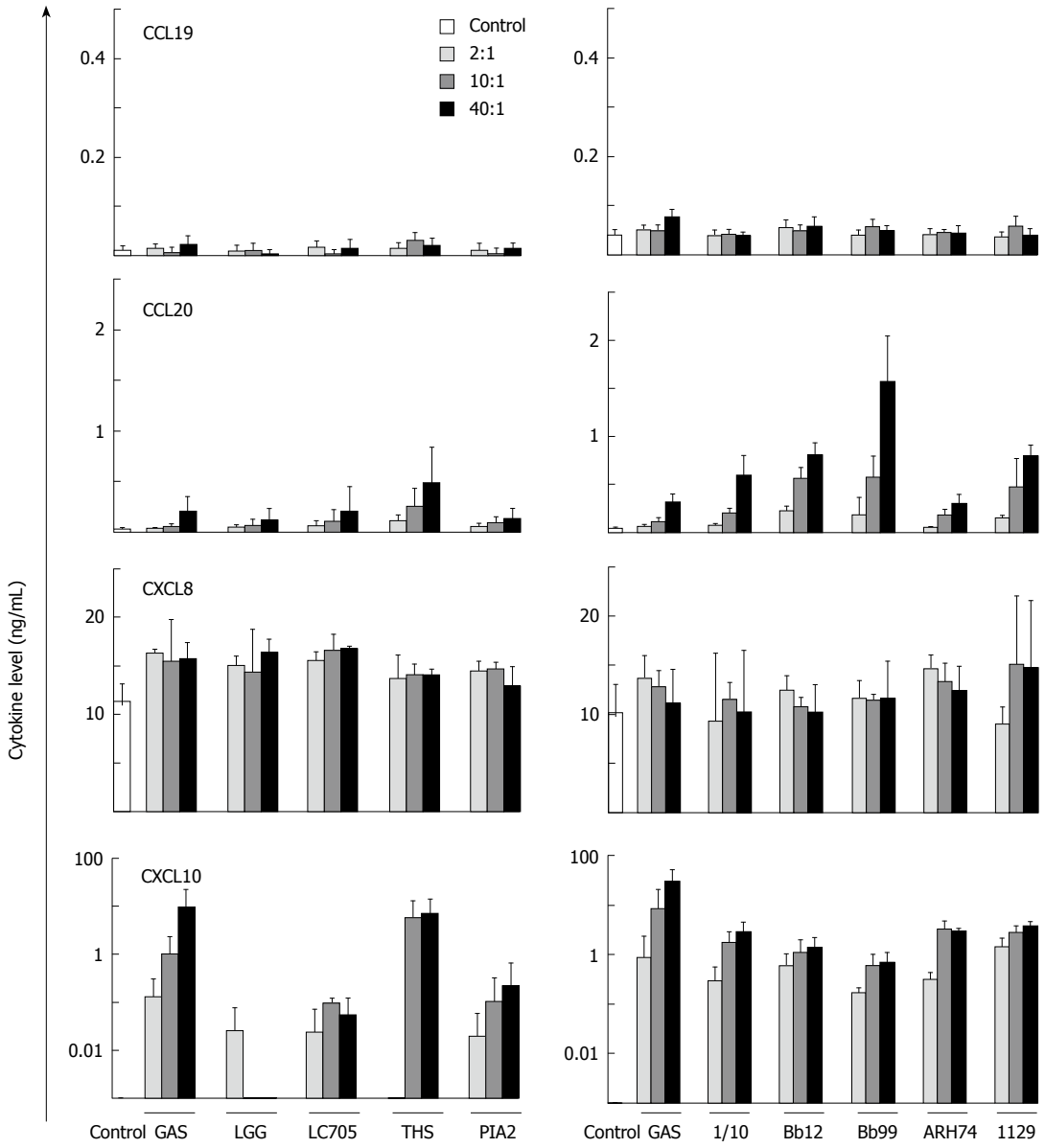


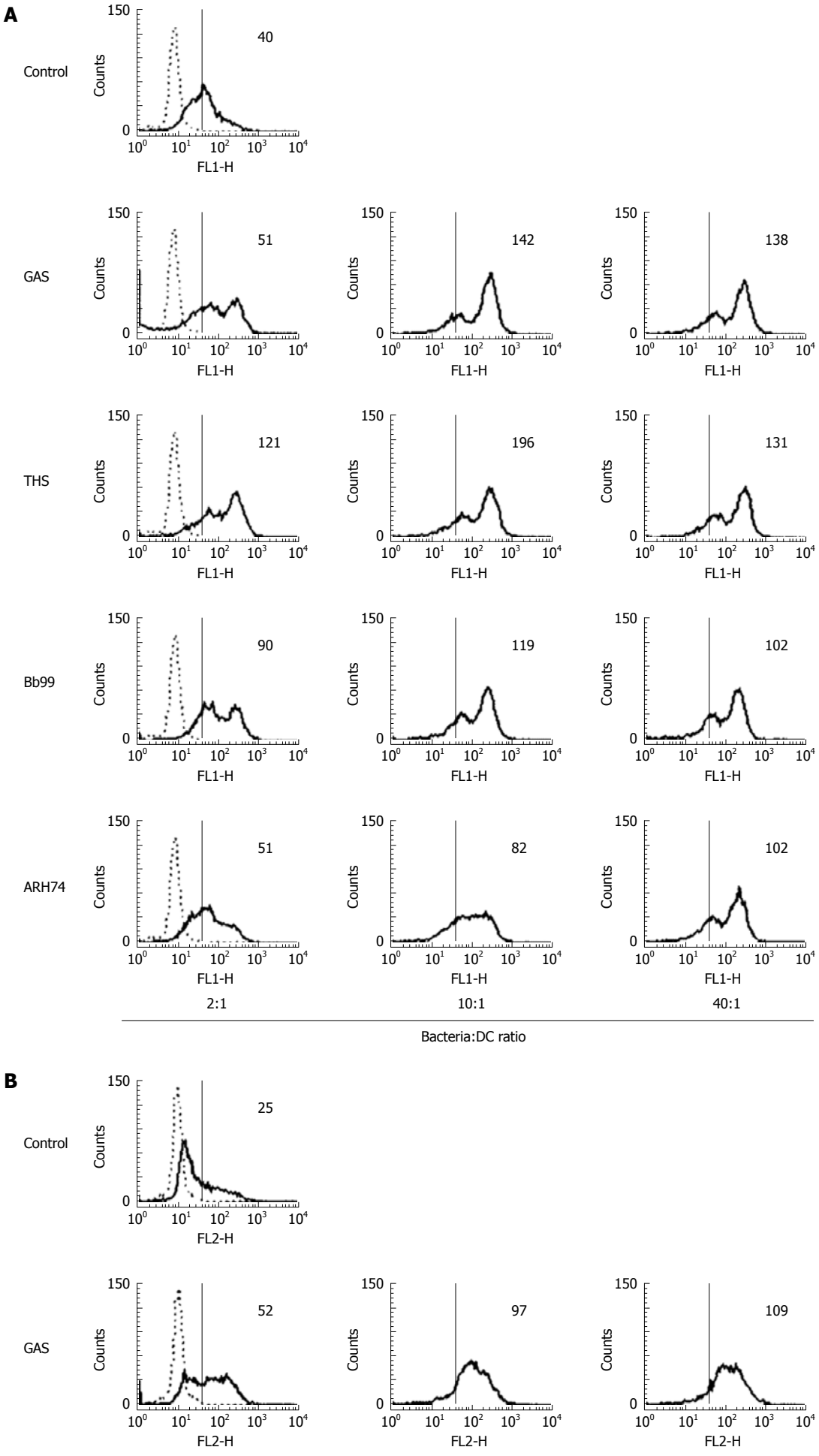
Figure 2 Probiotic bacteria induce chemokine production in moDCs. MoDCs were stimulated with bacteria:host cell ratio of 2:1, 10:1, or 40:1. Cell culture supernatants from four blood donors were collected at 24 h after bacterial stimulation and the chemokine levels were determined by ELISA. The data is shown as means and error bars indicate standard deviations. Note the differences in scales. The data is from a representative experiment out of two.

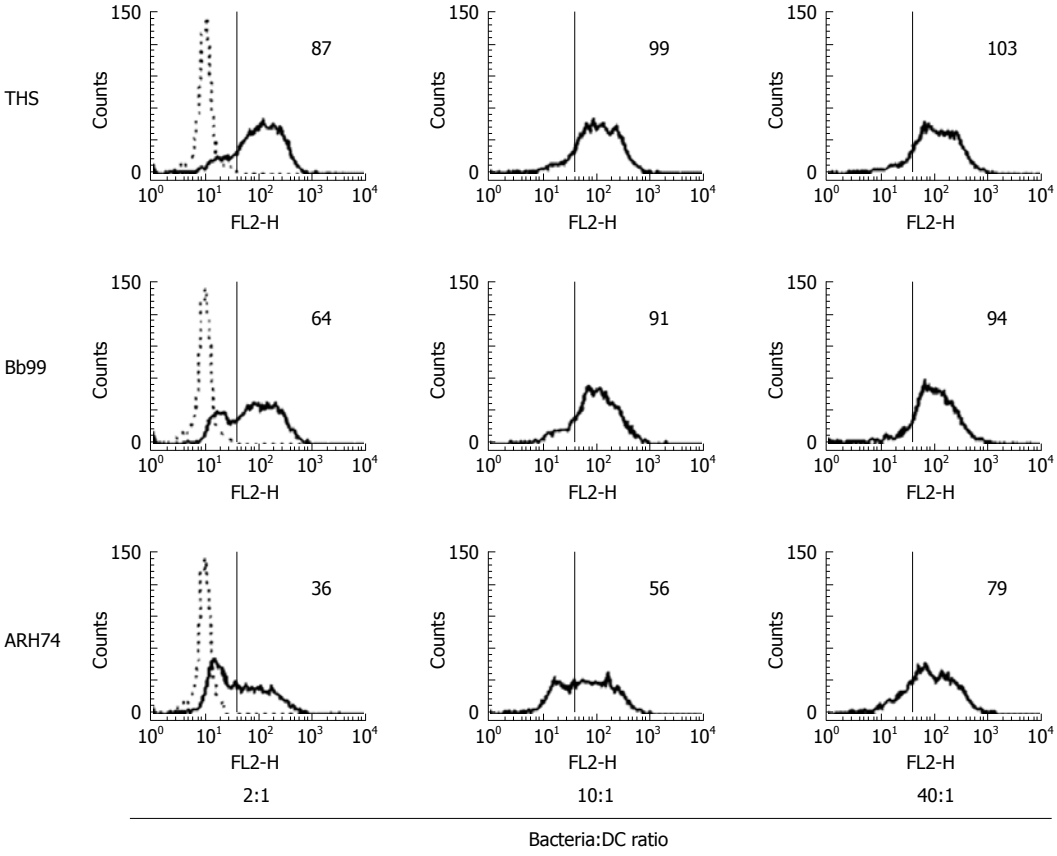
B. breve Bb99 stimulated moDCs. In moDCs stimulated with *S. thermophilus* THS and *L. lactis* subsp. *cremoris* ARH74 TNF-α mRNA levels were lower than in *B. breve* Bb99 stimulated moDCs. Cell culture supernatants were also collected and cytokine levels were determined by ELISA. The cytokine production patterns correlated with the kinetics and magnitude of bacteria induced mRNA expression profiles (Figure 4B). IL-12 and IFN-γ mRNAs were not detectable in cells stimulated with probiotic bacteria, and also protein levels remained lower than in *S. pyogenes* stimulated moDCs. IL-10 mRNA and protein expression was best induced by *B. breve* Bb99.

Pharmacological signaling inhibitors affect the cytokine production of probiotic bacteria-stimulated moDCs

In order to obtain insight into the mechanisms of probiotic bacteria-induced cytokine gene expression in human moDCs, we used different pharmacological inhibitors for MAPK, PI3K, NF-κB, and nuclear factor of activated T cells (NFAT) signaling pathways to study whether they interfere with cytokine production in bacteria-stimulated cells.

B. breve Bb99 was chosen for these experiments due to its ability to effectively induce cytokine production in moDCs. Cells were treated with the inhibitors 30 min prior to bacterial stimulation and the inhibitors were present throughout the stimulation experiment. The p38 MAPK inhibitor SB202190[39] decreased the production of TNF-α, IL-6, IFN-γ, and IL-10 production in a dose-dependent manner (Figure 5). The inhibitor of PI3 kinase, LY294002[40], abolished CXCL10, TNF-α, IFN-γ, and IL-10 production at a concentration of 50 μmol/L. Concentration 100 μmol/L of NF-κB inhibitor PDTC reduced CXCL10 and IFN-γ production, whereas no significant inhibition in the production of other


A
Control
GAS
THS
Bb99
ARH74
Counts
FL1-H
40
51
142
138
121
196
131
90
119
102
51
82
102
2:1
10:1
40:1
Bacteria:DC ratio
B
Control
GAS
FL2-H
25
52
97
109



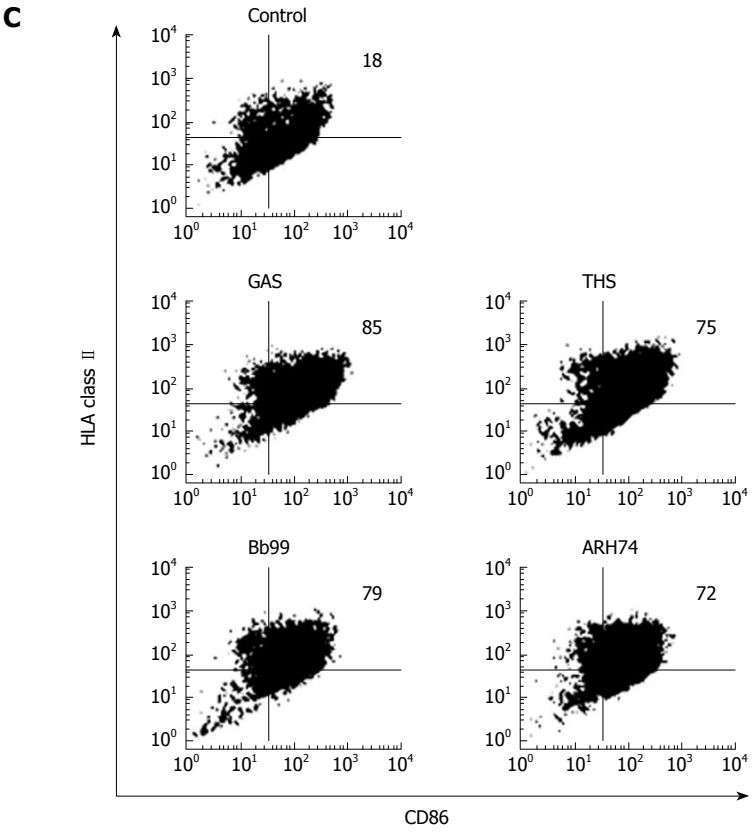


Figure 3 The effect of bacterial dose on the maturation of moDCs. MoDCs were stimulated with bacteria:host cell ratio of 2:1, 10:1, or 40:1. Cells from four different donors were collected, pooled, and stained with antibodies against (**A**) HLA class Ⅱ and (**B**) CD86. The expression of co-stimulatory molecules was analyzed by flow cytometry. The values represent mean fluorescence intensities (MFIs). Results from a representative experiment out of two are shown. Dotted lines indicate respective isotype controls. The staining profiles of HLA class Ⅱ/CD86 double-positive (percentages included in the profiles) cells (**C**). GAS: *S. pyogenes*; THS: *S. thermophilus* THS; Bb99: *B. breve* Bb99; ARH74: *L. lactis* subsp. *cremoris* ARH74.

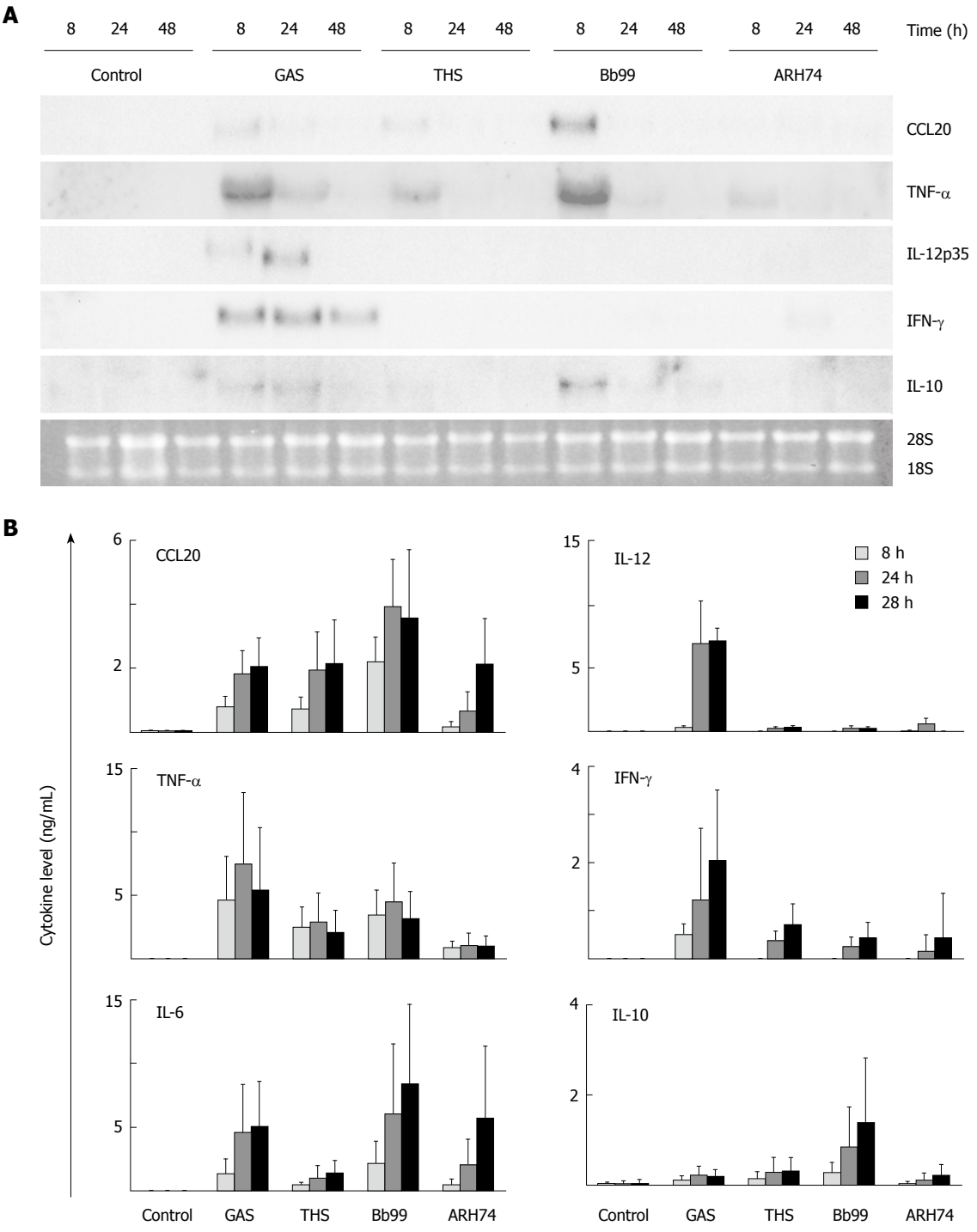


Figure 4 Kinetics of probiotic bacteria induced cytokine gene expression in human moDCs. MoDCs were stimulated with GAS and indicated probiotic bacteria with a bacteria:host cell ratio of 40:1. **A**: Cells were collected at 8, 24, and 48 h after stimulation and total cellular RNA was isolated. mRNA expression was analyzed with Northern blotting using CCL20, TNF-α, IL-12p35, IFN-γ, and IL-10 cDNA probes. Ethidium bromide staining was used to control equal sample loading and the integrity of RNA; **B**: Cell culture supernatants from the experiment described in panel A were collected and cytokine levels in the supernatants were determined by ELISA. The bars and error bars represent the means and standard deviations from four different blood donors. One representative experiment out of two is shown. See Figure 3 for abbreviations of bacteria.

cytokines was observed (Figure 5). The ERK MAPK inhibitor PD98059[41] had no effect on cytokine production in *B. breve* Bb99 stimulated moDCs. The NFAT inhibitor CsA had a weak inhibitory effect on Bb99-induced IFN-γ production. The production of CCL20 was enhanced in the presence of LY294002, PDTC, and CsA. Likewise, TNF-α production was enhanced in the presence of PDTC and CsA (Figure 5). The viability of inhibitor-treated moDCs was monitored with propidium iodide (PI) staining. Treatment of moDCs with signaling inhibitors LY294002 or PDTC had a minor effect on cell viability, and approximately 70% of cells were alive at 24 h after the use of these inhibitors. P38 or NFAT inhibitors did not have effects on cell viability. In untreated control cells 86% of the moDCs were viable (data not shown).

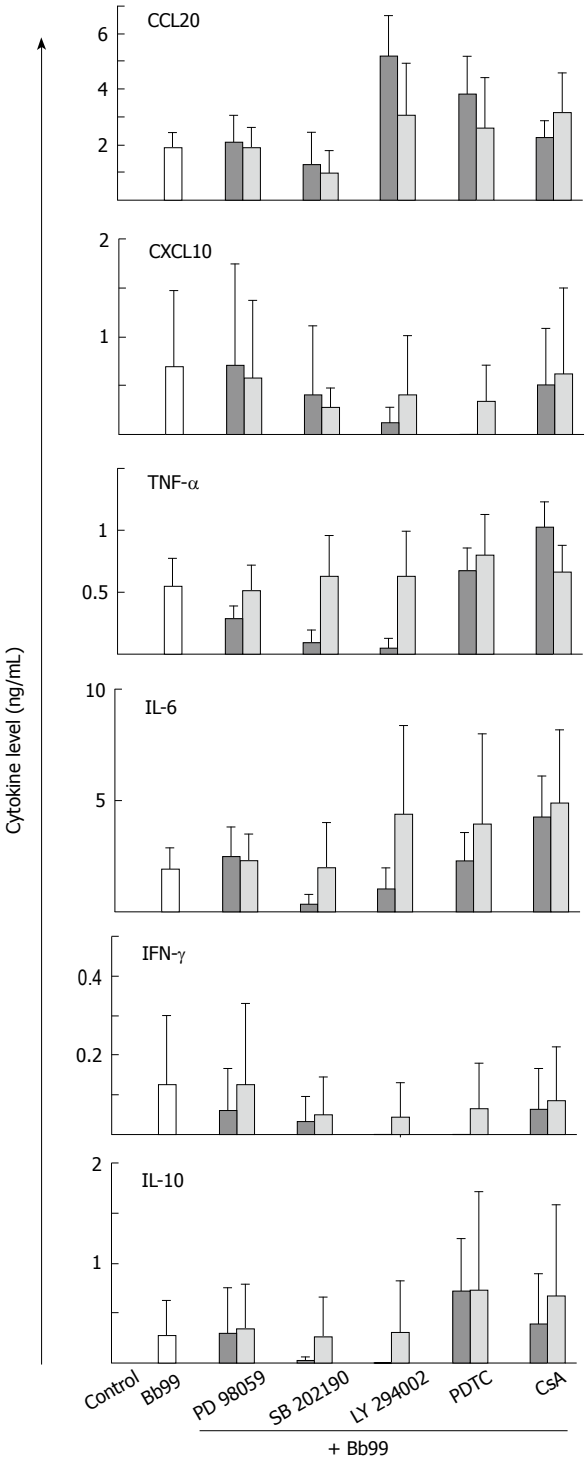


Figure 5 Pharmacological signaling inhibitors interfere with *Bifidobacterium breve* Bb99 induced cytokine production in moDCs. moDCs were left untreated or treated with different pharmacological inhibitors for 30 min prior to stimulation with Bb99 (bacteria:host cell ratio of 40:1). Cell culture supernatants from four blood donors were collected at 24 h after stimulation, and cytokine levels were determined by ELISA. The used inhibitors were: p38 MAP kinase inhibitor SB202190, PI3-kinase inhibitor LY294002, ERK MAP kinase inhibitor PD98059, NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC), and NFAT inhibitor cyclosporinA (CsA). Inhibitor concentrations decrease from left to right.

Cytokine priming regulates cytokine production in bacteria stimulated moDCs

Since *B. breve* Bb99 and other studied bacteria were able to induce cytokine production in moDCs (Figure 1) we analyzed whether proinflammatory cytokines could further contribute to probiotic bacteria-induced cytokine production and whether they would autoregulate their own production by positive or negative feedback mechanisms. MoDCs were pre-treated with IL-6, TNF-α, or IFN-γ for 16 h followed by stimulation with *B. breve* Bb99 or *S. pyogenes* for 24 h (Figure 6). IL-6 priming had a minor stimulatory effect on *S. pyogenes* induced IFN-γ and IL-10 production, whereas such an effect was not seen in *B. breve* Bb99 stimulated cells. TNF-α priming reduced IL-6, IL-10, IFN-γ, and CXCL10 production in *S. pyogenes* stimulated cells, and IL-6 and IL-10 production in *B. breve* Bb99 stimulated cells. In IFN-γ primed cells the production of IL-6, IL-10, and TNF-α was enhanced after *B. breve* Bb99 or *S. pyogenes* stimulation. In addition, the production of CCL20 and CXCL10 chemokines was enhanced in *B. breve* Bb99 stimulated cells after IFN-γ priming (Figure 6).

DISCUSSION

In the present study cytokine production profiles after probiotic bacterial stimulation were analyzed in a model system using *in vitro* cultured human moDCs. In the gastrointestinal tract bacteria are in close contact with gut epithelial cells and various types of immune cells that reside in lamina propria and the luminal side of the mucosal epithelium[42]. Mucosal DCs participate in sampling the gut microbiota by extending dendrites through the gut epithelium[15]. They have a central role in regulating both innate and adaptive immune responses during microbial infections. Therefore, it is important to study the interactions between DCs and potentially probiotic bacteria. Mucosal DCs primarily consist of myeloid DCs, which presumably are of monocyte origin[43].

We have compared the ability of two well characterized probiotics *L. rhamnosus* GG and *B. animalis* Bb12 and seven potentially probiotic bacteria to induce maturation of human moDCs. We observed that moDCs stimulated with these probiotic bacteria matured as well as moDCs stimulated with a significant human pathogen, *S. pyogenes*, as indicated by the expression of costimulatory molecules CD86 and HLA class Ⅱ. The results are well in line with previous studies which show that *S. pyogenes*, different lactobacilli, and bifidobacteria can induce DC maturation[19,33,44]. Our data shows that also *L. rhamnosus* LC705, *L. helveticus* 1129, *B. longum* 1/10, *B. breve* Bb99, *S. thermophilus* THS, *L. lactis* ARH74, and *L. mesenteroides* subsp. *cremoris* PIA2 with potential probiotic characteristics can enhance the expression of DC maturation markers. Lactobacilli seem to be able to induce DC maturation but cytokine production remains low after stimulation with *L. rhamnosus* strains LGG and LC705. Our data demonstrates that some lactobacilli are poor inducers of proinflammatory cytokines while others can stimulate cytokine production in human moDCs (Figures 1 and 2), as previously observed[44,45]. The ability of bacteria to induce moDC maturation but not cytokine production could mean that they stimulate the development of partially- or semi-mature DCs[33].

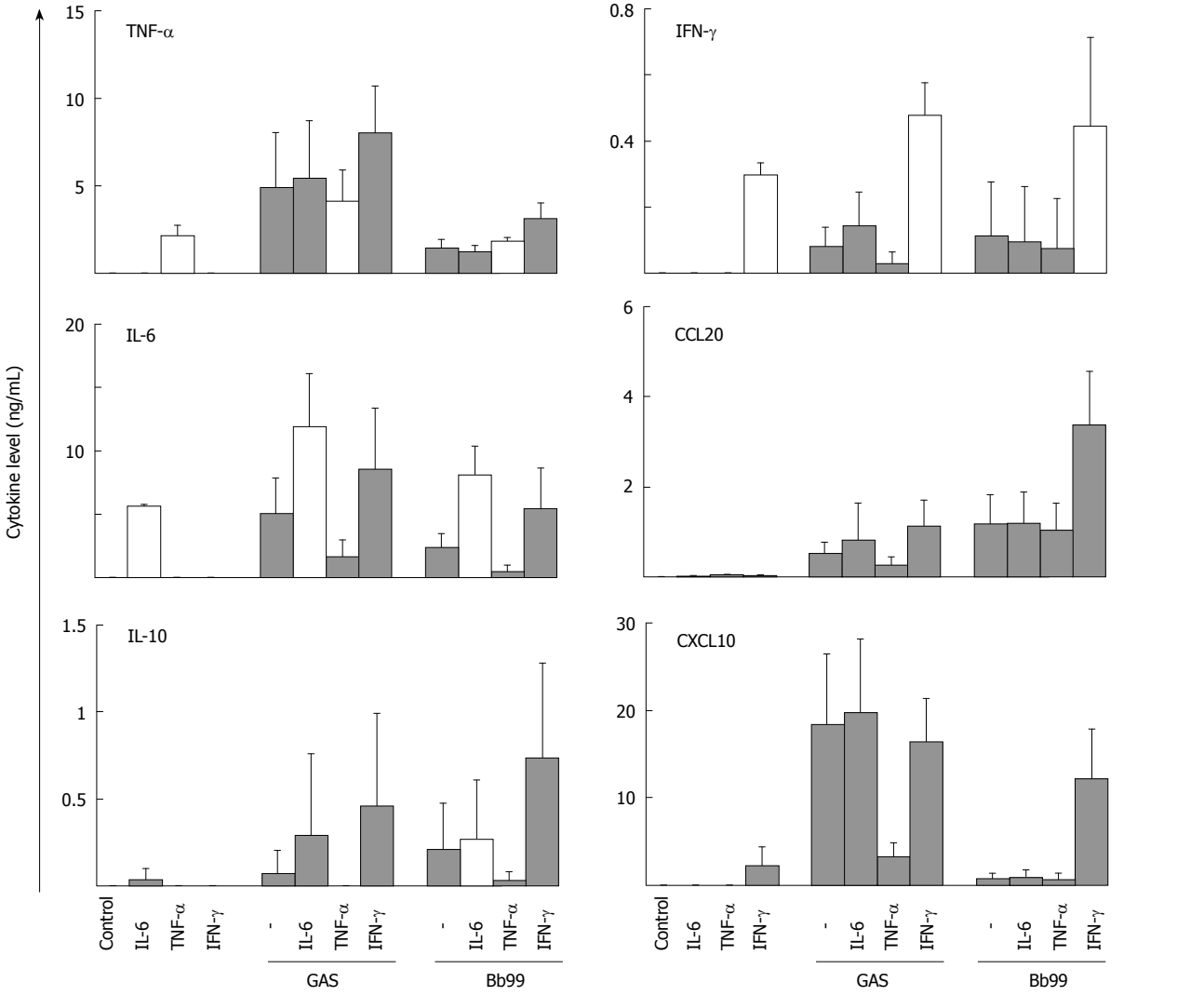


Figure 6 Pre-treatment of human moDCs with proinflammatory cytokines affects bacteria induced cytokine production. MoDCs were primed with IL-6, TNF-α, or IFN-γ for 16 h. Unprimed or cytokine primed cells were stimulated with GAS or Bb99 (bacteria:host cell ratio of 40:1). Cell culture supernatants were collected at 24 h after stimulation, and cytokine levels in supernatants were determined by ELISA. The experiment was done with cells obtained from four blood donors, and results of a representative experiment out of two are shown. The data is shown as the means, and the error bars represent standard deviations. White bars indicate cytokine priming. GAS; *Streptococcus pyogenes*; Bb99: *Bifidobacterium breve* Bb99.

This type of DCs may be involved in the induction of tolerance, as previously suggested[46].

The potentially probiotic and probiotic bacteria used in our study were previously analyzed by Kekkonen *et al*[29] in peripheral blood mononuclear cells (PBMC). In PBMC, *L. mesenteroides* subsp. *cremoris* PIA2 and *S. thermophilus* THS were potent inducers of IL-12 and IFN-γ production. However, in moDCs *S. thermophilus* THS was a potent inducer of IL-12 but not that of IFN-γ. *L. mesenteroides* subsp. *cremoris* PIA2 did not induce IL-12 or IFN-γ production in moDCs. This data demonstrates that *S. thermophilus* THS can induce a proinflammatory cytokine response in several primary cells analyzed. As shown in the present study, we are the first ones to demonstrate IFN-γ production in human moDCs in response to stimulation with potentially probiotic *B. animalis* subsp. *lactis* Bb12, *B. breve* Bb99, *L. lactis* ARH74, and *L. helveticus* 1129.

We observed that *B. animalis* subsp. *lactis* Bb12 and *B. breve* Bb99 were good inducers of IL-10 production in moDCs. *B. animalis* subsp. *lactis* Bb12 and *B. breve* Bb99 have been reported to induce IL-10 also in PBMCs[29] and other bifidobacteria in human colonic lamina propria DCs[45]. IL-10 has anti-inflammatory effects by decreasing IL-12 production and thereby also IFN-γ production which leads to the development of Th2 or Th3 type immune responses[19]. The ability of bifidobacteria to induce IL-10 production may be one factor contributing to their observed anti-inflammatory activities[45,47]. The proinflammatory cytokines produced by probiotic bacteria stimulated moDCs are suggested to induce low-grade inflammation that might protect allergy prone people from the development of allergy[48]. The ability of probiotics to induce distinct moDC cytokine production profiles in a genera or a strain-specific manner could be due to different molecules and structures expressed on the surface of these bacteria. Bacteria can also secrete active proteins or peptides that can stimulate host cells[49-51]. Recognition of these components by receptors on host cell surface can result

in the activation of diverse signaling pathways and trigger differential cytokine production.

Probiotic bacteria used in our study differentially induced moDC cytokine gene expression. *L. rhamnosus* strains LGG and LC705, and *L. mesenteroides* subsp. *cremoris* PIA2 were weak in inducing moDC cytokine responses. Bifidobacteria, *S. thermophilus* THS, *L. lactis* ARH74, and *L. helveticus* 1129 induced the production of proinflammatory cytokines TNF-α, IL-6, IL-12, and IFN-γ as well as anti-inflammatory IL-10. Pathogenic *S. pyogenes* and *S. thermophilus*, a streptococcus used in yogurt fermentation induced production of proinflammatory cytokines and chemokines TNF-α, IL-6, IL-12, CCL20, and CXCL10. IL-10 production was not observed suggesting that at least these two streptococcal species have a tendency to enhance inflammatory cytokine production. It has previously been shown that some streptococci or their components utilize TLR2 pathway to activate host cells[52,53]. Since these bacteria are good inducers of IL-12, that can not be efficiently triggered through TLR2[54], it is likely that other receptor/signaling systems apart from TLR2 are also activated. It is of interest that *L. lactis* subsp. *cremoris* ARH74 and *L. helveticus* 1129 were able to induce both strong inflammatory cytokine/chemokine and anti-inflammatory IL-10 responses.

The kinetics of bacteria-induced cytokine mRNA expression was studied with *S. thermophilus* THS, *B. breve* Bb99, and *L. lactis* subsp. *cremoris* ARH7. The expression of CCL20 and TNF-α mRNAs, and CCL20, TNF-α, and IL-6 protein production was detected at early time points after stimulation with these three probiotic bacteria. Fast TNF-α and IL-6 production enhances inflammatory responses. CCL20 attracts immature DCs to the site of infection where they can be activated[55]. The fact that *S. thermophilus* THS, *B. breve* Bb99, and *L. lactis* subsp. *cremoris* ARH74 can induce efficient TNF-α, IL-6, and CCL20 production suggests that they all can trigger efficient inflammatory responses. CCL19 is a chemokine produced by activated DCs that attracts immature T cells to the local lymph nodes where their activation takes place[55]. It was of interest that none of the bacteria used in this study could induce CCL19 mRNA or protein expression. The lack of CCL19 production could indicate that the immune response triggered by probiotic bacteria may be incomplete compared to moDC responses to pathogenic bacteria, such as *S. enterica* serovar Typhimurium which is known to induce CCL19 production[56]. Th1 type cytokines IL-12 and IFN-γ were produced at later time points after stimulation with *S. pyogenes* and probiotic bacteria as shown previously with *S. pyogenes*[33]. Our results are well in line with previous experiments done with human moDCs showing that TNF-α, IL-12, and CCL20 production is induced in response to *S. pyogenes* stimulation, while a probiotic *L. rhamnosus* GG was weak in activating moDC cytokine production[33].

We used pharmacological signaling inhibitors to analyze the host cell signaling pathways regulating probiotic bacteria-induced cytokine production. Our results show that p38 MAPK, PI3 kinase, and NF-κB signaling pathways play an essential although a differential role in the ability of probiotic bacteria to induce moDC cytokine gene expression. MAPK and PI3K pathways are involved in TNF-α, IL-6, IL-10, IFN-γ, and CXCL10 production in *B. breve* Bb99 stimulated moDC. Inhibition of NF-κB and NFAT pathways led to a more limited effect on CXCL10 and IFN-γ production induced by *B. breve*. Surprisingly, the use of some inhibitors resulted in enhanced *B. breve* Bb99 induced cytokine production. Blocking one signaling pathway likely leads to stimulation of an alternative signaling pathway thus increasing cytokine production[57].

Since *B. breve* Bb99 was efficient in inducing moDC cytokine production (Figure 1), we studied the effects of different cytokines on the ability of this bacterium to induce the production of other cytokines. IFN-γ priming enhanced *B. breve* Bb99 and *S. pyogenes* stimulated production of cytokines. The effects of IFN-γ are likely due to the enhanced expression of TLRs and other signaling components, which are under the transcriptional regulation of IFNs[58]. Since IFN-γ priming increased moDC responsiveness to *B. breve* Bb99 and *S. pyogenes* it may be that identical signaling pathways are involved in cytokine production in response to stimulation with non-pathogenic *B. breve* Bb99 and pathogenic *S. pyogenes*. TNF-α priming had an opposite effect on moDC cytokine production. TNF-α is involved in moDC maturation, which decreases the capacity of moDCs to take up microbes or their components[59]. Therefore, TNF-α primed cells could be less responsive to *B. breve* stimulation. However, further studies are needed to reveal the mechanisms and co-operativity of signaling pathways involved in cytokine production in bacteria-stimulated moDCs.

In the present study carried out with human primary DCs we compared nine potentially probiotic bacteria in their abilities to induce cytokine and chemokine production in moDCs. We demonstrate that these bacteria have strain-specific effects on moDC cytokine production and they all induce moDC maturation as efficiently as pathogenic bacteria. This data is valuable for selecting new probiotic bacteria. The knowledge of unique cytokine production profiles may help in targeting specific probiotic strains for clinical applications.

ACKNOWLEDGMENTS

Minja Miettinen is greatly appreciated for her critical comments on the manuscript. The authors thank Mari Aaltonen, Hanna Valtonen, and Juha Laukonmaa for their expert technical assistance.

COMMENTS

Background

Probiotic bacteria are widely used to relieve the symptoms of many disorders. However, the mechanisms that cause these beneficial actions are yet to be characterized. One of the factors contributing to the health promoting effects of probiotic bacteria could be their capacity to induce cytokine production that further regulates the development of innate and adaptive immune responses.

Innovations and breakthroughs

At present the cytokine production profiles of different probiotic strains, some of which have already been used in clinical trials, are poorly characterized. Also comparative data from same experimental model systems is limited. In this study we have systematically analyzed the ability of nine different potentially probiotic bacteria to induce maturation and cytokine production in human monocyte-derived dendritic cells (moDCs) in order to compare their capacities to activate innate and adaptive immune responses.

Applications

Data shows that probiotic bacteria induced the maturation of moDCs in a dose-dependent manner. They induced moDC maturation as efficiently as pathogenic bacteria but differed in a genera-specific manner in their ability to induce moDC cytokine gene expression. *S. thermophilus* induced the expression of pro-inflammatory (TNF-α, IL-12, IL-6, and CCL20) and Th1 type (IL-12 and IFN-γ) cytokines, while *B. breve* and *L. lactis* were also potent inducers of anti-inflammatory IL-10. Mitogen-activated protein kinase (MAPK) p38, phosphatidylinositol 3 (PI3) kinase, and nuclear factor-kappa B (NF-κB) signaling pathways were shown to be involved in bacteria-induced cytokine production. The cytokine profiles of probiotic bacteria can be useful in selecting new probiotic bacteria for *in vivo* trials and help to understand the mechanisms behind probiotic actions.

Peer review

This paper describes the induction of cytokines and DC maturation by different potentially probiotic bacteria. The study clearly describes the potential of probiotic bacteria to induce DC maturation, dose-dependent responses, cytokine production kinetics, and signaling pathways involved in cytokine gene expression. It's interesting.

REFERENCES

1 **Macfarlane GT**, Cummings JH. Probiotics, infection and immunity. *Curr Opin Infect Dis* 2002; **15**: 501-506
2 **Reid G**, Jass J, Sebulsky MT, McCormick JK. Potential uses of probiotics in clinical practice. *Clin Microbiol Rev* 2003; **16**: 658-672
3 **Salminen S**, Arvilommi H. Probiotics demonstrating efficacy in clinical settings. *Clin Infect Dis* 2001; **32**: 1577-1578
4 **Kajander K**, Hatakka K, Poussa T, Farkkila M, Korpela R. A probiotic mixture alleviates symptoms in irritable bowel syndrome patients: a controlled 6-month intervention. *Aliment Pharmacol Ther* 2005; **22**: 387-394
5 **Myllyluoma E**, Veijola L, Ahlroos T, Tynkkynen S, Kankuri E, Vapaatalo H, Rautelin H, Korpela R. Probiotic supplementation improves tolerance to Helicobacter pylori eradication therapy--a placebo-controlled, double-blind randomized pilot study. *Aliment Pharmacol Ther* 2005; **21**: 1263-1272
6 **Arvola T**, Laiho K, Torkkeli S, Mykkanen H, Salminen S, Maunula L, Isolauri E. Prophylactic Lactobacillus GG reduces antibiotic-associated diarrhea in children with respiratory infections: a randomized study. *Pediatrics* 1999; **104**: e64
7 **Szajewska H**, Kotowska M, Mrukowicz JZ, Armanska M, Mikolajczyk W. Efficacy of Lactobacillus GG in prevention of nosocomial diarrhea in infants. *J Pediatr* 2001; **138**: 361-365
8 **Kalliomaki M**, Salminen S, Poussa T, Arvilommi H, Isolauri E. Probiotics and prevention of atopic disease: 4-year follow-up of a randomised placebo-controlled trial. *Lancet* 2003; **361**: 1869-1871
9 **Schultz M**, Linde HJ, Lehn N, Zimmermann K, Grossmann J, Falk W, Scholmerich J. Immunomodulatory consequences of oral administration of Lactobacillus rhamnosus strain GG in healthy volunteers. *J Dairy Res* 2003; **70**: 165-173
10 **Majamaa H**, Isolauri E. Probiotics: a novel approach in the management of food allergy. *J Allergy Clin Immunol* 1997; **99**: 179-185
11 **Saxelin M**, Tynkkynen S, Mattila-Sandholm T, de Vos WM. Probiotic and other functional microbes: from markets to mechanisms. *Curr Opin Biotechnol* 2005; **16**: 204-211
12 **Schiffrin EJ**, Rochat F, Link-Amster H, Aeschlimann JM, Donnet-Hughes A. Immunomodulation of human blood cells following the ingestion of lactic acid bacteria. *J Dairy Sci* 1995; **78**: 491-497
13 **Macdonald TT**, Monteleone G. Immunity, inflammation, and allergy in the gut. *Science* 2005; **307**: 1920-1925
14 **Niess JH**, Brand S, Gu X, Landsman L, Jung S, McCormick BA, Vyas JM, Boes M, Ploegh HL, Fox JG, Littman DR, Reinecker HC. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* 2005; **307**: 254-258
15 **Rescigno M**, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, Granucci F, Kraehenbuhl JP, Ricciardi-Castagnoli P. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol* 2001; **2**: 361-367
16 **Sallusto F**, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* 1994; **179**: 1109-1118
17 **Quah BJ**, O'Neill HC. Maturation of function in dendritic cells for tolerance and immunity. *J Cell Mol Med* 2005; **9**: 643-654
18 **Sallusto F**, Lanzavecchia A. The instructive role of dendritic cells on T-cell responses. *Arthritis Res* 2002; **4** Suppl 3: S127-S132
19 **Christensen HR**, Frokiaer H, Pestka JJ. Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. *J Immunol* 2002; **168**: 171-178
20 **Iwasaki A**, Kelsall BL. Mucosal immunity and inflammation. I. Mucosal dendritic cells: their specialized role in initiating T cell responses. *Am J Physiol* 1999; **276**: G1074-G1078
21 **Mills KH**, McGuirk P. Antigen-specific regulatory T cells--their induction and role in infection. *Semin Immunol* 2004; **16**: 107-117
22 **Inohara N**, Nunez G. NODs: intracellular proteins involved in inflammation and apoptosis. *Nat Rev Immunol* 2003; **3**: 371-382
23 **Akira S**, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006; **124**: 783-801
24 **Janeway CA Jr**, Medzhitov R. Innate immune recognition. *Annu Rev Immunol* 2002; **20**: 197-216
25 **Iwasaki A**, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 2004; **5**: 987-995
26 **Armant MA**, Fenton MJ. Toll-like receptors: a family of pattern-recognition receptors in mammals. *Genome Biol* 2002; **3**: REVIEWS3011
27 **Takeda K**, Akira S. Microbial recognition by Toll-like receptors. *J Dermatol Sci* 2004; **34**: 73-82
28 **Yang Z**, Staaf M, Huttunen E, Widmalm G. Structure of a viscous exopolysaccharide produced by Lactobacillus helveticus K16. *Carbohydr Res* 2000; **329**: 465-469
29 **Kekkonen RA**, Kajasto E, Miettinen M, Veckman V, Korpela R, Julkunen I. Probiotic Leuconostoc mesenteroides ssp. cremoris and Streptococcus thermophilus induce IL-12 and IFN-gamma production. *World J Gastroenterol* 2008; **14**: 1192-1203
30 **Nordmark EL**, Yang Z, Huttunen E, Widmalm G. Structural studies of an exopolysaccharide produced by Streptococcus thermophilus THS. *Biomacromolecules* 2005; **6**: 105-108
31 **Yang Z**, Huttunen H, Staaf M, Widmalm G, Tenhu H. Separation, purification and characterization of extracellular polysaccharides produced by slime-forming Lactococcus lactis ssp. cremoris strains. *International Dairy Journal* 1999; **9**: 631-638
32 **Miettinen M**, Matikainen S, Vuopio-Varkila J, Pirhonen J, Varkila K, Kurimoto M, Julkunen I. Lactobacilli and streptococci induce interleukin-12 (IL-12), IL-18, and gamma interferon production in human peripheral blood mononuclear cells. *Infect Immun* 1998; **66**: 6058-6062
33 **Veckman V**, Miettinen M, Pirhonen J, Siren J, Matikainen

S, Julkunen I. Streptococcus pyogenes and Lactobacillus rhamnosus differentially induce maturation and production of Th1-type cytokines and chemokines in human monocyte-derived dendritic cells. *J Leukoc Biol* 2004; **75**: 764-771

34 **Miettinen M**, Vuopio-Varkila J, Varkila K. Production of human tumor necrosis factor alpha, interleukin-6, and interleukin-10 is induced by lactic acid bacteria. *Infect Immun* 1996; **64**: 5403-5405

35 **Sasaki DT**, Dumas SE, Engleman EG. Discrimination of viable and non-viable cells using propidium iodide in two color immunofluorescence. *Cytometry* 1987; **8**: 413-420

36 **Rossi DL**, Vicari AP, Franz-Bacon K, McClanahan TK, Zlotnik A. Identification through bioinformatics of two new macrophage proinflammatory human chemokines: MIP-3alpha and MIP-3beta. *J Immunol* 1997; **158**: 1033-1036

37 **Sareneva T**, Pirhonen J, Cantell K, Kalkkinen N, Julkunen I. Role of N-glycosylation in the synthesis, dimerization and secretion of human interferon-gamma. *Biochem J* 1994; **303** (Pt 3): 831-840

38 **Banchereau J**, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K. Immunobiology of dendritic cells. *Annu Rev Immunol* 2000; **18**: 767-811

39 **Nemoto S**, Xiang J, Huang S, Lin A. Induction of apoptosis by SB202190 through inhibition of p38beta mitogen-activated protein kinase. *J Biol Chem* 1998; **273**: 16415-16420

40 **Vlahos CJ**, Matter WF, Hui KY, Brown RF. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem* 1994; **269**: 5241-5248

41 **Alessi DR**, Cuenda A, Cohen P, Dudley DT, Saltiel AR. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. *J Biol Chem* 1995; **270**: 27489-27494

42 **McCracken VJ**, Lorenz RG. The gastrointestinal ecosystem: a precarious alliance among epithelium, immunity and microbiota. *Cell Microbiol* 2001; **3**: 1-11

43 **Bell SJ**, Rigby R, English N, Mann SD, Knight SC, Kamm MA, Stagg AJ. Migration and maturation of human colonic dendritic cells. *J Immunol* 2001; **166**: 4958-4967

44 **Zeuthen LH**, Christensen HR, Frokiaer H. Lactic acid bacteria inducing a weak interleukin-12 and tumor necrosis factor alpha response in human dendritic cells inhibit strongly stimulating lactic acid bacteria but act synergistically with gram-negative bacteria. *Clin Vaccine Immunol* 2006; **13**: 365-375

45 **Hart AL**, Lammers K, Brigidi P, Vitali B, Rizzello F, Gionchetti P, Campieri M, Kamm MA, Knight SC, Stagg AJ. Modulation of human dendritic cell phenotype and function by probiotic bacteria. *Gut* 2004; **53**: 1602-1609

46 **Lutz MB**, Schuler G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol* 2002; **23**: 445-449

47 **Chung F**. Anti-inflammatory cytokines in asthma and allergy: interleukin-10, interleukin-12, interferon-gamma. *Mediators Inflamm* 2001; **10**: 51-59

48 **Marschan E**, Kuitunen M, Kukkonen K, Poussa T, Sarnesto A, Haahtela T, Korpela R, Savilahti E, Vaarala O. Probiotics in infancy induce protective immune profiles that are characteristic for chronic low-grade inflammation. *Clin Exp Allergy* 2008; **38**: 611-618

49 **Tao Y**, Drabik KA, Waypa TS, Musch MW, Alverdy JC, Schneewind O, Chang EB, Petrof EO. Soluble factors from Lactobacillus GG activate MAPKs and induce cytoprotective heat shock proteins in intestinal epithelial cells. *Am J Physiol Cell Physiol* 2006; **290**: C1018-C1030

50 **Hoarau C**, Lagaraine C, Martin L, Velge-Roussel F, Lebranchu Y. Supernatant of Bifidobacterium breve induces dendritic cell maturation, activation, and survival through a Toll-like receptor 2 pathway. *J Allergy Clin Immunol* 2006; **117**: 696-702

51 **Yan F**, Cao H, Cover TL, Whitehead R, Washington MK, Polk DB. Soluble proteins produced by probiotic bacteria regulate intestinal epithelial cell survival and growth. *Gastroenterology* 2007; **132**: 562-575

52 **Yoshimura A**, Lien E, Ingalls RR, Tuomanen E, Dziarski R, Golenbock D. Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J Immunol* 1999; **163**: 1-5

53 **Schwandner R**, Dziarski R, Wesche H, Rothe M, Kirschning CJ. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *J Biol Chem* 1999; **274**: 17406-17409

54 **Pirhonen J**, Siren J, Julkunen I, Matikainen S. IFN-alpha regulates Toll-like receptor-mediated IL-27 gene expression in human macrophages. *J Leukoc Biol* 2007; **82**: 1185-1192

55 **Dieu MC**, Vanbervliet B, Vicari A, Bridon JM, Oldham E, Ait-Yahia S, Briere F, Zlotnik A, Lebecque S, Caux C. Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J Exp Med* 1998; **188**: 373-386

56 **Pietila TE**, Veckman V, Kyllonen P, Lahteenmaki K, Korhonen TK, Julkunen I. Activation, cytokine production, and intracellular survival of bacteria in Salmonella-infected human monocyte-derived macrophages and dendritic cells. *J Leukoc Biol* 2005; **78**: 909-920

57 **Puig-Kroger A**, Relloso M, Fernandez-Capetillo O, Zubiaga A, Silva A, Bernabeu C, Corbi AL. Extracellular signal-regulated protein kinase signaling pathway negatively regulates the phenotypic and functional maturation of monocyte-derived human dendritic cells. *Blood* 2001; **98**: 2175-2182

58 **Miettinen M**, Sareneva T, Julkunen I, Matikainen S. IFNs activate toll-like receptor gene expression in viral infections. *Genes Immun* 2001; **2**: 349-355

59 **Sallusto F**, Cella M, Danieli C, Lanzavecchia A. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J Exp Med* 1995; **182**: 389-400

S- Editor Li DL **L- Editor** Alpini GD **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5584

World J Gastroenterol 2008 September 28; 14(36): 5584-5589
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Fecal markers of inflammation used as surrogate markers for treatment outcome in relapsing inflammatory bowel disease

Michael Wagner, Christer GB Peterson, Peter Ridefelt, Per Sangfelt, Marie Carlson

Michael Wagner, Per Sangfelt, Marie Carlson, Department of Medical Sciences, Gastroenterology Research Group, University Hospital, SE-751 85 Uppsala, Sweden
Christer GB Peterson, Peter Ridefelt, Department of Medical Sciences, Clinical Chemistry, University Hospital, SE-751 85 Uppsala, Sweden
Author contributions: Sangfelt P and Carlson M contributed equally to this work; Sangfelt P, Peterson ChGB and Carlson M designed research; Wagner M, Sangfelt P, Peterson ChGB and Carlson M performed research; Ridefelt P contributed analytic tools; Wagner M, Sangfelt P, Peterson ChGB and Carlson M analyzed data; Wagner M, Sangfelt P, and Carlson M wrote the paper.
Supported by Grants from the Medical Faculty, Uppsala University, Uppsala, Sweden
Correspondence to: Marie Carlson, MD, PhD, Associate Professor, Department of Medical Sciences, Gastroenterology Research Group, University Hospital, SE-751 85 Uppsala, Sweden. marie.carlson@akademiska.se
Telephone: + 46-18-6114249 Fax: + 46-18-6113703
Received: June 13, 2008 Revised: August 14, 2008
Accepted: August 21, 2008
Published online: September 28, 2008

Abstract

AIM: To evaluate fecal calprotectin (FC) as a surrogate marker of treatment outcome of relapse of inflammatory bowel disease (IBD) and, to compare FC with fecal myeloperoxidase (MPO) and fecal eosinophil protein X (EPX).

METHODS: Thirty eight patients with IBD, comprising of 27 with ulcerative colitis (UC) and 11 with Crohn's disease (CD) were investigated before treatment (inclusion), and after 4 and 8 wk of treatment. Treatment outcomes were evaluated by clinical features of disease activity and endoscopy in UC patients, and disease activity in CD patients. In addition, fecal samples were analyzed for FC by enzyme-linked immunosorbent assay (ELISA), and for MPO and EPX with radioimmunoassay (RIA).

RESULTS: At inclusion 37 of 38 (97%) patients had elevated FC levels (> 94.7 μg/g). At the end of the study, 31 of 38 (82%) patients fulfilled predefined criteria of a complete response [UC 21/27 (78%); CD 10/11 (91%)]. Overall, a normalized FC level at the end of the study predicted a complete response in 100% patients, whereas elevated FC level predicted incomplete response in 30%. Normalized MPO or EPX levels predicted a complete response in 100% and 90% of the patients, respectively. However, elevated MPO or EPX levels predicted incomplete response in 23% and 22%, respectively.

CONCLUSION: A normalized FC level has the potential to be used as a surrogate marker for successful treatment outcome in IBD patients. However, patients with persistent elevation of FC levels need further evaluation. FC and MPO provide superior discrimination than EPX in IBD treatment outcome.

Key words: Fecal markers; Calprotectin; Myeloperoxidase; Eosinophil protein X treatment; Inflammatory bowel disease; Ulcerative colitis; Crohn's disease

Peer reviewer: Riina Salupere, MD, PhD, Division of Endocrinology and Gastroenterology, University of Tartu, L. Puusepa Street 6, Tartu 51014, Estonia

Wagner M, Peterson ChGB, Ridefelt P, Sangfelt P, Carlson M. Fecal markers of inflammation used as surrogate markers for treatment outcome in relapsing inflammatory bowel disease. *World J Gastroenterol* 2008; 14(36): 5584-5589 Available from: URL: http://www.wjgnet.com/1007-9327/14/5584.asp DOI: http://dx.doi.org/10.3748/wjg.14.5584

INTRODUCTION

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is a chronic, idiopathic inflammatory condition of the gut with a typically relapsing and remitting course. Exacerbations are characterized by symptoms of diarrhea, urgency of defecation and occasionally rectal bleeding and abdominal pain. The aim of treatment is to induce and maintain disease remission[1]. Currently, the most reliable method to assess intestinal inflammation is endoscopy with mucosal biopsy. These techniques are costly, invasive, time consuming and unpopular with patients. Furthermore, the site of inflammation is not always reached by endoscopy as in the case of small bowel disease in CD. Simple, inexpensive and objective tools for the assessment of mucosal inflammation are therefore desirable. Previous studies have indicated that fecal markers may be used in the differentiation of IBD from functional gastrointestinal disorders. However,

the usefulness of these markers in monitoring therapy of patients with disease relapse in IBD needs further evaluation[2-4].

A prominent feature of mucosal histology in patients with active IBD is infiltration by neutrophil granulocytes[5]. Calprotectin is a major protein of neutrophils and macrophages, accounting for about 60% of the cytosol of these cells[6]. Several workers have shown that elevated fecal calprotectin (FC) levels correlate with intestinal inflammation, both in adults and children[7-9], and has the ability to predict relapse in IBD[10,11]. Moreover, FC levels remain stable in stools, and stool samples are easy to collect[12-17].

Other fecal proteins of interest in monitoring IBD are myeloperoxidase (MPO) and eosinophil protein X (EPX). MPO is mainly derived from neutrophil granulocytes and has been observed both in the intestinal mucosa[18-20], and in gut lavage, and has the potential of monitoring treatment outcome[21]. EPX is released by activated eosinophil granulocytes, which are abundant in the mucosa in active IBD[22-25]. We have previously reported that fecal levels of EPX and MPO have the potential of monitoring therapy in UC[26].

The primary aim of the present study was to assess FC as a surrogate marker of treatment outcome of relapse in patients with IBD compared to the standard criteria of response. The second aim was to compare FC with fecal MPO and EPX, with respect to their applicability in monitoring treatment outcome.

MATERIALS AND METHODS

Patients

Forty adult patients (19 females and 21 males, mean age 40 years, range 21-70 years), seeking medical advice for symptoms of relapse of previously diagnosed IBD were consecutively recruited at the department of Gastroenterology, University Hospital, Uppsala, Sweden, between October 2002 and April 2003. Two patients were excluded: one patient had salmonella enteritis, while the other patient dropout after 8 wk of treatment. Before entry into the study, the patients had to fulfil standard diagnostic criteria of UC (n = 27) and CD (n = 11)[27]. Details of the clinical date of the study population are provided in Table 1.

At inclusion, all patients had mild to moderate disease activity, and the endoscopic examination was consistent with active disease. The exclusion criteria were: pregnant or lactating women, enteritis due to infections, and intestinal biopsies performed within 3 d before inclusion. The project was approved by the Ethical Committee of the Medical Faculty, Uppsala University, and all patients gave their written informed consent before participating in the study.

The patients were examined according to the study protocol at inclusion and after 4 and 8 wk of treatment (Table 2).

In patients with UC, a semi-quantitative four-grade (normal, mild, moderate and severe) scale was used for clinical and endoscopic score[28]. In patients with CD, the Harvey-Bradshaw's clinical activity index (HBI) was used[29]. Histopathology was graded as active or inactive inflammation, based on the number of neutrophil granulocytes in the mucosa as judged by an experienced pathologist, according to the criteria of Truelove and Witts[30]. At inclusion, 7 of 27 (26%) patients with UC were on no treatment, 14 (52%) were receiving systemic 5-aminosalicylic acid (5-ASA), and 6 (22%) were under treatment with different combinations of systemic 5-ASA, prednisone, azathioprine and topical prednisone. In CD patients, at inclusion 4 of 11 (36%) patient were on no treatment, 6 (54%) were receiving systemic 5-ASA, and one patient was under treatment with a combination of 5-ASA, azathioprine and Metronidazole.

Treatment of a relapse was individualized, according to standard recommendations for the management of IBD. Topical and/or systemic 5-ASA was administered to 26 of 27 (96%) patients with UC, and to 9 of 11 (82%) patients with CD. Topical and/or systemic prednisone was given to 22 of 27 (81%) UC patients, and 7 of 11 (81%) CD patients. Azathioprine was used in 3 of 27 (11%) UC patients and in 3 of 11 (27%) CD patients, while methotrexate was prescribed to one patient with CD.

Table 1 Clinical data of the study population

	UC	CD
Number of subjects	27	11
Gender (female/male)	15/12	3/8
Age (yr, mean)	42.5 (21-66)	34.6 (21-70)
Disease duration (yr, mean)	9.3 (0-38)	5.6 (0-18)
Extent of disease:		
Colon	9[1]	8
Left side colitis	14	-
Proctitis	4	-
Ileocolitis	-	1
Prior surgery	1	1
Treatment at inclusion:		
No treatment	7	4
5-ASA (topical/systemic)	19	4
Azathioprine	1	1
Prednisone (topical/systemic)	5	-
Metronidazole	-	1

[1]Extensive colitis means disease extending from proximal to the splenic flexure.

Table 2 Study protocol

Procedure	Inclusion	4 wk	8 wk
Stool sample	Done	Done	Done
Clinical score	Done	Done	Done
Endoscopy and biopsy	Done[1]	Done[2]	Done[3]
Histopathology	Done[1]	Done[2]	Done[3]

[1]Not done in 2 CD patients; [2]Not done in 4 CD patients; [3]Not done in 2 CD patients.

Definition of treatment outcome

In UC, a complete response to treatment was defined as return of the clinical and endoscopic scores to normal. A partial response was defined as reduction in both the clinical and endoscopic scores, but their failure to return

Table 3 FC levels (μg/g, mean, 10-90th percentile) in patients with a relapse of IBD at inclusion, and after 4 and 8 wk of treatment

	FC inclusion	FC 4 wk	FC 8 wk	*P*
All patients	5430 (151-14170)	1920 (61-4150)	1720 (38-3390)	< 0.01[1]
UC	5600 (210-14170)	1730 (61-2620)	1820 (40-3390)	< 0.01[1]
CD	5010 (151-9180)	2440 (124-7910)	1460 (36-5030)	= NS[1]

[1]Friedmans Test for 3 variables.

to normal. A non-response was defined as a decrease in only clinical or endoscopical score or an unchanged or increased clinical and/or endoscopic score. In CD, a complete response was defined as decrease in HBI score to ⩽ 5 points; partial response was a decrease in the clinical score, but not < 6 points; and a non-response was defined as an unchanged or increased HBI score.

Fecal inflammation markers

Stool samples were collected in screw-capped plastic containers at inclusion (visit 1), and after four (visit 2) and 8 wk of treatment (end of study, visit 3). Stool samples were kept at 4℃ for up to 2 d before freezing at -70℃. Stool extracts for EPX and MPO measurement were prepared as described previously[31]. Calprotectin in fecal extracts was analyzed, using the calprotectin enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (Calprest; Eurospital SpA, Trieste, Italy). Before testing, the supernatants were thawed, diluted 1:50 with assay buffer and then analyzed with Calprest. Calprotectin was expressed as microgram per gram of feces. According to the manufacturer, a calprotectin level > 50 μg/g is pathological. FC was determined in samples from 44 apparently healthy adults, and the normal range was 9.2-94.5 μg/g (5-95th percentile). EPX and MPO were determined using radioimmunoassay (RIA) (Pharmacia Diagnostics, Uppsala, Sweden), and the intra- and inter-assay variations were less than 9% for both assays. Marker levels in faeces were adjusted for fecal water content, as described previously[31], and were expressed as μg/g semidry faeces. The concentration of fecal markers in healthy adults for fecal MPO (1.3-8.8 μg/g, 5-95th percentile) and fecal EPX (0.2-1.7 μg/g, 5-95th percentile) (median age 44 years, range 18-73, n = 44) have been reported recently[31].

Statistical analysis

Non-parametric tests of Kruskal-Wallis ANOVA and the Mann-Whitney *U*-test were used for unpaired comparisons. For paired analyses we used Friedman ANOVA and Wilcoxon matched pairs test. Spearman rank order correlations were used to express relationship between variables. $P < 0.05$ was considered as significant. All calculations were performed on a personal computer by means of the statistical software Statistica (Statsoft Inc, Tulsa, Oklahoma USA). We used the 95th percentile concentration level of respective fecal marker as the cut-off level for normal and elevated values i.e. concentration was set at 94.5 μg/g for FC, at 8.8 μg/g for MPO, and at 1.7 μg/g for EPX.

RESULTS

FC levels at inclusion and during the study period

At inclusion, 37 of 38 (97%) patients manifested elevated FC levels. There was no difference in the FC levels between UC and CD at any time point during the study. Overall, a significant decline in FC levels was seen during the study ($P < 0.01$) in patients with UC ($P < 0.01$), but not in CD ($P = 0.367$) (Table 3).

Analysis based on the extent of the disease showed that patients with proctitis had lower levels of FC ($P < 0.01$) compared with more extensive disease. This difference was noted in patients with UC ($P < 0.05$), but was not analyzed in CD since only one patient had proctitis.

FC levels in correlation to clinical scores

In patients with UC, there was no correlation between the clinical score and FC levels at inclusion, and at the end of the study (8 wk of treatment). However, correlation was noted after 4 wk of treatment ($R = 0.0424$, $P < 0.01$).

In CD patients, there was no correlation between FC and the HBI score at inclusion, and after 4 wk of treatment, but correlation was present at the end of the study (8 wk of treatment) ($R = 0.7804$, $P < 0.01$).

FC levels in relation to endoscopical score

In patients with UC, the endoscopic score was calculated and correlated with FC levels. No correlation was seen at inclusion, but after 4 and 8 wk of treatment a correlation was noted ($R = 0.5045$, $P < 0.01$) and ($R = 0.6659$, $P < 0.01$), respectively.

FC levels in correlation to histopathology

At inclusion, all patients with UC (27/27) showed histological signs of active disease, whereas 12 of 26 (one missing value) and 13 of 27 showed inactive histology after 4 and 8 wk of treatment, respectively. Patients with UC with histologic evidence of active inflammation, demonstrated higher levels of FC compared to UC patients with inactive inflammation after 4 and 8 wk of treatment ($P < 0.05$). In CD patients, no difference was found during the study period.

FC in relation to treatment outcome

In UC patients, a complete response was observed in 14 of 27 (52%) and 21 of 27 (78%) patients after 4 and 8 wk of treatment, respectively. Two patients were classified as partial responders and four patients as non responders at the end of the study. In complete responders, there was a significant decline in FC levels ($P < 0.01$), which was not observed in partial or non responders (Figure 1). The decline in FC levels was already significant after 4 wk of treatment in complete responders ($P < 0.01$).

Table 4 Correlation between fecal FC, MPO and EPX at inclusion, and after 4 and 8 wk of treatment in patients with UC and CD

Patients	Fecal marker	Inclusion	4 wk	8 wk
All	FC and MPO	$R = 0.884$, $P < 0.01$	$R = 0.941$, $P < 0.01$	$R = 0.924$, $P < 0.01$
All	FC and EPX	$R = 0.592$, $P < 0.01$	$R = 0.724$, $P < 0.01$	$R = 0.642$, $P < 0.01$
UC	FC and MPO	$R = 0.868$, $P < 0.01$	$R = 0.927$, $P < 0.01$	$R = 0.940$, $P < 0.01$
UC	FC and EPX	$R = 0.556$, $P < 0.01$	$R = 0.762$, $P < 0.01$	$R = 0.602$, $P < 0.01$
CD	FC and MPO	$R = 0.936$, $P < 0.01$	$R = 0.818$, $P < 0.01$	$R = 0.903$, $P < 0.01$
CD	FC and EPX	$R = 0.654$, $P < 0.05$	$R = 0.636$, $P < 0.05$	$R = 0.854$, $P < 0.01$

All: $n = 38$; UC: $n = 27$; CD: $n = 11$.

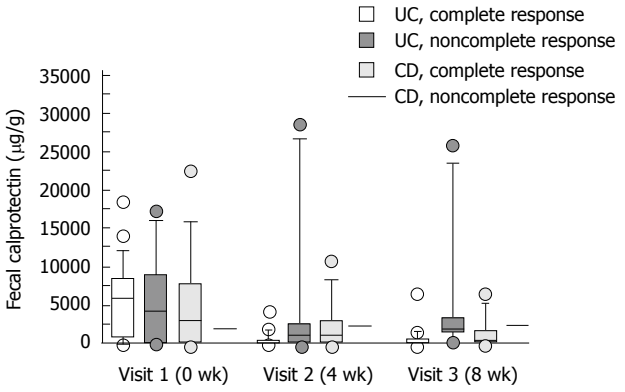


Figure 1 FC levels (box displays median, 25-75th percentile and lines 10-90th percentile with dots displaying outliers), and treatment outcome in terms of complete and incomplete response in patients with UC and CD treated for 8 wk.

Table 5 PPV and NPV of a complete response after 8 wk of treatment in UC or CD calculated by using fecal marker levels above the 95th percentile as the cut-off value

Fecal marker	Patients	PPV (95% CI)	NPV (95% CI)
FC	All $n = 37$[1]	30 (13-53)	100 (77-100)
FC	UC $n = 27$	38 (13-65)	100 (71-100)
FC	CD $n = 10$[1]	14 (2-58)	100 (30-100)
MPO	All $n = 37$[1]	23 (10-42)	100 (59-100)
MPO	UC $n = 27$	27 (10-50)	100 (48-100)
MPO	CD $n = 10$[1]	12 (2-53)	100 (19-100)
EPX	All $n = 37$[1]	22 (8-42)	90 (56-98)
EPX	UC $n = 27$	25 (8-49)	85 (42-98)
EPX	CD $n = 10$[1]	14 (3-58)	100 (30-100)

[1]One missing value. FC cut-off value 94.5 μg/g, MPO cut-off value 8.8 μg/g, EPX cut-off value 1.7 μg/g.

In CD patients, a complete response was observed in 9 of 11 (81%) and 10 of 11 (91%) patients after 4 and 8 wk of treatment, respectively. There was a trend in the decline in FC levels in complete responders but the difference was not significant ($P = 0.13$) (Figure 1). One CD patient, who was a partial responder, manifested increasing levels of FC during the study (2030, 2220, 2460 μg/g, respectively).

FC in correlation with MPO and EPX

There was a good correlation between FC, and both MPO and EPX, at all the study visits in the group as a whole, as well as on sub-group analyses of patients with UC and CD (Table 4).

FC, MPO and EPX as predictors of treatment outcome

In order to predict treatment outcome, positive predictive value (PPV) and negative predictive value (NPV) were calculated at the end of the study (after 8 wk of treatment). All patients with a normalized FC value at the end of the study fulfilled the predefined criteria of a complete response, irrespective of the diagnosis of UC or CD. However, an elevated FC value was noted in 10 of 21 patients with UC and 6 of 9 patients with CD who fulfilled criteria for complete response at the end of the study. The same pattern was observed in patients with UC and CD with regard to MPO, however, for EPX this was only seen in CD patients (Table 5).

DISCUSSION

In the present study, we evaluated FC as a surrogate marker of treatment outcome in IBD. FC levels were monitored before and during treatment of patients with a relapse of IBD. In both UC and CD, patients with normalized FC level after 8 wk of treatment fulfilled predefined criteria of a complete response. However, elevated FC levels at the end of the study did not rule out response to treatment. As described by other workers, we also observed elevated FC levels in all but one patient presenting with disease relapse[8,9].

It has been reported that FC levels in UC patients reflects the degree of inflammation rather than the extent of the disease[8]. By contrast, in the present study, we observed higher FC levels in UC patients at inclusion with extensive disease compared to those with proctitis.

Endoscopic findings have been shown to correlate with FC levels in patients with IBD[32]. In the present study, no such correlation was observed in UC patients at inclusion, however, a correlation was noted after 4 and 8 wk of treatment. It is well known that there are difficulties in endoscopic grading of inflammation due to inter-observer variations[33], and with the use of different scoring systems. In the present study, we used a four-grade score[28], which may be inadequate in distinguishing between mild and moderate inflammation.

In patients with UC, the clinical activity index and FC levels showed a correlation after 4 wk of treatment, but not at inclusion or at the end of the study. In patients with CD, clinical activity and FC showed a correlation only at the end of the study. These weak correlations between clinical indices and FC levels are in accordance with previous reports[9,34,35]. By contrast, a recent study reported significantly higher FC levels in patients with severe disease compared to those with moderate

disease[7]. Since our patients manifested only mild to moderate disease, the present study cannot clarify this matter.

The histological findings in present study confirmed previous reports that patients with active UC manifest higher FC levels compared to those with inactive disease[8,32]. However, this finding was not seen in CD patients. These observations should be interpreted with caution, since the number of patients was small. One may speculate that one explanation for these findings may be that biopsies are more prone to sampling error in CD because of the patchy mucosal inflammation in CD compared to UC. Using FC values below ULN (95th percentile of the normal range) as a negative predictor of active disease after 8 wk of treatment, revealed a NPV of 100%. This is in line with previous reports showing that normalisation of FC predicts mucosal healing in patients with IBD[4].

However, using an elevated level of FC as a positive predictor to detect ongoing active disease or treatment failure after 8 wk of treatment, showed a PPV of 38% in UC and only of 14% in CD. In children with active IBD treated with prednisone, FC levels declined in line with clinical improvement but seldom fell within the normal range[36]. A persistently high level of FC suggests ongoing inflammation in clinically silent disease and may predict relapse of IBD[11,37]. It is possible that our patients who responded to therapy but continued to have elevated FC, may have normalized the FC levels if the study had been prolonged. Another explanation is that these patients may relapse again soon after termination of the study. After the completion of the present study, we followed our patients retrospectively but did not find any relapsers over the following 3 mo (data not shown). At present, there is insufficient data to conclude that patients without clinical or endoscopic signs of active IBD but with elevated FC levels, may benefit from more aggressive anti-inflammatory treatment.

In the present study, fecal MPO and EPX were also evaluated in order to monitor treatment outcome compared with FC levels. A recent study showed that MPO and EPX may be used to detect active UC[26]. In the present study, we observed a close correlation between FC, MPO and EPX, and treatment outcome; FC and MPO provided better assessment of treatment outcome compared to EPX in patients with UC. Interestingly, normalized levels of EPX after 8 wk of treatment indicated a complete response, especially in patients with CD.

In conclusion, normalized FC level may be used as a surrogate marker for treatment response in patients with IBD. However, the significance of persistent elevation of FC needs further evaluation.

ACKNOWLEDGMENTS

We are grateful to Ingrid Stolt for expert handling of samples and for skilful technical assistance, and to Maria Lampinen for valuable views on the manuscript.

COMMENTS

Background

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic, idiopathic inflammatory conditions of the gut with a typically relapsing and remitting course. Exacerbations are characterized by symptoms of diarrhea, urgency of defecation, and occasionally rectal bleeding and abdominal pain. At present, the most reliable method of assessing intestinal inflammation is endoscopy with biopsy. These techniques are costly, invasive, time consuming and unpopular with the patients. Furthermore, the site of inflammation is not always reached by endoscopy as in patients with CD of the small bowel. Simple, inexpensive and objective tools for the assessment of mucosal inflammation are therefore desirable. Previous studies have indicated that fecal markers may be used in the differentiation of inflammatory bowel disease (IBD) from functional gastrointestinal disorders. However, the usefulness of these markers in monitoring therapy of patients with relapse of IBD needs further evaluation.

Research frontiers

The present study suggests that a normalized fecal calprotectin (FC) level may be used as a surrogate marker for successful treatment outcome in IBD patients. However, patients with persistently elevated FC levels need further evaluation.

Innovations and breakthroughs

The present study confirmed previous observations that patients with a relapse of IBD had elevated fecal markers, and normalized FC levels may be used as a surrogate marker for treatment response in IBD. We evaluated patients diagnosed previously with UC or CD before starting treatment, and after 4 and 8 wk of treatment. Treatment outcome, based on clinical activity and endoscopic findings in UC patients, and clinical activity in CD patients, were evaluated together with fecal samples analyzed for FC, and compared with fecal myeloperoxidase (MPO) and eosinophil protein X (EPX). It was observed that FC and MPO provided better assessment of treatment outcome compared to EPX in patients with UC. Interestingly, normalized levels of EPX after 8 wk of treatment indicated a complete response, especially in patients with CD.

Applications

These findings suggest that fecal markers can be used as surrogate markers for successful treatment outcome in IBD patients. Fecal markers are simple, inexpensive and objective tools for the assessment of mucosal inflammation.

Peer review

This study provides good data on material and methods. The results are based on sufficient experimental evidence. The discussion is well organized, and the conclusions are reliable and valuable.

REFERENCES

1 **Hanauer SB**. Inflammatory bowel disease: epidemiology, pathogenesis, and therapeutic opportunities. *Inflamm Bowel Dis* 2006; **12** Suppl 1: S3-S9

2 **Desai D**, Faubion WA, Sandborn WJ. Review article: biological activity markers in inflammatory bowel disease. *Aliment Pharmacol Ther* 2007; **25**: 247-255

3 **von Roon AC**, Karamountzos L, Purkayastha S, Reese GE, Darzi AW, Teare JP, Paraskeva P, Tekkis PP. Diagnostic precision of fecal calprotectin for inflammatory bowel disease and colorectal malignancy. *Am J Gastroenterol* 2007; **102**: 803-813

4 **Roseth AG**, Aadland E, Grzyb K. Normalization of faecal calprotectin: a predictor of mucosal healing in patients with inflammatory bowel disease. *Scand J Gastroenterol* 2004; **39**: 1017-1020

5 **Saverymuttu SH**, Camilleri M, Rees H, Lavender JP, Hodgson HJ, Chadwick VS. Indium 111-granulocyte scanning in the assessment of disease extent and disease activity in inflammatory bowel disease. A comparison with colonoscopy, histology, and fecal indium 111-granulocyte excretion. *Gastroenterology* 1986; **90**: 1121-1128

6 **Johne B**, Fagerhol MK, Lyberg T, Prydz H, Brandtzaeg P, Naess-Andresen CF, Dale I. Functional and clinical aspects of the myelomonocyte protein calprotectin. *Mol Pathol* 1997; **50**: 113-123

7 **Langhorst J**, Elsenbruch S, Mueller T, Rueffer A, Spahn

G, Michalsen A, Dobos GJ. Comparison of 4 neutrophil-derived proteins in feces as indicators of disease activity in ulcerative colitis. *Inflamm Bowel Dis* 2005; **11**: 1085-1091
8 **Roseth AG**, Aadland E, Jahnsen J, Raknerud N. Assessment of disease activity in ulcerative colitis by faecal calprotectin, a novel granulocyte marker protein. *Digestion* 1997; **58**: 176-180
9 **Tibble J**, Teahon K, Thjodleifsson B, Roseth A, Sigthorsson G, Bridger S, Foster R, Sherwood R, Fagerhol M, Bjarnason I. A simple method for assessing intestinal inflammation in Crohn's disease. *Gut* 2000; **47**: 506-513
10 **Costa F**, Mumolo MG, Bellini M, Romano MR, Ceccarelli L, Arpe P, Sterpi C, Marchi S, Maltinti G. Role of faecal calprotectin as non-invasive marker of intestinal inflammation. *Dig Liver Dis* 2003; **35**: 642-647
11 **Tibble JA**, Sigthorsson G, Bridger S, Fagerhol MK, Bjarnason I. Surrogate markers of intestinal inflammation are predictive of relapse in patients with inflammatory bowel disease. *Gastroenterology* 2000; **119**: 15-22
12 **Carroccio A**, Iacono G, Cottone M, Di Prima L, Cartabellotta F, Cavataio F, Scalici C, Montalto G, Di Fede G, Rini G, Notarbartolo A, Averna MR. Diagnostic accuracy of fecal calprotectin assay in distinguishing organic causes of chronic diarrhea from irritable bowel syndrome: a prospective study in adults and children. *Clin Chem* 2003; **49**: 861-867
13 **Dale I**, Brandtzaeg P, Fagerhol MK, Scott H. Distribution of a new myelomonocytic antigen (L1) in human peripheral blood leukocytes. Immunofluorescence and immunoperoxidase staining features in comparison with lysozyme and lactoferrin. *Am J Clin Pathol* 1985; **84**: 24-34
14 **Roseth AG**, Fagerhol MK, Aadland E, Schjonsby H. Assessment of the neutrophil dominating protein calprotectin in feces. A methodologic study. *Scand J Gastroenterol* 1992; **27**: 793-798
15 **Roseth AG**, Schmidt PN, Fagerhol MK. Correlation between faecal excretion of indium-111-labelled granulocytes and calprotectin, a granulocyte marker protein, in patients with inflammatory bowel disease. *Scand J Gastroenterol* 1999; **34**: 50-54
16 **Summerton CB**, Longlands MG, Wiener K, Shreeve DR. Faecal calprotectin: a marker of inflammation throughout the intestinal tract. *Eur J Gastroenterol Hepatol* 2002; **14**: 841-845
17 **Fagerberg UL**, Loof L, Lindholm J, Hansson LO, Finkel Y. Fecal calprotectin: a quantitative marker of colonic inflammation in children with inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 2007; **45**: 414-420
18 **Carlson M**, Raab Y, Seveus L, Xu S, Hallgren R, Venge P. Human neutrophil lipocalin is a unique marker of neutrophil inflammation in ulcerative colitis and proctitis. *Gut* 2002; **50**: 501-506
19 **Izzo RS**, Witkon K, Chen AI, Hadjiyane C, Weinstein MI, Pellecchia C. Interleukin-8 and neutrophil markers in colonic mucosa from patients with ulcerative colitis. *Am J Gastroenterol* 1992; **87**: 1447-1452
20 **Sugi K**, Saitoh O, Hirata I, Katsu K. Fecal lactoferrin as a marker for disease activity in inflammatory bowel disease: comparison with other neutrophil-derived proteins. *Am J Gastroenterol* 1996; **91**: 927-934
21 **Sangfelt P**, Carlson M, Thorn M, Loof L, Raab Y. Neutrophil and eosinophil granule proteins as markers of response to local prednisolone treatment in distal ulcerative colitis and proctitis. *Am J Gastroenterol* 2001; **96**: 1085-1090
22 **Bischoff SC**, Wedemeyer J, Herrmann A, Meier PN, Trautwein C, Cetin Y, Maschek H, Stolte M, Gebel M, Manns MP. Quantitative assessment of intestinal eosinophils and mast cells in inflammatory bowel disease. *Histopathology* 1996; **28**: 1-13
23 **Raab Y**, Fredens K, Gerdin B, Hallgren R. Eosinophil activation in ulcerative colitis: studies on mucosal release and localization of eosinophil granule constituents. *Dig Dis Sci* 1998; **43**: 1061-1070
24 **Carlson M**, Raab Y, Peterson C, Hallgren R, Venge P. Increased intraluminal release of eosinophil granule proteins EPO, ECP, EPX, and cytokines in ulcerative colitis and proctitis in segmental perfusion. *Am J Gastroenterol* 1999; **94**: 1876-1883
25 **Levy AM**, Gleich GJ, Sandborn WJ, Tremaine WJ, Steiner BL, Phillips SF. Increased eosinophil granule proteins in gut lavage fluid from patients with inflammatory bowel disease. *Mayo Clin Proc* 1997; **72**: 117-123
26 **Peterson CG**, Sangfelt P, Wagner M, Hansson T, Lettesjo H, Carlson M. Fecal levels of leukocyte markers reflect disease activity in patients with ulcerative colitis. *Scand J Clin Lab Invest* 2007; **67**: 810-820
27 **Garland CF**, Lilienfeld AM, Mendeloff AI, Markowitz JA, Terrell KB, Garland FC. Incidence rates of ulcerative colitis and Crohn's disease in fifteen areas of the United States. *Gastroenterology* 1981; **81**: 1115-1124
28 **Binder V**. A comparison between clinical state, macroscopic and microscopic appearances of rectal mucosa, and cytologic picture of mucosal exudate in ulcerative colitis. *Scand J Gastroenterol* 1970; **5**: 627-632
29 **Harvey RF**, Bradshaw JM. A simple index of Crohn's-disease activity. *Lancet* 1980; **1**: 514
30 **Truelove SC**, Richards WC. Biopsy studies in ulcerative colitis. *Br Med J* 1956; **1**: 1315-1318
31 **Peterson CG**, Eklund E, Taha Y, Raab Y, Carlson M. A new method for the quantification of neutrophil and eosinophil cationic proteins in feces: establishment of normal levels and clinical application in patients with inflammatory bowel disease. *Am J Gastroenterol* 2002; **97**: 1755-1762
32 **D'Inca R**, Dal Pont E, Di Leo V, Ferronato A, Fries W, Vettorato MG, Martines D, Sturniolo GC. Calprotectin and lactoferrin in the assessment of intestinal inflammation and organic disease. *Int J Colorectal Dis* 2007; **22**: 429-437
33 **Baron JH**, Connell AM, Lennard-Jones JE. Variation between Observers in Describing Mucosal Appearances in Proctocolitis. *Br Med J* 1964; **1**: 89-92
34 **Gomes P**, du Boulay C, Smith CL, Holdstock G. Relationship between disease activity indices and colonoscopic findings in patients with colonic inflammatory bowel disease. *Gut* 1986; **27**: 92-95
35 **Korelitz BI**. An indicator of Crohn's disease activity. A need unfulfilled. *J Clin Gastroenterol* 1986; **8**: 220-222
36 **Kolho KL**, Raivio T, Lindahl H, Savilahti E. Fecal calprotectin remains high during glucocorticoid therapy in children with inflammatory bowel disease. *Scand J Gastroenterol* 2006; **41**: 720-725
37 **Costa F**, Mumolo MG, Ceccarelli L, Bellini M, Romano MR, Sterpi C, Ricchiuti A, Marchi S, Bottai M. Calprotectin is a stronger predictive marker of relapse in ulcerative colitis than in Crohn's disease. *Gut* 2005; **54**: 364-368

S- Editor Li DL **L- Editor** Anand BS **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5590

World J Gastroenterol 2008 September 28; 14(36): 5590-5594
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Resection and primary anastomosis with or without modified blow-hole colostomy for sigmoid volvulus

Sacid Coban, Mehmet Yilmaz, Alpaslan Terzi, Fahrettin Yildiz, Dincer Ozgor, Cengiz Ara, Saim Yologlu, Vedat Kirimlioglu

Sacid Coban, Department of General Surgery, Gaziantep University School of Medicine, Gaziantep 27310, Turkey
Mehmet Yilmaz, Dincer Ozgor, Cengiz Ara, Vedat Kirimlioglu, Department of General Surgery, Inonu University School of Medicine, Malatya 44280, Turkey
Alpaslan Terzi, Fahrettin Yildiz, Department of General Surgery, Harran University School of Medicine, Sanliurfa 63200, Turkey
Saim Yologlu, Department of Biostatistics, Inonu University School of Medicine, Malatya 44280, Turkey
Author contributions: Coban S and Yilmaz M contributed equally to this work; Coban S, Yilmaz M, Ozgor D, Ara C and Kirimlioglu V designed and performed the research; Terzi A, Yildiz F and Yologlu S analyzed data; and Coban S, Terzi A, Yildiz F wrote the paper.
Correspondence to: Sacid Coban, MD, Department of General Surgery, Gaziantep University School of Medicine, Gaziantep 27310, Turkey. sacitcoban@yahoo.com
Telephone: +90-342-3601200-76330 Fax: +90-342-3601013
Received: June 9, 2008 Revised: August 11, 2008
Accepted: August 18, 2008
Published online: September 28, 2008

Abstract

AIM: To evaluate the efficacy of resection and primary anastomosis (RPA) and RPA with modified blow-hole colostomy for sigmoid volvulus.

METHODS: From March 2000 to September 2007, 77 patients with acute sigmoid volvulus were treated. A total of 47 patients underwent RPA or RPA with modified blow-hole colostomy. Twenty-five patients received RPA (Group A), and the remaining 22 patients had RPA with modified blow-hole colostomy (Group B). The clinical course and postoperative complications of the two groups were compared.

RESULTS: The mean hospital stay, wound infection and mortality did not differ significantly between the groups. Superficial wound infection rate was higher in group A (32% *vs* 9.1%). Anastomotic leakage was observed only in group A, with a rate of 6.3%. The difference was numerically impressive but was statistically not significant.

CONCLUSION: RPA with modified blow-hole colostomy provides satisfactory results. It is easy to perform and may become a method of choice in patients with sigmoid volvulus. Further studies are required to further establish its role in the treatment of sigmoid volvulus.

Key words: Acute sigmoid volvulus; Anastomosis; Primary resection; Surgical technique

Peer reviewer: María IT López, Professor, Experimental Biology, University of Jaen, araje de las Lagunillas s/n, Jaén 23071, Spain

Coban S, Yilmaz M, Terzi A, Yildiz F, Ozgor D, Ara C, Yologlu S, Kirimlioglu V. Resection and primary anastomosis with or without modified blow-hole colostomy for sigmoid volvulus. *World J Gastroenterol* 2008; 14(36): 5590-5594 Available from: URL: http://www.wjgnet.com/1007-9327/14/5590.asp DOI: http://dx.doi.org/10.3748/wjg.14.5590

INTRODUCTION

The epidemiology and clinical presentation of sigmoid volvulus are well established. Although the clinical manifestations of acute volvulus are often clear-cut, a diagnostic dilemma is not uncommon. Sigmoid volvulus is the third most common cause of large-bowel obstruction in the western world, after cancer and diverticular disease[1]. It accounts for 4% of all cases of large-bowel obstruction in the United States and United Kingdom[1,2]. Sigmoid volvulus is relatively more common in Eastern Europe, India and Africa, accounting for 50% of all cases of intestinal obstruction[1,2].

The precise etiology of sigmoid volvulus remains speculative, and several etiological factors have been suggested including chronic constipation, high fiber diet, bowel habit, high altitude, and enemas containing ginger, pepper and herbal extracts[3]. Patients with sigmoid volvulus present with abdominal distention, pain, nausea, vomiting, and obstipation, while peritoneal signs are noted infrequently[4]. The colon is distended often to enormous proportions, particularly when the patient is symptomatic. Plain abdominal X-rays are often diagnostic of volvulus. Air-fluid levels are present, and a "bird's beak" deformity is often seen at the site of the torsion[5].

Sigmoid volvulus has been described since ancient times, but its treatment continues to evolve[6,7]. Several therapeutic approaches such as: resection, non-operative

reduction with the help of a colonoscope, sigmoidopexy and mesosigmoidoplasty have been employed[8]. There is no consensus with regard to the optimal surgical management in patient with an acute presentation. Proximal decompressive blow-hole colostomy has been used in patients with toxic megacolon secondary to inflammatory bowel disease and *clostridium difficile* colitis, with a view to avoid manipulating the colon and to tide over the critical phase[9]. This procedure is also performed in hemodynamically unstable patients with malignant bowel obstruction[10-12].

Resection and primary anastomosis (RPA) has emerged as the treatment of choice for sigmoid volvulus over the past two centuries[6,7]. Particularly in elderly and hemodynamically unstable patients, anastomotic leakage may occur due to co-morbid risk factors with this approach. In such situations, a blow-hole colostomy may play a protective role in avoiding anastomotic leakage.

The aim of the present study was to compare the results of RPA with or without modified blow-hole colostomy in an unprepared bowel in patients with acute sigmoid volvulus.

MATERIALS AND METHODS

From March 2000 to September 2007, 77 patients with acute sigmoid volvulus were treated in the department of general surgery, school of medicine, Inonu University. Colonoscopic derotation was attempted in 27 patients, and was successful in 19 patients. In 10 of the 19 non-operatively reduced patients, semi-elective one-stage resection was performed; the remaining 9 patients refused surgery after non-operative decompression. Hartmann's operation was performed in 11 patients who were not in stable condition. After excluding these 30 cases, the remaining 47 patients who underwent RPA with or without a modified blow-hole colostomy were included in this study.

The diagnosis of sigmoid volvulus was made on the basis of clinical features and plain abdominal radiographs. Laparotomy was performed in all patients after active fluid resuscitation and correction of electrolyte imbalance was obtained. Seftriacson 1000 mg and metronidazole 500 mg were administered intravenously at the time of induction of anesthesia, and were continued every 12 h after the operation for 5 d in patients with viable bowel and for 7 d in those with gangrenous bowel.

None of the patients included in the study were treated with preoperative decompression techniques. At laparotomy, the distended bowel was decompressed by using a rectal tube, and any residual feces was milked digitally into the segment of the bowel to be resected. Although the bowel was unprepared, on-table lavage was not performed in any patient. Informed consent was obtained from each patient prior to the surgery. In 25 patients, RPA (Group A) was performed and in the remaining 22 patients, a modified blow-hole colostomy was performed with RPA (Group B). All the anastomoses were inverting and two-layered.

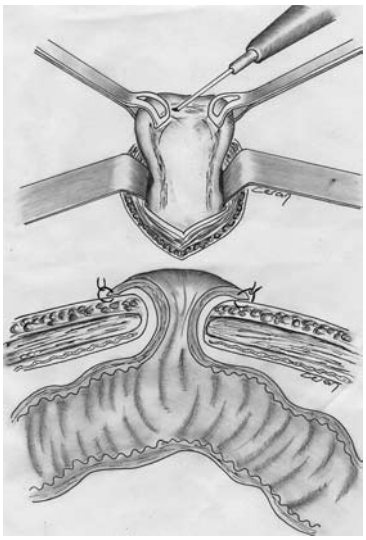


Figure 1 The cut edges of the colon were sutured to the skin with 3/0 vicryl, without fascial or peritoneal sutures. Therefore, the procedure was called "modified blow-hole colostomy technique".

The clinical course and postoperative complications were documented. Wound infection was defined as spontaneous discharge of pus from the wound or a wound that requires drainage. Anastomotic leak was defined as the presence of fecal fistula or the presence of feces in the drain.

Surgical technique of modified blow-hole colostomy

In group B, a proximal stoma was performed to protect the anastomoses. A 3 cm longitudinal incision was made through the tenia libera, and into the transverse colon. An abdominal wall aperture intended for the colostomy was made in the right upper quadrant using a rectus splitting incision. The collapse of the colon allowed it to reach the incision, and facilitated performing a skin-level colostomy. In the blow-hole colostomy technique which has been previously reported, the omentum and seromuscular layers of the colon were attached to the peritoneum and the rectus fascia with interrupted or continuous sutures[9]. In the present study, the cut edges of the colon were sutured to the skin with 3/0 vicryl without fascial or peritoneal sutures. As a result, we call it a "modified blow-hole colostomy technique" (Figure 1). In these patients, oral intake was started on postoperative day 1. On postoperative day 10, the anastomotic integrity was checked using water-soluble radiological studies, and if intact, colostomy closure was performed.

Statistical analysis

Statistical analyses were performed using SPSS for Windows version 11.0 program. Continuous variables were reported as mean ± SD. Categorical variables were reported as percent. Normality for continued variables in groups was determined by the Shapiro Wilk test. The variables showed normal distribution ($P > 0.05$). Therefore, unpaired *t*-test was used for comparison of variables (age and length of hospital stay) between the two groups. Fisher's Exact and Pearson χ^2 tests were used for comparison of categorical variables between the groups. $P < 0.05$ was considered as significant.

Table 1 Operative procedure, morbidity and deaths in 47 patients

Procedure	No. of patients	Wound infection	Anastomotic dehiscence	Deaths
RPA (Group A)	25 (16V, 9G)	6V, 2G	2V, 1G	1V, 1G
RPA with modified blow-hole colostomy (Group B)	22 (17V, 4G, 1P)	2V	-	1V, 1G
Total	47 (33V, 13G, 1P)	10 (8V, 2G)	3 (2V, 1G)	4 (2V, 2G)

V: Viable; G: Gangrenous; P: Perforation; RPA: Resection and primary anastomosis.

Table 2 Comparison of confounding variables between Group A (RPA) and Group B (RPA with modified blow-hole colostomy)

Variable	Group A (n = 25)	Group B (n = 22)	Statistical test and significance
Age (yr)	66.12 ± 14.17	62.27 ± 16.26	Unpaired t-test, P = 0.391
Sex: Female ratio	4/25 (16%)	3/22 (13.6%)	Fisher's exact test, χ^2-test, P = 0.574
Hospital stay (d)	13.68 ± 8.47	16.72 ± 4.90	Unpaired t-test, P = 0.145
Wound infection	8 (32%)	2 (9.1%)	χ^2-test, P = 0.11
Anastomotic leak	3 (12%)	-	Fisher's exact test, χ^2-test, P = 0.142
Mortality	2 (8%)	2 (10%)	Fisher's exact test, χ^2-test, P = 0.645

RESULTS

Forty-seven consecutive patients (7 women and 40 men), who had undergone RPA (25 patients) or RPA with modified blow-hole colostomy (22 patients) were evaluated. There was no significant difference between the two groups in the mean age or the sex ratio. The operative procedures and postoperative outcomes are shown in Table 1. Nine (36%) patients in group A and 5 (22%) in group B had gangrenous bowel. The mean hospital stay, wound infection and mortality did not differ between the groups (Table 2). However, superficial wound infection was almost four times more common in the group A (32% *vs* 9.1%), and nearly two times more common in patients with a viable colon. All the infected wounds healed with conservative measures. Three patients (12%) developed anastomotic leak in group A; two had viable colon and one had gangrenous sigmoid colon. At re-laparotomy, Hartmann's procedure was performed in patients with anastomotic dehiscence. No anastomotic leak was observed in group B. The time to resumption of oral intake was postoperative day 4 in group A, and day 1 in group B, due to the presence of a protective stoma. All patients in group B, had stoma closure performed on postoperative day 10, after radiological studies were carried out. Development of leak or wound infection secondary to stoma closure was not observed in any patient.

The mortality rates were identical in the two groups. One patient died of myocardial infarction and one of sepsis resulting from anastomotic dehiscence in group A on the 1st and 4th postoperative day respectively. In group B, one patient died on the 6th postoperative day due to pulmonary embolism, and one patient died secondary to multi-system organ failure on the 16th postoperative day.

DISCUSSION

The management of sigmoid volvulus involves relief of obstruction and prevention of recurrence. Several operative procedures have been used in the emergency management of sigmoid volvulus. However, permanent cure involves resection of the sigmoid colon, with or without anastomosis[7,13,14]. Less extensive procedures are not always successful and are contraindicated if gangrene or compound volvulus is present[15]. Colonoscopic detrortion and laparotomy with detorsion and colopexy are associated with appreciable morbidity[16]. A recent study reported on the use of laparoscopic recto-sigmoidectomy following colonoscopic decompression in nine patients[17]. Although further studies with larger number of patients are required, this technique appears to be a good option, but can only be applied in decompressed patients.

Traditional surgical teaching has dictated that a primary anastomosis should not be undertaken in an unprepared, obstructed bowel[18-20]. A number of studies on sigmoid volvulus explored the feasibility of one-stage resection using on-table lavage[13,21-24]. The advantages of this approach include single stage procedure, no need for a colostomy, possible lower morbidity and mortality, and shorter hospital stay. The disadvantages include prolonged operative time, need for several liters of irrigation solution, and risk of contamination. However, clinical and experimental evidence supports the view that a clean bowel has an important advantage in surgery of the left colon and rectum, which are parts of the bowel containing solid feces, with a high bacterial count[19,25]. Therefore, an emergency RPA of an unprepared left colon is a controversial subject. Traditionally, obstruction of the left colon is managed by a multi-stage defunctioning colostomy and resection. However, there is a growing acceptance of one-stage primary resection and anastomosis with the use of on-table antegrade irrigation[24,26-28]. However, several studies have suggested that on-table lavage may not be necessary for a safe emergency RPA of unprepared left colon[26-29]. Most experts agree that temporary proximal fecal diversion or decompression can reduce the risk of sepsis resulting from anastomotic leakage[30,31]. All patients in the two study groups, had RPA after intra-operative decompression without any on-table lavage.

Symptomatic anastomotic leak is the most important postoperative complication following emergency colorectal resection with intestinal anastomosis. De *et al*[26] reported 197 patients who had a single stage primary anastomosis without colonic lavage for left-sided colonic obstruction due to acute sigmoid volvulus; only 2 (1.01%) patients developed symptomatic anastomotic leak. In a similar prospective study by

Raveenthiran[29], 57 consecutive patients with acute sigmoid volvulus had emergency RPA without on-table lavage or caecostomy. The anastomotic leak was seen in 10% patients, with higher leak rate in patients with gangrenous colon. Factors such as acute anemia, shock and peri-operative whole blood transfusion are believed to be associated with major anastomotic leaks in patients with gangrenous colon. Due to these co-morbid risk factors, particularly in patients presenting with gangrenous sigmoid volvulus, the addition of a modified blow-hole colostomy appears to be a promising procedure in order to avoid anastomotic leak. Early diet consumption is another advantage of this procedure and may improve the postoperative course. In the present study, anastomotic leakage occurred in 3 (6.3%) patients in group A, compared to none in group B. The difference was numerically impressive but not statistically significant. Comparison of hospital stay, mortality and wound infection did not reveal any significant difference between the two groups. The lower rate of wound infection in group B is a controversial subject. In our opinion, this is probably due to the beneficial effect of early dietary consumption. Previous studies have clearly shown the advantages of early enteral nutrition in surgical patients in reducing septic complications and the overall morbidity compared to parenteral nutrition[32,33].

In conclusion, RPA with modified blow-hole colostomy provides satisfactory results in patients with sigmoid volvulus. This procedure is safe and effective in preventing anastomotic leaks, and may become the treatment of choice in patients with sigmoid volvulus. Further studies are required to definitively establish its role in sigmoid volvulus.

COMMENTS

Background

Although the diagnosis of sigmoid volvulus is not difficult, the emergent surgical approach to sigmoid volvulus is a subject of much debate. There is a growing acceptance of one-stage primary resection and anastomosis of sigmoid colon. However, with this approach, anastomotic leakage may occur, particularly in elderly and hemodynamically unstable patients. Blow-hole colostomy can play a protective role to avoid anastomotic leakage. In the present study, we compared the results of resection and primary anastomosis (RPA), with or without the use of a modified blow-hole colostomy in unprepared bowel in patients with acute sigmoid volvulus.

Research frontiers

Anastomotic leakage is the most important postoperative complication following emergency colorectal resection with intestinal anastomosis. Temporary proximal fecal diversion or decompression can reduce the risk of sepsis from anastomotic leakage. The present study showed that RPA with modified blow-hole colostomy in unprepared bowel with sigmoid volvulus is effective in preventing anastomotic leakage.

Innovations and breakthroughs

This is a new technique for use in patients with sigmoid volvulus. We believe that this technique will decrease the rate of complications such as anastomotic leakage, especially in high risk patients.

Applications

The present study has shown that RPA with modified blow-hole colostomy for sigmoid volvulus is a safe procedure, which enables successful decompression and avoids high mortality rates, particularly in elderly and hemodynamically unstable patients. Future studies will be required to verify the effectiveness and safety of this technique.

Peer review

The authors compared the efficacy of two different type of surgery in patients with sigmoid volvulus. The observations are interesting.

REFERENCES

1 **Welch GH**, Anderson JR. Acute volvulus of the sigmoid colon. *World J Surg* 1987; **11**: 258-262
2 **Wertkin MG**, Aufses AH Jr. Management of volvulus of the colon. *Dis Colon Rectum* 1978; **21**: 40-45
3 **Avots-Avotins KV**, Waugh DE. Colon volvulus and the geriatric patient. *Surg Clin North Am* 1982; **62**: 249-260
4 **Oncu M**, Piskin B, Calik A, Yandi M, Alhan E. Volvulus of the sigmoid colon. *S Afr J Surg* 1991; **29**: 48-49
5 **Geer DA**, Arnaud G, Beitler A, Holcomb J, Homan J, James L, Martin D, McWhirt E, Napoli P. Colonic volvulus. The Army Medical Center experience 1983-1987. *Am Surg* 1991; **57**: 295-300
6 **Ballantyne GH**. Review of sigmoid volvulus. Clinical patterns and pathogenesis. *Dis Colon Rectum* 1982; **25**: 823-830
7 **Madiba TE**, Thomson SR. The management of sigmoid volvulus. *J R Coll Surg Edinb* 2000; **45**: 74-80
8 **Peoples JB**, McCafferty JC, Scher KS. Operative therapy for sigmoid volvulus. Identification of risk factors affecting outcome. *Dis Colon Rectum* 1990; **33**: 643-646
9 **Remzi FH**, Oncel M, Hull TL, Strong SA, Lavery IC, Fazio VW. Current indications for blow-hole colostomy:ileostomy procedure. A single center experience. *Int J Colorectal Dis* 2003; **18**: 361-364
10 **Huang WS**, Liu KW, Lin PY, Hsieh CC, Wang JY. Delayed ischemic gangrene change of distal limb despite optimal decompressed colostomy constructed in obstructed sigmoid colon cancer: a case report. *World J Gastroenterol* 2006; **12**: 993-995
11 **Poon RT**, Law WL, Chu KW, Wong J. Emergency resection and primary anastomosis for left-sided obstructing colorectal carcinoma in the elderly. *Br J Surg* 1998; **85**: 1539-1542
12 **Lau PW**, Lo CY, Law WL. The role of one-stage surgery in acute left-sided colonic obstruction. *Am J Surg* 1995; **169**: 406-409
13 **Sule AZ**, Iya D, Obekpa PO, Ogbonna B, Momoh JT, Ugwu BT. One-stage procedure in the management of acute sigmoid volvulus. *J R Coll Surg Edinb* 1999; **44**: 164-166
14 **Kuzu MA**, Aslar AK, Soran A, Polat A, Topcu O, Hengirmen S. Emergent resection for acute sigmoid volvulus: results of 106 consecutive cases. *Dis Colon Rectum* 2002; **45**: 1085-1090
15 **Ballantyne GH**, Brandner MD, Beart RW Jr, Ilstrup DM. Volvulus of the colon. Incidence and mortality. *Ann Surg* 1985; **202**: 83-92
16 **Lal SK**, Morgenstern R, Vinjirayer EP, Matin A. Sigmoid volvulus an update. *Gastrointest Endosc Clin N Am* 2006; **16**: 175-187
17 **Cartwright-Terry T**, Phillips S, Greenslade GL, Dixon AR. Laparoscopy in the management of closed loop sigmoid volvulus. *Colorectal Dis* 2008; **10**: 370-372
18 **Reilly PMJ**, Jones B, Bulkley GB. Volvulus of the colon.In Cameron JL., ed. Current Surgical Therapy. St Louis: Decker Inc, 1992: 170-174
19 **Irvin TT**, Goligher JC. Aetiology of disruption of intestinal anastomoses. *Br J Surg* 1973; **60**: 461-464
20 **Smith SR**, Connolly JC, Gilmore OJ. The effect of faecal loading on colonic anastomotic healing. *Br J Surg* 1983; **70**: 49-50
21 **Gurel M**, Alic B, Bac B, Keles C, Akgun Y, Boylu S. Intraoperative colonic irrigation in the treatment of acute sigmoid volvulus. *Br J Surg* 1989; **76**: 957-958
22 **Gibney EJ**. On-table lavage in the management of sigmoid volvulus: a review. *West Afr J Med* 1992; **11**: 223-225

23 **Keller A**, Aeberhard P. Emergency resection and primary anastomosis for sigmoid volvulus in an African population. *Int J Colorectal Dis* 1990; **5**: 209-212
24 **Sule A**, Obepka PO, Iya D, Ogbonna B, Momoh J. Intraoperative colonic irrigation in the management of left sided large bowel emergencies in Jos University Teaching Hospital, Nigeria. *East Afr Med J* 2000; **77**: 613-617
25 **Nwafo DC**. Selective primary suture of the battle-injured colon: an experience of the Nigerian Civil War. *Br J Surg* 1980; **67**: 195-197
26 **De U**, Ghosh S. Single stage primary anastomosis without colonic lavage for left-sided colonic obstruction due to acute sigmoid volvulus: a prospective study of one hundred and ninety-seven cases. *ANZ J Surg* 2003; **73**: 390-392
27 **Naaeder SB**, Archampong EQ. One-stage resection of acute sigmoid volvulus. *Br J Surg* 1995; **82**: 1635-1636
28 **Duthie GS**, Foster ME, Price-Thomas JM, Leaper DJ. Bowel preparation or not for elective colorectal surgery. *J R Coll Surg Edinb* 1990; **35**: 169-171
29 **Raveenthiran V**. Restorative resection of unprepared left-colon in gangrenous vs. viable sigmoid volvulus. *Int J Colorectal Dis* 2004; **19**: 258-263
30 **Pakkastie TE**, Ovaska JT, Pekkala ES, Luukkonen PE, Jarvinen HJ. A randomised study of colostomies in low colorectal anastomoses. *Eur J Surg* 1997; **163**: 929-933
31 **Karanjia ND**, Corder AP, Holdsworth PJ, Heald RJ. Risk of peritonitis and fatal septicaemia and the need to defunction the low anastomosis. *Br J Surg* 1991; **78**: 196-198
32 **Meguid MM**, Campos AC, Hammond WG. Nutritional support in surgical practice: Part II. *Am J Surg* 1990; **159**: 427-443
33 **Moore FA**, Feliciano DV, Andrassy RJ, McArdle AH, Booth FV, Morgenstein-Wagner TB, Kellum JM Jr, Welling RE, Moore EE. Early enteral feeding, compared with parenteral, reduces postoperative septic complications. The results of a meta-analysis. *Ann Surg* 1992; **216**: 172-183

S- Editor Li DL **L- Editor** Anand BS **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5595

World J Gastroenterol 2008 September 28; 14(36): 5595-5600
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Pancreatic guidewire placement for achieving selective biliary cannulation during endoscopic retrograde cholangio-pancreatography

Kei Ito, Naotaka Fujita, Yutaka Noda, Go Kobayashi, Takashi Obana, Jun Horaguchi, Osamu Takasawa, Shinsuke Koshita, Yoshihide Kanno

Kei Ito, Naotaka Fujita, Yutaka Noda, Go Kobayashi, Takashi Obana, Jun Horaguchi, Osamu Takasawa, Shinsuke Koshita, Yoshihide Kanno, Department of Gastroenterology, Sendai City Medical Center, 5-22-1, Tsurugaya, Miyagino-ku, Sendai, Miyagi 983-0824, Japan

Author contributions: Ito K designed the study, performed the research and wrote the paper; Fujita N reviewed the paper; Noda Y, Kobayashi G, Obana T, Horaguchi J and Takasawa O performed endoscopic procedures and analyzed data; Koshita S and Kanno Y analyzed data.

Correspondence to: Kei Ito, Department of Gastroenterology, Sendai City Medical Center, 5-22-1, Tsurugaya, Miyagino-ku, Sendai, Miyagi 983-0824, Japan. keiito@openhp.or.jp

Telephone: +81-22-2521111 Fax: +81-22-2529431

Received: June 10, 2008 Revised: August 18, 2008
Accepted: August 25, 2008
Published online: September 28, 2008

Abstract

AIM: To investigate the frequency and risk factors for acute pancreatitis after pancreatic guidewire placement (P-GW) in achieving cannulation of the bile duct during endoscopic retrograde cholangio-pancreatography (ERCP).

METHODS: P-GW was performed in 113 patients in whom cannulation of the bile duct was difficult. The success rate of biliary cannulation, the frequency and risk factors of post-ERCP pancreatitis, and the frequency of spontaneous migration of the pancreatic duct stent were investigated.

RESULTS: Selective biliary cannulation with P-GW was achieved in 73% of the patients. Post-ERCP pancreatitis occurred in 12% (14 patients: mild, 13; moderate, 1). Prophylactic pancreatic stenting was attempted in 59% of the patients. Of the 64 patients who successfully underwent stent placement, three developed mild pancreatitis (4.7%). Of the 49 patients without stent placement, 11 developed pancreatitis (22%: mild, 10; moderate, 1). Of the five patients in whom stent placement was unsuccessful, two developed mild pancreatitis. Univariate and multivariate analyses revealed no pancreatic stenting to be the only significant risk factor for pancreatitis. Spontaneous migration of the stent was observed within two weeks in 92% of the patients who had undergone pancreatic duct stenting.

CONCLUSION: P-GW is useful for achieving selective biliary cannulation. Pancreatic duct stenting after P-GW can reduce the incidence of post-ERCP pancreatitis, which requires evaluation by means of prospective randomized controlled trials.

Key words: Endoscopic retrograde cholangio-pancreatography; Pancreatic duct stenting; Acute pancreatitis; Post-endoscopic retrograde cholangio-pancreatography pancreatitis; Biliary cannulation

Peer reviewer: William Dickey, Altnagelvin Hospital, Londonderry, BT47 6SB, Northern Ireland, United Kingdom

Ito K, Fujita N, Noda Y, Kobayashi G, Obana T, Horaguchi J, Takasawa O, Koshita S, Kanno Y. Pancreatic guidewire placement for achieving selective biliary cannulation during endoscopic retrograde cholangio-pancreatography. *World J Gastroenterol* 2008; 14(36): 5595-5600 Available from: URL: http://www.wjgnet.com/1007-9327/14/5595.asp DOI: http://dx.doi.org/10.3748/wjg.14.5595

INTRODUCTION

Endoscopic retrograde cholangio-pancreatography (ERCP), which was first reported in the late 1960's[1], is a well-accepted technique for evaluating pancreato-biliary diseases. Although selective cannulation of the bile duct is mandatory for therapeutic ERCP of biliary diseases, anatomical difficulties or papillary spasm sometimes preclude it. Numerous techniques have been developed for selective biliary cannulation[2,3]. Pancreatic guidewire placement (P-GW) has been reported to be effective in patients with difficult cannulation of the bile duct[4-7]. Since P-GW is usually attempted in patients with difficult cannulation of the bile duct, this technique entails a possible increased risk of post-ERCP pancreatitis. The aim of this study was to investigate the frequency and risk factors for acute pancreatitis after P-GW in achieving cannulation of the bile duct during ERCP.

MATERIALS AND METHODS

Study population

Between December, 2001 and January, 2008, 3955 ERCPs were performed at Sendai City Medical Center. P-GW was performed in patients with pancreato-biliary diseases in whom cannulation of the bile duct was difficult and guidewire insertion into the pancreatic duct was achieved. Difficult cannulation of the bile duct was defined as unsuccessful cannulation with a cannula or a sphincterotome within 15 min. Wire-guided cannulation was not performed in this study. Patients in whom an insertion of a guidewire into the pancreatic duct could not be achieved were excluded from the study. Written informed consent was obtained from all patients before the procedure. This study was approved by the Institutional Review Board of Sendai City Medical Center. Prophylactic pancreatic stenting was performed in selected patients after P-GW. Indication for pancreatic duct stenting was determined by each operator during the procedure. Established criteria for pancreatic duct stenting after P-GW was not determined in this study.

Endoscopic procedures and patient care after the procedure

Before endoscopic procedures, all patients were given a standard premedication consisting of intravenous administration of pentazocine (7.5-15 mg) and diazepam (3-10 mg) or midazolam (3-10 mg), the doses depending on age and tolerance. The procedures were carried out with side-viewing duodenoscopes (JF200, 230, 240, 260V: Olympus Optical Co., Tokyo, Japan). When selective biliary cannulation with a cannula or a sphincterotomy was regarded as difficult, P-GW was attempted. A 0.025-inch or 0.035-inch guidewire (Jagwire: Microvasive, Boston Scientific Corp., Natick, MA, USA) was inserted into the pancreatic duct via the cannula in order to stabilize the papilla of Vater and straighten the bile duct terminal. Alongside the guidewire, a second cannula was passed into the same working channel of the scope with the two-devices-in-one-channel method[8], and cannulation of the bile duct was attempted. After successful cannulation of the bile duct, additional procedures such as endoscopic sphincterotomy, biliary stent placement, intraductal ultrasonography, biopsy of the bile duct, and so on were performed as necessary. Pancreatic duct stenting was performed for prophylaxis of post-ERCP pancreatitis. The pancreatic duct stent used was a 5 Fr, 4-cm-long stent with a single duodenal pigtail (Pit-stent: Cathex, Co., Ltd., Tokyo, Japan). All procedures were performed by operators with experience in more than 500 ERCPs. Abdominal radiographs were obtained to assess the stent position after deployment within 14 d after the ERCP. Endoscopic stent removal was performed if necessary.

Patients continued to fast after the procedure for a minimum of 24 h with drip infusion of 2000 mL and remained in the hospital for at least 48 h. They received 8-h infusion of protease inhibitor (nafamostat mesilate, 20 mg/d) and antibiotics (SBT/CPZ, 2 g/d) for 2 d. Serum amylase levels were measured before the procedure and 3 h, 6 h and 24 h afterwards. The reference range was 42-130 IU/L for the amylase. Symptoms (abdominal pain, nausea, *etc.*) and physical findings (abdominal tenderness) were clinically evaluated.

Main outcome measurements

The success rate of bile duct cannulation, the frequency and risk factors of post-ERCP pancreatitis, and the frequency of spontaneous migration of the pancreatic duct stent were investigated.

A diagnosis of post-ERCP pancreatitis was made based on the presence of abdominal pain 24 h after the procedure and an increase in serum amylase level greater than three times the upper normal limit[9]. The severity of pancreatitis was classified as mild if prolongation of fasting was within 1-3 d, moderate for fasting 4-10 d long, and severe for fasting more as 10 d, as well as in cases of hemorrhagic pancreatitis, phlegmon, or pseudocyst.

Statistical analysis

Fisher's exact probability test was used for statistical analyses where appropriate. Variables found to be possibly significant ($P < 0.5$) in univariate analysis were chosen for entry into a multiple logistic regression. A P value less than 0.05 was regarded as significant. Statistical analysis was performed with StatMate Ⅲ (ATMS Co. Ltd., Tokyo, Japan) and StatView Ver.5.0 (SAS Institute, Cary, NC, USA).

RESULTS

Table 1 shows the characteristics and diagnoses of the patients. Selective biliary cannulation with P-GW was achieved in 73% of the patients (82 patients, Figure 1). Of the remaining 31 patients with unsuccessful biliary cannulation, eight underwent precut sphincterotomy in the same session, biliary cannulation being subsequently achieved in two of them. The other patients underwent a second ERCP including precut sphincterotomy, percutaneous biliary drainage, surgical treatment, or substitute diagnostic procedures. Selective biliary cannulation was finally achieved in 86% of the patients (97 patients).

Prophylactic pancreatic duct stenting was attempted in 59% of the patients (69 patients). Although stenting was successful in all patients, five developed migration of the stent during the procedure.

Post-ERCP pancreatitis occurred in 12% (14 patients: mild, 13; moderate, 1). All of them improved with conservative therapy in a few days. Of the 64 patients who successfully underwent pancreatic duct stenting, three developed mild pancreatitis (4.7%). Of the remaining 49 patients without a stent, 11 developed pancreatitis (22%: mild, 10; moderate, 1). Of the five patients with stent migration, two developed mild pancreatitis (40%).

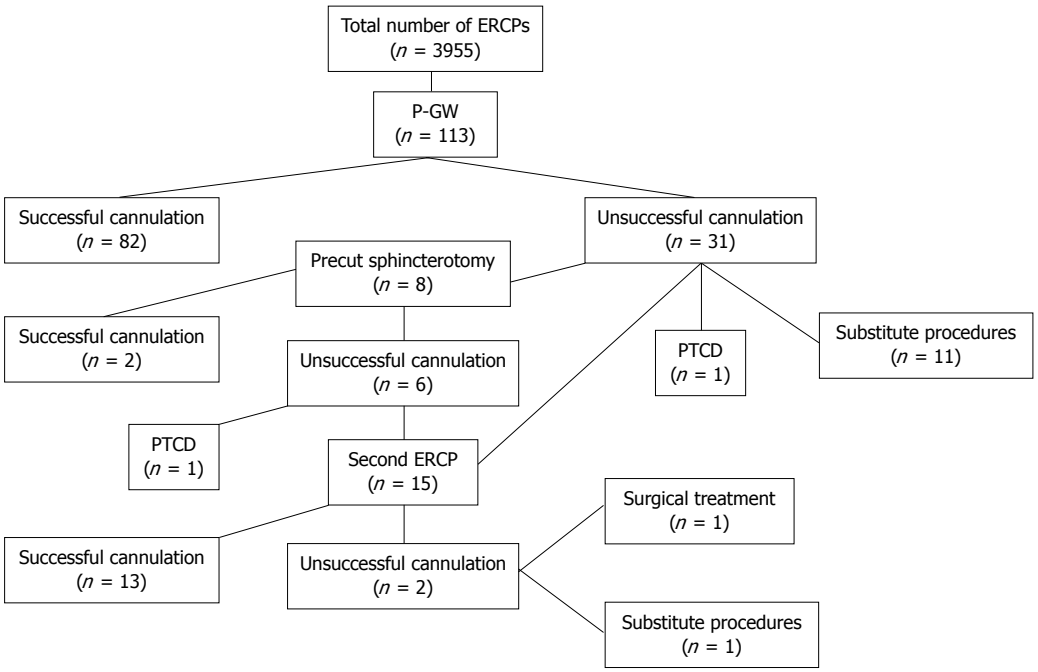


Figure 1 Flow chart showing clinical course of the patients. P-GW: Pancreatic guidewire placement; PTCD: Percutaneous transhepatic cholangio-drainage.

Table 1 Characteristics and diagnosis of the patients (*n* = 113)

Characteristics and diagnosis	Number of patients
Mean age (range, yr)	70 (33-90)
Male/Female	63/50
History of pancreatitis	8 (7%)
History of post-ERCP pancreatitis	0%
Normal bilirubin level	67 (59%)
Duodenal diverticulum	32 (28%)
Pancreatic duct stenosis	7 (6%)
Mean procedure time (range, min)	46 (17-98)
Procedure time > 45 min	52 (46%)
Therapeutic ERCP	75 (66%)
Pancreatic duct opacification	100 (100%)
Pancreatic duct stenting	64 (57%)
Biliary sphincterotomy	39 (35%)
Precut	8 (7.1%)
Intraductal ultrasonography	25 (22%)
Biliary stent placement	24 (21%)
Papillary balloon dilatation	2 (1.8%)
Cytology of bile/pancreatic juice	11 (9.7%)
Biopsy of the bile duct/pancreatic duct	10 (8.8%)
Choledocholithiasis	42 (37%)
Cholecystolithiasis	26 (23%)
Cholangiocarcinoma	9 (8.0%)
Pancreatic cancer	8 (7.1%)
Chronic pancreatitis	5 (4.4%)
Intraductal papillary mucinous neoplasm	4 (3.5%)
Ampullary cancer/adenoma	4 (3.5%)

Post-ERCP pancreatitis occurred in 13% of the 31 patients with unsuccessful biliary cannulation. Of the patients with unsuccessful biliary cannulation, pancreatic duct stenting was performed in 24 patients. The frequency of post-ERCP pancreatitis of the patients without pancreatic duct stenting was significantly higher than that of the patients with pancreatic duct stenting (43% *vs* 4.2%, $P = 0.041$; odds ratio, 17; 95% CI, 1.4-210).

Hyperamylasemia at 24 h after ERCP was observed in 68% (68 patients) of the 100 patients with normal serum amylase level before the procedure. Of these patients, the peak serum amylase level was seen at 3 h after ERCP in 8.8% (6 patients), at 6 h in 50% (34), and at 24 h in 41% (28). All of them showed a decrease in serum amylase level at 48-72 h after ERCP. Of the 14 patients who developed post-ERCP pancreatitis, none showed a peak serum amylase level at 3 h after ERCP, six patients showed a peak at 6 h, and eight patients showed a peak at 24 h after ERCP.

Univariate analysis revealed no pancreatic stenting to be the only significant risk factor for post-ERCP pancreatitis ($P = 0.011$; odds ratio 5.9; 95% CI, 1.5-22; Table 2). Multivariate analysis also revealed no pancreatic stenting to be the only significant risk factor for post-ERCP pancreatitis ($P = 0.017$, Table 3).

Among the 64 patients who underwent pancreatic duct stenting, five patients who required additional ERCP due to various reasons underwent endoscopic removal of the stent at the time of the procedure. Estimation of stent passage was not performed in six patients. Spontaneous migration of the pancreatic stent was observed within two weeks in 92% (49/53) of the patients who had undergone pancreatic duct stenting. In the four patients without passage of a pancreatic stent, two underwent additional endoscopy for removal of the stent. The other two patients refused additional endoscopy. No complications concerning spontaneous passage of the stent occurred.

DISCUSSION

Selective biliary cannulation is mandatory for therapeutic ERCPs in patients with biliary diseases. Since difficulties in selective cannulation are sometimes encountered due to anatomical constraints or papillary spasm, numerous techniques (papillotome cannulation,

Table 2 Risk factors of post-ERCP pancreatitis (univariate analysis) *n* (%)

Risk factors	Pancreatitis (+) (*n* = 14)	Pancreatitis (-) (*n* = 99)	*P*	OR (95% CI)
Age (< 50 yr)	2 (14)	3 (3)	0.22	5.3 (0.81-35)
Female gender	8 (57)	42 (42)	0.45	1.8 (0.58-5.6)
History of pancreatitis	0	8 (8)	0.58	-
Normal bilirubin level	12 (86)	55 (56)	0.063	4.8 (1.0-23)
Duodenal diverticulum	3 (21)	29 (29)	0.77	0.66 (0.17-2.5)
Pancreatic duct stenosis	0	7 (7)	0.66	-
Procedure time > 45 min	5 (36)	47 (47)	0.59	0.61 (0.19-2.0)
Therapeutic ERCP	7 (50)	68 (69)	0.28	0.46 (0.15-1.4)
PD opacification	14 (100)	99 (100)	-	-
No PD stenting	11 (79)	38 (38)	0.011	5.9 (1.5-22)
Failure in PD stenting	2 (14)	3 (3)	0.22	5.3 (0.81-35)
Unsuccessful BD cannulation	4 (29)	27 (27)	0.83	1.1 (0.31-3.7)
Biliary sphincterotomy	3 (21)	36 (36)	0.42	0.48 (0.12-1.8)
Precut	2 (14)	6 (6)	0.57	2.6 (0.47-14)
Intraductal ultrasonography	3 (21)	22 (22)	0.78	0.95 (0.24-3.7)
Biliary stent placement	2 (14)	22 (22)	0.74	0.58 (0.12-2.8)
Papillary balloon dilatation	1 (7)	1 (1)	0.58	7.5 (0.44-128)
Cytology of bile/pancreatic juice	1 (7)	10 (10)	0.89	0.68 (0.081-5.8)
Biopsy of the BD/PD	1 (7)	9 (9)	0.79	0.77 (0.090-6.6)
Choledocholithiasis	5 (36)	37 (37)	0.86	0.93 (0.29-3.0)
Cholecystolithiasis	5 (36)	21 (21)	0.39	2.1 (0.62-6.8)
Cholangiocarcinoma	1 (7)	8 (8)	0.68	0.88 (0.10-7.6)
Pancreatic cancer	0	8 (8)	0.58	-
Chronic pancreatitis	0	5 (5)	0.87	-
Intraductal papillary mucinous neoplasm	1 (7)	3 (3)	0.99	2.5 (0.24-25)
Ampullary cancer/adenoma	0	4 (4)	0.99	-

ERCP: Endoscopic retrograde cholangiopancreatography; PD: Pancreatic duct; BD: Bile duct; OR: Odds ratio; CI: Confidence interval.

Table 3 Risk factors of post-ERCP pancreatitis (multivariate analysis)

	P
Age (< 50 yr)	0.9
Female gender	0.97
Normal bilirubin level	0.12
Therapeutic ERCP	0.63
Biliary sphincterotomy	0.81
No PD stenting	0.017
Unsuccessful BD cannulation	0.65
Cholecystolithiasis	0.44

PD: Pancreatic duct; ERCP: Endoscopic retrograde cholangio-pancreatography; BD: Bile duct.

precut sphincterotomy, or guidewire cannulation) and pharmacologic aids (cholecystokinin or nitroglycerine spray) have been developed for this purpose[2,3].

Dumonceau *et al*[4] first reported P-GW for selective biliary cannulation in a patient with surgically altered anatomy. Gotoh *et al* reported a second case of successful biliary cannulation with P-GW in a patient with a tortuous common channel[5]. P-GW serves several functions advantageous for selective bile duct cannulation, including opening a stenotic papillary orifice, stabilizing the papilla and lifting it up towards the working channel, straightening the pancreatic duct and the common channel, draining the pancreatic duct, potentially minimizing repeated injections into the pancreatic duct, and providing access for placement of a pancreatic stent if necessary[2].

Maeda *et al*[7] carried out a randomized controlled trial with 53 patients in whom conventional cannulation failed within 10 min, these patients being were assigned to either P-GW (n = 27) or continuation of ordinary techniques (n = 26). Successful biliary cannulation was achieved in 93% of the patients with P-GW compared with 58% in the group of continuation of ordinary techniques (P = 0.0085). They reported a significantly high frequency of hyperamylasemia in the P-GW group, with no incidence of acute pancreatitis. We retrospectively estimated the efficacy and safety of P-GW for biliary cannulation. Although the success rate of biliary cannulation with P-GW in our study was inferior to that of their study (73% *vs* 93%), the final cannulation rate (86%) in our study was comparable. Patients included in our study were only 2.9% of the patients who underwent ERCP during the same period, and thus they were considered to be extremely difficult cases for biliary cannulation, thus allowing the assessment of success rate of biliary cannulation to be acceptable. It is often emphasized that precut sphincterotomy, which is generally followed by conventional sphincterotomy, should be performed only by expert endoscopists. On the other hand, P-GW can be performed even by endoscopists with less experience in both diagnostic and therapeutic ERCPs without the need for sphincterotomy. Although it entails a potential risk of failure to place a guidewire deeply in a tortuous main duct, P-GW is worth attempting in patients with

difficult biliary cannulation before precut sphincterotomy or other invasive techniques are undertaken.

Post-ERCP pancreatitis occurred in 12% of the patients in our study, in contrast to 0% in Maeda's study[7]. This is thought to be due to the number of patients included, differences in study population, and definitions of post-ERCP pancreatitis. Deviere commented in his review that Maeda's data are not sufficient to claim that the risk of pancreatitis will not be increased by this technique because of the small number of patients and the use of confounding medications such as isosorbide dinitrate and urinastatin[10]. One of the major mechanisms of post-ERCP pancreatitis is insufficient pancreatic duct drainage as a result of papillary edema after repetitive manipulation or contrast injections. Therefore, difficult cannulation of the bile duct is given as a risk factor[11]. Since P-GW is attempted in patients with difficult biliary cannulation, it entails a possible increased risk of this particular complication. The efficacy of prophylactic pancreatic stent placement to prevent post-ERCP pancreatitis has been reported by prospective randomized controlled trials[12-16]. Fazel *et al*[13] reported that pancreatic stent (5 Fr, 2 cm or 5 Fr nasopancreatic catheter) insertion reduced the frequency of post-ERCP pancreatitis from 28% to 5% in patients at high risk for this complication following difficult cannulation, sphincter of Oddi manometry, and/or sphincterotomy. In our study, post-ERCP pancreatitis occurred in 4.7% of patients with pancreatic duct stenting in contrast to 22% of patients without stenting. Non-performance of pancreatic duct stenting was found to be the only significant risk factor for post-ERCP pancreatitis by univariate and multivariate analysis. Based on these results, we suggest that pancreatic duct stenting is indispensable for P-GW in order to prevent post-ERCP pancreatitis. Of course, our study had some limitations. First, it was not a randomized or prospective study. Second, routine administration of protease inhibitor might have influenced the frequency of post-ERCP pancreatitis. Third, the usefulness of P-GW was not compared with other methods such as precut sphincterotomy, guidewire cannulation, and so on. Forth, repeated attempts at entering the pancreatic duct with a wire might increase pancreatitis risk. Unfortunately, this study cannot evaluate the risk as patients in whom insertion of a guidewire into the pancreatic duct cannot be achieved were excluded. Further randomized controlled trials are necessary for the evaluation of these points. Nevertheless, our study yielded significant information as regards prophylaxis of post-ERCP pancreatitis by pancreatic duct stenting in patients who undergo P-GW.

In conclusion, P-GW is useful for achieving selective cannulation of the bile duct. Pancreatic duct stenting after P-GW can reduce the incidence of post-ERCP pancreatitis, which should be verified by prospective randomized controlled trials.

COMMENTS

Background

Selective biliary cannulation is mandatory for therapeutic endoscopic retrograde cholangio-pancreatography (ERCP) in patients with biliary diseases. Since difficulties in selective cannulation are sometimes encountered due to anatomical constraints or papillary spasm, numerous techniques (papillotome cannulation, precut sphincterotomy, or guidewire cannulation) and pharmacologic aids (cholecystokinin or nitroglycerine spray) have been developed for this purpose.

Research frontiers

Pancreatic guidewire placement (P-GW) is reported to be effective in patients with difficult cannulation of the bile duct. However, this technique entails a possible increased risk of post-ERCP pancreatitis.

Innovations and breakthroughs

In a single-center, retrospective study, selective biliary cannulation with P-GW was achieved in 73% of the patients. Post-ERCP pancreatitis occurred in 12% after P-GW. Univariate and multivariate analyses revealed no pancreatic stenting to be the only significant risk factor for pancreatitis. Pancreatic duct stenting after P-GW may reduce the incidence of post-ERCP pancreatitis.

Applications

P-GW is useful for achieving selective biliary cannulation. Pancreatic duct stenting after P-GW can reduce the incidence of post-ERCP pancreatitis, which requires evaluation by means of prospective randomized controlled trials.

Peer review

It's an interesting paper. The authors clarified that pancreatic P-GW is useful for achieving selective biliary cannulation in patients with difficult cannulation of the bile duct.

REFERENCES

1 **McCune WS**, Shorb PE, Moscovitz H. Endoscopic cannulation of the ampulla of vater: a preliminary report. *Ann Surg* 1968; **167**: 752-756

2 **Freeman ML**, Guda NM. ERCP cannulation: a review of reported techniques. *Gastrointest Endosc* 2005; **61**: 112-125

3 **Artifon EL**, Sakai P, Cunha JE, Halwan B, Ishioka S, Kumar A. Guidewire cannulation reduces risk of post-ERCP pancreatitis and facilitates bile duct cannulation. *Am J Gastroenterol* 2007; **102**: 2147-2153

4 **Dumonceau JM**, Deviere J, Cremer M. A new method of achieving deep cannulation of the common bile duct during endoscopic retrograde cholangiopancreatography. *Endoscopy* 1998; **30**: S80

5 **Gotoh Y**, Tamada K, Tomiyama T, Wada S, Ohashi A, Satoh Y, Higashizawa T, Miyata T, Ido K, Sugano K. A new method for deep cannulation of the bile duct by straightening the pancreatic duct. *Gastrointest Endosc* 2001; **53**: 820-822

6 **Hayashi H**, Maeda S, Hosokawa O, Dohden K, Hattori M, Tanikawa Y, Watanabe K, Ibe N, Tatsumi S. A technique for selective cannulation of the common bile duct in endoscopic retrograde cholangiopancreatography. Insertion of guidewire into the pancreatic duct. *Gastroenterol Endoscopy* 2001; **43**: 828-832

7 **Maeda S**, Hayashi H, Hosokawa O, Dohden K, Hattori M, Morita M, Kidani E, Ibe N, Tatsumi S. Prospective randomized pilot trial of selective biliary cannulation using pancreatic guide-wire placement. *Endoscopy* 2003; **35**: 721-724

8 **Fujita N**, Noda Y, Kobayashi G, Kimura K, Yago A. ERCP for intradiverticular papilla: two-devices-in-one-channel method. Endoscopic Retrograde Cholangiopancreatograph. *Gastrointest Endosc* 1998; **48**: 517-520

9 **Cotton PB**, Lehman G, Vennes J, Geenen JE, Russell RC, Meyers WC, Liguory C, Nickl N. Endoscopic sphincterotomy complications and their management: an attempt at consensus. *Gastrointest Endosc* 1991; **37**: 383-393

10 **Deviere J**. Using the pancreas for common bile duct cannulation? *Endoscopy* 2003; **35**: 750-751

11 **Freeman ML**, DiSario JA, Nelson DB, Fennerty MB, Lee JG, Bjorkman DJ, Overby CS, Aas J, Ryan ME, Bochna GS, Shaw MJ, Snady HW, Erickson RV, Moore JP, Roel JP. Risk factors for post-ERCP pancreatitis: a prospective, multicenter

study. *Gastrointest Endosc* 2001; **54**: 425-434

12 **Tarnasky PR**, Palesch YY, Cunningham JT, Mauldin PD, Cotton PB, Hawes RH. Pancreatic stenting prevents pancreatitis after biliary sphincterotomy in patients with sphincter of Oddi dysfunction. *Gastroenterology* 1998; **115**: 1518-1524

13 **Fazel A**, Quadri A, Catalano MF, Meyerson SM, Geenen JE. Does a pancreatic duct stent prevent post-ERCP pancreatitis? A prospective randomized study. *Gastrointest Endosc* 2003; **57**: 291-294

14 **Harewood GC**, Pochron NL, Gostout CJ. Prospective, randomized, controlled trial of prophylactic pancreatic stent placement for endoscopic snare excision of the duodenal ampulla. *Gastrointest Endosc* 2005; **62**: 367-370

15 **Tsuchiya T**, Itoi T, Sofuni A, Itokawa F, Kurihara T, Ishii K, Tsuji S, Kawai T, Moriyasu F. Temporary pancreatic stent to prevent post endoscopic retrograde cholangiopancreatography pancreatitis: a preliminary, single-center, randomized controlled trial. *J Hepatobiliary Pancreat Surg* 2007; **14**: 302-307

16 **Sofuni A**, Maguchi H, Itoi T, Katanuma A, Hisai H, Niido T, Toyota M, Fujii T, Harada Y, Takada T. Prophylaxis of post-endoscopic retrograde cholangiopancreatography pancreatitis by an endoscopic pancreatic spontaneous dislodgement stent. *Clin Gastroenterol Hepatol* 2007; **5**: 1339-1346

S- Editor Zhong XY **L- Editor** Rippe RA **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5601

World J Gastroenterol 2008 September 28; 14(36): 5601-5605
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Prolapse gastropathy syndrome may be a predictor of pathologic acid reflux

Jin-Soo Kim, Hyung-Keun Kim, Young-Seok Cho, Hiun-Suk Chae, Byung-Wook Kim, Jin-Il Kim, Sok-Won Han, Kyu-Yong Choi

Jin-Soo Kim, Hyung-Keun Kim, Young-Seok Cho, Hiun-Suk Chae, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, Uijeongbu St. Mary's Hospital, Gyeonggi-do 480-717, South Korea
Byung-Wook Kim, Kyu-Yong Choi, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, Our Lady of Mercy Hospital, Gyeonggi-do 403-720, South Korea
Jin-Il Kim, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, Yeoeuido St. Mary's Hospital, Seoul 150-713, South Korea
Sok-Won Han, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, Holy Family Hospital, Gyeonggi-do 420-717, South Korea
Author contributions: Chae HS, Kim BW, Kim JI, Han SW, Choi KY designed study and revised manuscript; Kim HK, Cho YS analyzed data; Kim JS mainly performed study and wrote the paper.
Correspondence to: Hiun-Suk Chae, MD, Department of Internal Medicine, Uijeongbu St. Mary's Hospital, 65-1 Geumo-dong, Uijeongbu city, Kyunggido 480-717, South Korea. chs@catholic.ac.kr
Telephone: +82-31-8203019 Fax: +82-31-8472719
Received: April 2, 2008 Revised: August 24, 2008
Accepted: September 1, 2008
Published online: September 28, 2008

Abstract

AIM: To assess the occurrence of gastric acid reflux into the esophagus in endoscopically confirmed prolapse gastropathy syndrome (PGS).

METHODS: Using ambulatory esophageal pH measurement (BRAVO™ wireless esophageal pH monitoring system), twenty-six patients with PGS were compared with twenty-one patients with erosive esophagitis (EE) as controls. We assessed several reflux parameters, including the percentage of total time at pH < 4, and the DeMeester score.

RESULTS: There were no statistical differences between the PGS group and the EE group as to mean age, sex ratio and pH recording time. The EE group showed more severe reflux than the PGS group, as evaluated in terms of the longest duration of reflux, the number of reflux episodes, the number of reflux episodes lasting > 5 min, the total time with pH < 4 during acid reflux episodes, and the DeMeester score, but none of these parameters showed statistically significant difference. Although 53.8% (14/26) of the PGS group and 76.2% (16/21) of the EE group demonstrated pathologic acid reflux (DeMeester score > 14.72), there was no statistically significant difference between the two groups in the incidence of pathologic acid reflux (P = 0.11).

CONCLUSION: There was no statistically significant difference in pathologic acid reflux between the PGS and EE group. These data suggest that endoscopically diagnosed PGS might be a predictor of pathologic acid reflux.

Key words: Prolapse gastropathy syndrome; Pathologic acid reflux; Erosive esophagitis; Ambulatory esophageal pH monitoring; Retching

Peer reviewer: Tomohiko Shimatani, Assistant Professor, Department of General Medicine, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 7348551, Japan

Kim JS, Kim HK, Cho YS, Chae HS, Kim BW, Kim JI, Han SW, Choi KY. Prolapse gastropathy syndrome may be a predictor of pathologic acid reflux. *World J Gastroenterol* 2008; 14(36): 5601-5605 Available from: URL: http://www.wjgnet.com/1007-9327/14/5601.asp DOI: http://dx.doi.org/10.3748/wjg.14.5601

INTRODUCTION

In 1984, Shepherd[1] proposed the term "prolapse gastropathy syndrome (PGS)", defining the diagnostic criteria for PGS as persistent and recurrent retching symptoms combined with hematemesis or abdominal pain, an endoscopic finding of prolapse of tense, inflamed, congested gastric mucosa into the esophageal lumen during retching, and an unusually strong gag and retch at endoscopy. PGS has been considered as one of the causes of upper gastrointestinal bleeding[2-6]. In addition, it has been regarded as esophageal pseudotumor[7] and retrograde gastroesophageal intussusception[8], combined with several severe complications such as Mallory-Weiss syndrome and esophageal rupture[9]. Besides the above conditions, it is thought that gastric acid might be regurgitated into the esophagus along with prolapsed gastric mucosa.

However, there have been no studies of pathologic gastric acid reflux into the esophagus associated with PGS. Therefore, we investigated whether gastric acid into the esophagus may occur in endoscopically confirmed PGS.

MATERIALS AND METHODS

Subjects

Subjects were selected from patients undergoing upper endoscopy at the gastrointestinal endoscopy center of Uijeongbu St. Mary's Hospital as part of a medical checkup or because of upper abdominal discomfort between May 2005 and May 2006. All patients with endoscopically confirmed PGS and erosive esophagitis (EE) were considered for inclusion in the study. Written informed consent for participation in the study was obtained from all patients. We prospectively enrolled 47 patients in two groups. The inclusion criteria for the PGS (n = 26) group were endoscopically confirmed prolapse of inflamed gastric mucosa into the esophageal lumen during retching and symptoms of recurrent retching and nausea over 3 mo. Patients were excluded if they had endoscopically confirmed esophagitis or hiatus hernia, or were alcohol or drug abusers, pregnant, or obese (BMI > 25). The inclusion criterion for the EE group (n = 21) was evidence of grade A or B (Los Angeles Classification) esophagitis at endoscopy. There was no specific exclusion criterion for the EE group. Endoscopic procedures were performed with videoendoscopes (GIF-Q260, GIF-XQ260, Olympus Optical Co. Ltd, Tokyo, Japan, EG-450WR5, Fujinon Corporation, Saitama, Japan).

48-hour esophageal pH measurement

All patients were studied after discontinuation of histamine-receptor antagonists, proton pump inhibitors, and prokinetics for at least 7 d. The BRAVO wireless esophageal pH monitoring system (Medtronic, Minneapolis, MN, USA) was used to measure esophageal pH in both groups within one week of gastroscopy. During gastroscopy, all subjects were assessed in order to locate the pH electrode exactly between the squamocolumnar junction and the incisors. According to the manufacturer's instructions, the pH electrode was passed through the mouth and positioned 6 cm above the squamocolumnar junction. In order to maintain this position, a vacuum pump was connected to apply suction to the esophageal wall. Successful capture of the esophageal mucosa was assumed when the vacuum gauge on the pump stabilized at a value of > 510 mmHg for 30 s. Before the pH capsule was inserted, it was calibrated with the receiver in pH buffer solutions of pH 7.01 and pH 1.07 at room temperature. During the 48 h pH-recording period, patients were asked to keep a detailed diary of activity, food intake, symptoms, wake and sleep periods and posture. Upon completion of the study, the pH monitoring tracings were analyzed using PolygramTM NET software (Medtronic, version 4.01). For each patient, the following reflux parameters were determined: percentage of total time at pH < 4; upright time at pH < 4; supine time at pH < 4; the total number of reflux episodes; the number of reflux episodes longer than five minutes; and the mean duration of reflux episodes. A DeMeester score > 14.72 was defined as pathologic acid reflux. If the pH capsule detached within 16 h, it was reinserted and recorded again 2 wk later.

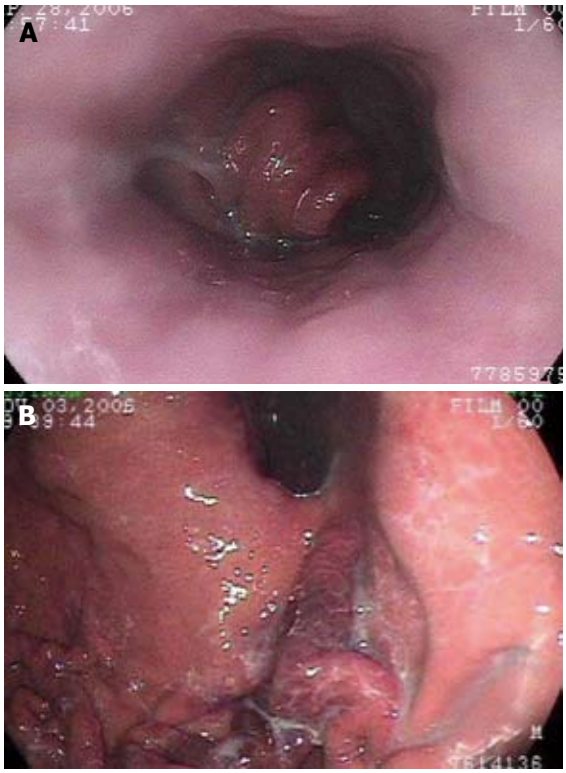


Figure 1 Endoscopic finding of the prolapse gastropathy syndrome group. **A**: Inflamed gastric mucosal prolapse viewed during retching; **B**: Congested and inflamed gastric mucosa secondary to prolapse gastropathy viewed in the gastric fundus.

Statistical analysis

Data presented in this manuscript are expressed as mean ± SD. Comparisons between groups were performed using unpaired Student's t test, Fisher's exact test and chi-square test. A P value < 0.05 was considered as indicating statistical significance. All statistical analyses were performed using SPSS 11.0 for Microsoft Windows (Chicago, IL, USA).

RESULTS

Forty-seven patients were included in the study, 26 patients with PGS and a control group of 21 patients with EE. The patients in the PGS group had a characteristic gastric mucosal prolapse due to retching (Figure 1A) and severe mucosal congestion and localized mucosal inflammation in the fundus (Figure 1B). Among the patients of the EE group, 17 (80%) showed grade A esophagitis and 4 (20%) showed grade B. The two groups were comparable in terms of mean age (41.5 ± 6.7 years for the PGS group and 46.9 ± 11.7 years for the EE group) and gender ratio (male/female, 15/11 and 15/6,

Table 1 Clinical characteristics and reflux parameters of patients in the PGS and the erosive esophagitis (EE) groups

	PGS group (n = 26)	EE group (n = 21)	P value
Age (yr)	41.5 ± 6.7	46.9 ± 11.7	0.08
Gender (male/female)	15/11	15/6	0.33
Mean recording time (h)	41.9 ± 5.9	40.9 ± 6.1	0.57
Number of reflux episodes	77.3 ± 46.4	98.2 ± 56.5	0.17
Number of long reflux episodes (> 5 min)	5.6 ± 4.0	8.5 ± 6.8	0.1
Duration of longest reflux episode (min)	19.6 ± 13.6	24.3 ± 15.7	0.28
Time at pH < 4 (min)	123.2 ± 93.6	173.7 ± 105.4	0.09
Total time of pH < 4 (%)	5.1 ± 4.5	6.9 ± 3.9	0.16
Erect time of pH < 4 (%)	5.6 ± 4.8	7.1 ± 4.2	0.08
Supine time of pH < 4 (%)	4.3 ± 3.2	5.0 ± 4.1	0.09
DeMeester score	15.9 ± 12.5	20.7 ± 10.8	0.17
Early detachment of Bravo wireless capsule	2 (7.7%)	0 (0%)	0.49

respectively) ($P > 0.05$). Data from pH recording of the two groups are shown in Table 1. The mean recording time for the two groups was 41.9 ± 6.7 h for the PGS group and 40.9 ± 6.1 h for the EE group ($P > 0.05$). The results of parameters of reflux in the PGS and EE groups showed no statistically significant differences in the percentage of total time at pH < 4 (5.1% ± 4.5% and 6.9% ± 3.9%, respectively), the total number of reflux episodes (77.3 ± 46.4 and 98.2 ± 56.5, respectively), the number of reflux episodes longer than five minutes (5.6 ± 4.0 and 8.5 ± 6.8), the duration of the longest reflux (19.6 ± 13.6 min and 24.3 ± 15.7 min), total duration of time with pH < 4 (123.2 ± 93.6 min and 173.7 ± 105.4 min), or DeMeester score (15.9 ± 12.5 and 20.7 ± 10.8). As far as the incidence of pathologic acid reflux (based on DeMeester score > 14.72) is concerned, there was no significant difference between the PGS group (14/26 or 53.8%) and the EE group (16/21 or 76.2%) ($P > 0.05$). Three patients developed complications related to the pH recording procedure. One patient in the PGS group experienced severe epigastric pain 48 h after placement of the pH capsule that resolved spontaneously. In two patients (7.7%) of the PGS group, early detachment of the pH capsule occurred as identified by a persistent decrease of pH to close to 2 followed by a rise above pH 7 after passage through the pylorus. For these patients, capsules were reinserted 2 wk later and pH recording was successful over 40 h.

DISCUSSION

Retrograde prolapse of an area of gastric mucosal from the proximal stomach into the distal esophagus can occur during retching or vomiting and can result in prolapse gastropathy and bleeding. In 1984, Shepherd[1] proposed the term "prolapse gastropathy syndrome" and reported 22 patients who presented with epigastric pain alone or with hematemesis associated with a previous history of recurrent early morning retching or postprandial retching. He defined the characteristic endoscopic finding in these patients as a knuckle of inflamed and sometimes bleeding gastric mucosa in the proximal stomach several centimeters distal to the gastroesophageal junction that repeatedly prolapsed into the esophageal lumen during retching[2,10,11]. The incidence of PGS has been reported to be 0.4%-2.4% in the general population, and it has been described in almost 2% of adults who undergo endoscopy for evaluation of hematemesis[1,12,13]. The mechanism of PGS is obscure although various factors such as relaxation of the esophagogastric junction, excessive redundancy of the gastric mucosa, retrograde peristalsis of the stomach and repeated regurgitation and vomiting have been suggested[7,14]. In this study, we investigated whether acid reflux into the esophagus may occur in PGS patients, because it is likely that regurgitation of acid accompanies the prolapsed mucosa. PGS patients were selected according to Shepherd's diagnostic criteria[1]. Patients with endoscopically confirmed esophagitis or hiatus hernia, who were alcohol or drug abusers, pregnant women, or obese (BMI > 25), were excluded to eliminate other factors that may contribute to pathologic acid reflux. In addition, a wireless ambulatory pH monitoring device was used rather than a traditional catheter-based esophageal pH monitoring system, because more throat and swallow discomfort, which might induce retching and pathologic acid reflux in PGS patients, has been reported with the catheter type than with the wireless monitoring device[15,16]. EE patients were chosen as a positive control for acid reflux because nonerosive gastroesophageal reflux disease (NERD) is often not related to pathologic acid reflux[17-20].

Although, based on the DeMeester score, the positive rate of pathologic acid reflux in the EE group was higher than in the PGS group (76.2% *vs* 53.8%), our data show a meaningful rate of pathologic reflux in PGS patients, based also on the fact that we excluded other possible factors leading to reflux. In previous reports, hiatus hernia, which has been regarded as predictor of gastroesophageal reflux disease (GERD)[21,22] and NERD, revealed incidences of acid reflux of 26%-57%[23,24] and 40%-63%[25,26], respectively. By comparison with these previous reports, the incidence of pathologic acid reflux in the PGS group is substantial.

With respect to complications associated with wireless pH recording, epigastric pain developed in one PGS patient 48 h after placement of the pH capsule that spontaneously resolved, and might be associated

with esophageal ulceration after placement of the pH capsule[27]. In addition, the pH capsule detached early in two patients of PGS group (7.7%, 2/26) but there was no case of detachment in the EE group. The detachment rate of the pH capsule in the PGS group was higher than in previous studies [3.2% (7/217), 3.7% (3/80)][28,29]. The early detachment of the pH capsule may result from the prolapsed mucosa striking the attached pH capsule, meaning that an increased possibility of early detachment should be considered in PGS. However, further studies are necessary to confirm this.

Until now, there has been no report of the long-term prognosis for PGS patients, and it is not known whether long-lasting exposure of the esophagus to acid in PGS would progress to EE, esophageal stricture, Barrett's esophagus or other complications of GERD. Moreover, it is unknown whether a proton pump inhibitor or other medication is necessary to inhibit acid reflux in PGS patients. However there have been reports of a relationship between prolonged acid exposure and the degree of esophageal injury[30,31], while recent cohort studies with prolonged follow-up showed progression from NERD to EE and from uncomplicated to complicated GERD[32,33]. We therefore recommend future studies to observe disease progression and response to medication in PGS over a long duration.

In conclusion, there was no statistically significant difference between PGS and EE groups in reflux parameters and presence of pathologic acid reflux. By comparison with previous reports of acid reflux in hiatus hernia and NERD, the incidence of pathologic acid reflux in the PGS group is substantial. This result suggests that PGS might be a predictor of pathologic acid reflux.

COMMENTS

Background

Prolapse gastropathy syndrome (PGS) occurs in patients with prolonged retching and vomiting. This disease is a clinical syndrome involving the invagination of part of the gastric mucosa into the lower esophagus, resulting in well demarcated hemorrhagic mucosa and severe mucosal congestion. Excessive gastric redundancy and prolapse of the mucosa may contribute to extended acid reflux of the esophagus. We aimed at investigating the acid reflux parameters in PGS compared with endoscopically confirmed erosive esophagitis.

Research frontiers

There have been several studies and case reports about PGS which are focused on upper gastrointestinal bleeding.

Innovations and breakthroughs

It seems to be that gastric acid might be regurgitated into the esophagus along with prolapsed gastric mucosa. However, there has been no study of acid reflux associated with PGS. This study is the first one designed to investigate the presence of pathologic acid reflux in PGS using the BRAVO wireless esophageal pH monitoring system to prevent acid reflux caused by pH monitoring probe of conventional method.

Applications

Pathologic acid reflux in PGS may contribute to patient symptoms and progression to erosive esophagitis. Based on this study, the effectiveness of proton pump inhibitors in PGS will be considerable.

Peer review

This is the first report designed to investigate the presence of pathologic acid reflux in PGS. It concludes that the parameters of pathologic acid reflux in PGS are not different from those of erosive esophagitis. This result suggests that PGS may be a predictor of pathologic acid reflux.

REFERENCES

1 **Shepherd HA**, Harvey J, Jackson A, Colin-Jones DG. Recurrent retching with gastric mucosal prolapse. A proposed prolapse gastropathy syndrome. *Dig Dis Sci* 1984; **29**: 121-128
2 **Byfield F**, Ligresti R, Green PH, Finegold J, Garcia-Carrasquillo RJ. Hematemesis due to prolapse gastropathy: an emetogenic injury. *Gastrointest Endosc* 1998; **48**: 527-529
3 **Bishop PR**, Nowicki MJ, Parker PH. Vomiting-induced hematemesis in children: Mallory-Weiss tear or prolapse gastropathy? *J Pediatr Gastroenterol Nutr* 2000; **30**: 436-441
4 **Pohl JF**, Melin-Aldana H, Rudolph C. Prolapse gastropathy in the pediatric patient. *J Pediatr Gastroenterol Nutr* 2000; **30**: 458-460
5 **Thomas E**, Khatak KG. Hemorrhage due to retrograde prolapse of stomach. An endoscopic diagnosis. *Am J Gastroenterol* 1979; **71**: 477-480
6 **Axon AT**, Clarke A. Haematemesis: a new syndrome? *Br Med J* 1975; **1**: 491-492
7 **Rudnick JP**, Ferrucci JT Jr, Eaton SB Jr, Dreyfuss JR. Esophageal pseudotomor: retrograde prolapse of gastric mucosa into the esophagus. *Am J Roentgenol Radium Ther Nucl Med* 1972; **115**: 253-256
8 **Palmer ED**. Mucosal prolapse at the esophagogastric junction. *Am J Gastroenterol* 1955; **23**: 530-537
9 **Miller G**, Savary M, Gloor F. [Gastro-esophageal prolapse as a cause of mucosal changes in the gastric fundus and esophagus. Bleeding hiatus hernia, Mallory-Weiss syndrome, esophageal rupture] *Dtsch Med Wochenschr* 1974; **99**: 553-556
10 **Blum SD**, Weiss A, Weiselberg HM, Siegel WB. Retrograde prolapse of gastric mucosa into the esophagus. *Gastroenterology* 1961; **41**: 408-411
11 **Young GP**, Thomas RJ, Wall AJ. Retrograde gastric mucosal prolapse as a cause of haematemesis. *Med J Aust* 1976; **2**: 488-489
12 **Yoruk G**, Aksoz K, Buyrac Z, Unsal B, Yildiz C. Is prolapse gastropathy a cause of upper gastrointestinal bleeding? *Turk J Gastroenterol* 2003; **14**: 106-110
13 **Kuwano H**, Morita M, Mori M, Matsuura H, Sugimachi K, Haraguchi Y. Endoscopic evidence of gastric mucosal prolapse with retching and vomiting. *Gastrointest Endosc* 1991; **37**: 585-586
14 **Feldman M**. Retrograde extrusion or prolapse of the gastric mucosa into the esophagus. *Am J Med Sci* 1951; **222**: 54-60
15 **Pandolfino JE**, Richter JE, Ours T, Guardino JM, Chapman J, Kahrilas PJ. Ambulatory esophageal pH monitoring using a wireless system. *Am J Gastroenterol* 2003; **98**: 740-749
16 **des Varannes SB**, Mion F, Ducrotte P, Zerbib F, Denis P, Ponchon T, Thibault R, Galmiche JP. Simultaneous recordings of oesophageal acid exposure with conventional pH monitoring and a wireless system (Bravo). *Gut* 2005; **54**: 1682-1686
17 **Martinez SD**, Malagon IB, Garewal HS, Cui H, Fass R. Non-erosive reflux disease (NERD)--acid reflux and symptom patterns. *Aliment Pharmacol Ther* 2003; **17**: 537-545
18 **Trimble KC**, Pryde A, Heading RC. Lowered oesophageal sensory thresholds in patients with symptomatic but not excess gastro-oesophageal reflux: evidence for a spectrum of visceral sensitivity in GORD. *Gut* 1995; **37**: 7-12
19 **Rodriguez-Stanley S**, Robinson M, Earnest DL, Greenwood-Van Meerveld B, Miner PB Jr. Esophageal hypersensitivity may be a major cause of heartburn. *Am J Gastroenterol* 1999; **94**: 628-631
20 **Tobey NA**, Carson JL, Alkiek RA, Orlando RC. Dilated intercellular spaces: a morphological feature of acid reflux-

-damaged human esophageal epithelium. *Gastroenterology* 1996; **111**: 1200-1205
21 **Jones MP**, Sloan SS, Rabine JC, Ebert CC, Huang CF, Kahrilas PJ. Hiatal hernia size is the dominant determinant of esophagitis presence and severity in gastroesophageal reflux disease. *Am J Gastroenterol* 2001; **96**: 1711-1717
22 **Campos GM**, Peters JH, DeMeester TR, Oberg S, Crookes PF, Mason RJ. The pattern of esophageal acid exposure in gastroesophageal reflux disease influences the severity of the disease. *Arch Surg* 1999; **134**: 882-887; discussion 887-888
23 **Johnsson F**, Joelsson B, Gudmundsson K, Greiff L. Symptoms and endoscopic findings in the diagnosis of gastroesophageal reflux disease. *Scand J Gastroenterol* 1987; **22**: 714-718
24 **Ott DJ**, Glauser SJ, Ledbetter MS, Chen MY, Koufman JA, Gelfand DW. Association of hiatal hernia and gastroesophageal reflux: correlation between presence and size of hiatal hernia and 24-hour pH monitoring of the esophagus. *AJR Am J Roentgenol* 1995; **165**: 557-559
25 **Lind T**, Havelund T, Carlsson R, Anker-Hansen O, Glise H, Hernqvist H, Junghard O, Lauritsen K, Lundell L, Pedersen SA, Stubberod A. Heartburn without oesophagitis: efficacy of omeprazole therapy and features determining therapeutic response. *Scand J Gastroenterol* 1997; **32**: 974-979
26 **Shi G**, Bruley des Varannes S, Scarpignato C, Le Rhun M, Galmiche JP. Reflux related symptoms in patients with normal oesophageal exposure to acid. *Gut* 1995; **37**: 457-464
27 **Wells CD**, Heigh RI, Burdick GE, Moirano MM, Fleischer DE. Symptomatic esophageal ulceration caused by a Bravo wireless pH probe and subsequent endoscopic removal of the probe using a retrieval net. *Endoscopy* 2006; **38** Suppl 2: E97
28 **Bhat YM**, McGrath KM, Bielefeldt K. Wireless esophageal pH monitoring: new technique means new questions. *J Clin Gastroenterol* 2006; **40**: 116-121
29 **Remes-Troche JM**, Ibarra-Palomino J, Carmona-anchez RI, Valdovinos MA. Performance, tolerability, and symptoms related to prolonged pH monitoring using the Bravo system in Mexico. *Am J Gastroenterol* 2005; **100**: 2382-2386
30 **Bremner RM**, Crookes PF, DeMeester TR, Peters JH, Stein HJ. Concentration of refluxed acid and esophageal mucosal injury. *Am J Surg* 1992; **164**: 522-526; discussion 526-527
31 **Vaezi MF**, Richter JE. Role of acid and duodenogastroesophageal reflux in gastroesophageal reflux disease. *Gastroenterology* 1996; **111**: 1192-1199
32 **Pace F**, Bollani S, Molteni P, Bianchi Porro G. Natural history of gastro-oesophageal reflux disease without oesophagitis (NERD)--a reappraisal 10 years on. *Dig Liver Dis* 2004; **36**: 111-115
33 **Ruigomez A**, Garcia Rodriguez LA, Wallander MA, Johansson S, Graffner H, Dent J. Natural history of gastro-oesophageal reflux disease diagnosed in general practice. *Aliment Pharmacol Ther* 2004; **20**: 751-760

S- Editor Zhong XY **L- Editor** Negro F **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5606

World J Gastroenterol 2008 September 28; 14(36): 5606-5611
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Tumor suppress genes screening analysis on 4q in sporadic colorectal carcinoma

Li-Xin Jiang, Jie Xu, Zhao-Wen Wang, Da-Peng Li, Zhi-Hai Peng, Jian-Jun Gao, Lin He, Hai-Tao Zheng

Li-Xin Jiang, Jie Xu, Hai-Tao Zheng, Department of Abdomen Surgery Department, the Affiliated Yantai Yuhuangding Hospital of Qingdao University Medical College, Yantai 264000, Shandong Province, China
Zhao-Wen Wang, Da-Peng Li, Zhi-Hai Peng, Department of General Surgery, the Affiliated Shanghai First People Hospital of Shanghai Jiaotong University, Shanghai 200080, China
Jian-Jun Gao, Lin He, Shanghai Bio-x Center, Shanghai Jiaotong University, Shanghai 200031, China
Author contributions: He L, Peng ZH designed research; Jiang LX, Xu J, Zheng HT performed research; Gao JJ contributed new reagents; Wang ZW and Li DP analyzed data; and Jiang LX, Zheng HT wrote the paper.
Supported by The National Natural Science Foundation of China, No. 30080016 and No. 30470977
Correspondence to: Hai-Tao Zheng, Department of Abdomen Surgery Department, the Affiliated Yantai Yuhuangding Hospital of Qingdao University Medical College, Yantai 264000, Shangdong Province, China. zhenghaitao1972@126.com
Telephone: +86-535-6691999-81707 Fax: +86-535-6691999
Received: July 20, 2008 Revised: August 26, 2008
Accepted: September 3, 2008
Published online: September 28, 2008

Abstract

AIM: To search candidate tumor suppressor genes (TSGs) on chromosome 4q through detecting high loss of heterozygosity (LOH) regions in sporadic colorectal carcinoma in Chinese patients.

METHODS: Thirteen fluorescent labeled polymorphic microsatellite markers were analyzed in 83 cases of colorectal carcinoma and matched normal tissue DNA by polymerase chain reaction (PCR). PCR products were electrophoresed on an ABI 377 DNA sequencer. Genescan 3.7 and Genotype 3.7 software were used for LOH scanning and analysis. Comparison between LOH frequency and clinicopathological factors were performed by χ^2 test.

RESULTS: Data were collected on all informative loci. The average LOH frequency on 4q was 28.56%. The D4S2915 locus showed highest LOH frequency (36.17%). Two obvious deletion regions were detected: one between D4S3000 and D4S2915 locus (4q12-21.1), another flanked by D4S407 and D4S2939 locus (4q25-31.1). None case showed complete deletion of 4q, most cases displayed interstitial deletion pattern solely. Furthermore, compared with clinicopathological features, a significant relationship was observed between LOH frequencies on D4S3018 locus. In tumors larger than 5 cm in diameter, LOH frequency was significantly higher than tumors that were less than 5 cm (56% *vs* 13.79%, $P = 0.01$). On D4S1534 locus, LOH was significantly associated with liver metastasis (80% *vs* 17.25%, $P = 0.012$). No relationship was detected on other locus compared with clinicopathological features.

CONCLUSION: By high resolution deletion mapping, two high frequency regions of LOH (4q12-21.1 and 4q25-31.1) were detected, which may contribute to locate TSGs on chromosome 4q involved in carcinogenesis and progression of sporadic colorectal carcinoma.

Key words: Loss of heterozygosity; Colorectal carcinoma; Chromosome; 4q; Tumor suppressor gene

Peer reviewer: Yoshiharu Motoo, MD, PhD, FACP, FACG, Professor and Chairman, Department of Medical Oncology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan

Jiang LX, Xu J, Wang ZW, Li DP, Peng ZH, Gao JJ, He L, Zheng HT. Tumor suppress genes screening analysis on 4q in sporadic colorectal carcinoma. *World J Gastroenterol* 2008; 14(36): 5606-5611 Available from: URL: http://www.wjgnet.com/1007-9327/14/5606.asp DOI: http://dx.doi.org/10.3748/wjg.14.5606

INTRODUCTION

Colorectal Cancer (CRC) is one of the most common malignant tumors threatening people's health[1,2]. After lung cancer in men and breast cancer in women, it is the most common cause of cancer related death. Improvement in its prognosis will not be achieved without a better understanding of its etiology and tumor molecular biology. In recent years, the genetic basis of human tumors has been increasingly elucidated. As a modal for both multistep and multipathway carcinogenesis, colorectal neoplastic progression provides paradigms of both oncogenes and tumor suppressor gene (TSG) in epithelial tumors[3,4]. The latter changes predominate. In addition to the allelic loss noted on chromosome 5q, 17p and 18q, many other chromosome losses can be observed in colorectal

carcinoma. Regions from chromosome 1q, 4p, 6p, 6q, 8p, 9p and 22q were lost in 25%-50% of the cases studied[3].

Chromosome losses in colorectal tumor were first detected by cytogenety, later, by probes of restriction fragment length polymorphisms (RFLPs), and now by loss of heterozygosity (LOH) in analyzing allelic loss so far. The loss of TSGs is believed to be one of the key steps to carcinogenesis of CRC[5]. The loss of one allelic at a specific locus is caused by deletion mutation or loss of a chromosome from a chromosome pair[6]. When this occurs at a TSG locus where one of the allelic is already abnormal, it can result in neoplastic transformation. The LOH analysis, based on polymorphic microsatellite DNA, has currently become an effective and powerful tool currently to find informative loci and then to find candidate TSGs[7,8].

Many studies reported frequent loss on 4q in couples of carcinoma. But much less studies has been done on 4q in colorectal carcinoma. In this study, we performed LOH analysis using high resolution microsatellite markers on chromosome 4q in 83 sporadic colorectal carcinoma cases, in an effort to identify additional TSGs involved in colorectal tumorigenesis.

MATERIALS AND METHODS

Patient sample and DNA extraction

This study was based on eighty-three consecutively collected tumors, including 40 males and 43 females, from unrelated patients with CRC, treated at the Surgical Department in Shanghai First People's Hospital, China. The patients' ages ranged from 31 to 84 years with a median of 66. The cancerous tissue and adjacent normal tissue were fresh frozen. These tissues were cut into cubes of approximately 2 mm^3 and immediately frozen in liquid nitrogen. DNA was extracted using standard methods with proteinase K digestion and phenol/chloroform purification[9]. All patients were confirmed by pathology, and were staged by Duke's criterion. Each patient gave his or her informed consent for the use of his or her tissue in this study.

Microsatellite markers and polymerase chain reaction (PCR)

Initially, 83 cases of CRC were analyzed by PCR using thirteen microsatellite markers which map to 4q. DNA samples were analyzed as tumor and matched normal pairs using primers of the following microsatellite loci (hereditary location): centromere--D4S3000 (4q12)--D4S3018 (4q13.2)--D4S2915 (4q21.1)--D4S1534 (4q21.23)--D4S2986 (4q23)--D4S407 (4q28.1)--D4S430 (4q28.1)--D4S2939 (4q31.1)--D4S3046 (4q32.2)--D4S2979 (4q32.3)--D4S415 (4q34.3)--D4S1535 (4q35.1)-qter (Figure 1). The average hereditary distance from genothon map is 10.4cM (centi-Morgan)[10].

LOH analysis

A portion of each PCR product (0.5 μL) was combined with 0.1 μL of Genescan 500 size standard (PE Applied Biosystems Foster city CA, USA) and 0.9 μL of formamide loading buffer. After denaturation at 96℃ for 5 min, products were electrophoresed on 5% polyacrylamide gels on an ABI 377 DNA sequencer (PE Applied Biosystems Foster city CA, USA) for 3 h. Genotype 3.7 software displays individual gel lanes as electropherograms with a given size, height, and area for each detected fluorescent peak. Stringent criteria were used to score the samples. Alleles were defined as the two highest peaks within the expected size range. A ratio of T1:T2/N1:N2 of less than 0.67 or greater than 1.50 was scored as a LOH (Figure 2). Most amplification of normal DNA produced two PCR products indicating heterozygosity. A single fragment amplified from normal DNA (homozygosity) and those PCR reactions in which fragments were not clearly amplified were scored as not informative. The LOH frequency of a locus is equal to the ratio of the number between allelic loss and informative cases. The average LOH frequency of chromosome 4q is the average value of each locus LOH frequency.

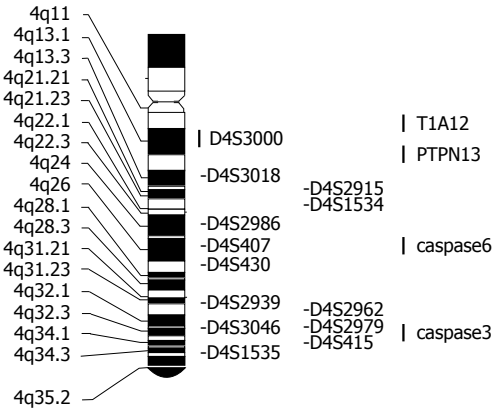


Figure 1 Microsatellite markers on chromosome 4q and putative TSGs.

Statistical analysis

Date was statistically analyzed using the SPSS software package, version 11.5 (SPSS Inc). Comparisons between LOH and clinicopathological data were performed by χ^2 test. $P < 0.05$ was considered as statistically significant.

RESULTS

LOH analysis of CRC on 4q

Eighty-three CRCs were analyzed for LOH at thirteen marker loci spanning chromosome 4q. All loci got informative messenger (Table 1). Tumors exhibiting wide microsatellite instability were excluded from deletion mapping. The average LOH frequency on 4q was 28.56%. Seventy-two samples (86.75%) showed LOH event on at least one locus. The D4S2915 locus showed highest LOH frequency (36.17%). Two distinct region of frequent allelic loss were observed on chromosome 4q, one between D4S3000 and D4S2915 locus (4q12-21.1), another flanked by D4S407 and D4S2939 locus (4q25-31.1). None case showed complete deletion of 4q,

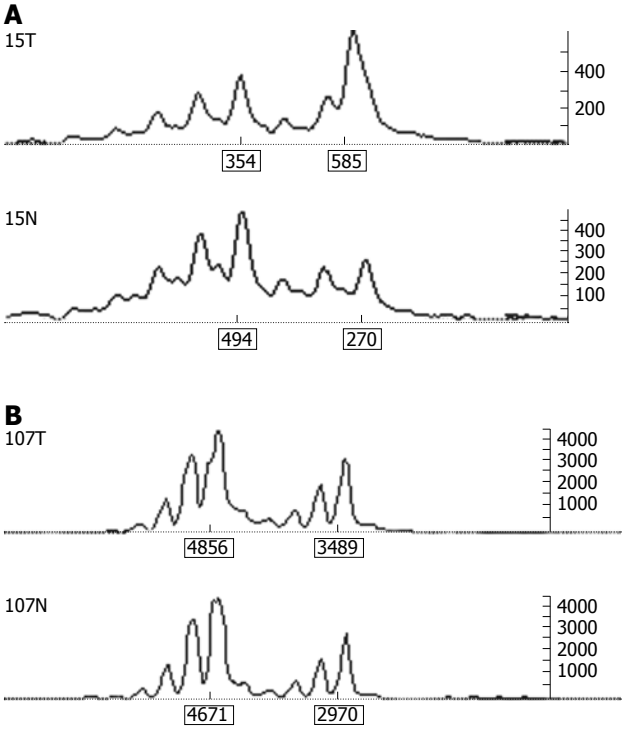


Figure 2 LOH demonstrations. **A**: The typical peak of LOH: Allele ratio = (T1/T2)/(N1/N2) = (354/585)/(494/270) = 0.33 < 0.67; **B**: The normal peak: Allele ratio = (T1/T2)/(N1/N2) = (4856/3489)/(4671/2970) = 0.67 < 0.89 < 1.5. T: Tumor; N: Normal.

most cases displayed interstitial deletion pattern solely. It can be speculated that putative TSGs may locate on 4q12-21.1 and 4q25-31.1.

Relationship between clinicopathological features and LOH on 4q

Compared with clinicopathological features, by statistics, a significant relationship was observed between LOH frequency on D4S3018 and tumor diameter. LOH frequency in tumors larger than 5 cm in diameter (56%, 14/25) was significantly higher than tumors that were less than 5 cm in diameter (13.79%, 4/29; $P = 0.01$). On 4S1534 locus, LOH frequency was 80% (4/5) in cases with liver metastasis, which was significantly higher than that without liver metastasis (17.25%, 5/29) ($P = 0.012$). No relationship was detected on other locus compared with these clinicopathological features.

DISCUSSION

Inactivation of TSGs appears to be one of the genetic mechanisms involved in the development of CRC[3,11,12]. Deletion of TSGs occurs frequently in human malignances. Such events can be detected using markers from the region of genomes that include a TSG. Allelic loss detected as LOH have been proved useful for mapping regions of DNA that contain TSGs. LOH at specific chromosomal regions strongly suggests the existence of TSGs at the relevant segment.

Studies conducted thus for have focused on known TSG, particularly, APC, p53, DCC, MCC, Smad4 and so on. The genetic events that led to the development and progression of CRC on chromosome 4q have not been elucidated. There were a lot of evidence that multiple genetic abnormalities on 4q contribute to development of a serials of tumors, and that supported the presence of TSGs in the long arm of chromosome 4.

Table 1 LOH frequency statistics result on 4q

Marker	Location	LOH case	Normal case	Informative case	Distance (cm)	LOH rate
D4S3000	4q12	22	42	78.05	-	34.38
D4S3018	4q13.2	18	36	65.06	9.7	33.33
D4S2915	4q21.1	17	30	56.63	10.6	36.17
D4S1534	4q21.23	9	25	40.96	7.5	26.47
D4S2986	4q23	7	39	55.42	10.6	15.22
D4S407	4q25	17	35	62.56	10.7	32.69
D4S430	4q28.1	15	35	60.24	11.1	30
D4S2939	4q31.1	21	38	71.08	14.4	35.59
D4S2962	4q31.23	16	43	71.08	12.1	27.12
D4S3046	4q32.2	16	39	66.27	13.3	29.09
D4S2979	4q32.3	7	25	36.78	12.6	21.87
D4S415	4q34.3	14	36	60.24	6.6	28
D4S1535	4q35.1	14	45	71.08	14	23.73

Loss of chromosome 4q has been found in the majority of breast carcinoma[13], cervical cancer (4q21-q25)[14]. In oral squamous cell carcinoma[15] and relapsed childhood acute lymphoblastic leukemia[16], 75% and 20% cases showed LOH on at least one of the loci, with frequent loss region centered on 4q25 in the former. In esophageal adenocarcinoma, Hammoud *et al*[17] found LOH frequency was 54.5%-65% on 4q21-35 with a lower resolution allelotyping studying. Rumpel *et al*[18] found LOH was 80% on at least one marker on 4q, and discerned three non-overlapping areas (4q21.1-22, 4q32-33, 4q35), whose total spanning length is quite coincident with Hammoud[17]. Hurst *et al*[19] found that in bladder cancer, 4q loss was the most frequent loss (83%) in all arms of chromosome by microarry-based comparative genome hybridization, with the minimal region of deletion at 4q13.1 and 4q31.3. This result was compatible with previous report of LOH analysis in bladder tumors[20,21]. In malignant mesothelioma and small cell lung cancer, two non-overlapping loss region on 4q were at 4q33-34 (> 80%) and 4q25-26 (> 60%)[22]. In primary small cell lung cancer, Cho *et al*[23] defined five commonly deleted region all more than 50% on 4q, namely, 4q24, 4q27-28.3, 4q33, 4q34.1 and 4q34.3-35.2. In the research of hepatocellular carcinoma, much work has been done. Piao *et al*[24] made a detailed deletion mapping and identified seven independent, frequently lost regions. In fact, a high frequency of LOH on chromosome 4q in hepatocellular carcinoma has been reported by several study groups[25-27]. Recent years, LOH frequency was found 33.3% (at 4q26-27)[28], 50%[29], and 38%[30] for at least two adjacent markers in hepatocellular carcinoma. These study results strongly suggested that 4q may locate more than one putative TSG.

The research of LOH on 4q in colorectal carcinoma was much less in this decade. LOH at telomere markers have been reported less than 10% on 4q[31]. Analysis of

mid-position microsatellite markers on 4q arm showed moderate of allelic imbalances (< 25%) in colorectal tumors[32]. Comparative genomic hybridization analysis of a serial of colorectal adenomas and carcinomas identified chromosome 4 losses as of the recurrent alterations observed in malignant stages[33]. Arribas *et al*[34] found frequent loss on 4q21-28 in CRC by using AP-PCR method. However, detailed mapping of the long arm of chromosome 4 has not been reported in colorectal carcinoma thus far.

With high resolution markers, we performed detailed LOH analysis and observed two obvious deletion regions: one between D4S3000 and D4S2915 locus (4q12-21.1), another flanked by D4S407 and D4S2939 locus (4q25-31.1). The first region included 4q12-21.1, 4q13.1[19], furthermore, the region was adjacent with 4q21-25[14], 4q21-35[17], 4q21.1-22[18], 4q33-34[22] detected before. The second region was adjacent with 4q21-25[14], 4q25[15], 4q31.3[19], covered by 4q21-35[17], and completely spanned 4q32-33[18], 4q25-26[22], 4q27-28.3, 4q33[23], 4q26-27[28], partially overlapped with 4q21-28[34], 4q24-26[35] as well. Because of different LOH judge criteria and different density markers used, the minimal deletion region in different tumors displayed discrepancy.

LOH on chromosome 4q seems to be correlated with aggression features and a late genetic event involved with the progression rather than the initiation of cancer. In hepatocellular carcinoma, a study by Okabe *et al*[36] showed that the high rate of LOH on 4q are associated with a poor differentiation of tumors, vascular invasion and intrahepatic metastasis in hepatocellular carcinoma. A similar finding was made by Konishi *et al*[37], LOH was found in 71% of poorly differentiated hepatocellular carcinoma. Rashid *et al*[29] found 4q was preferentially lost in hepatocellular carcinoma containing p53 mutation, that is, LOH of 4q was present in 75% of hepatocellular carcinoma with, but only 25% of hepatocellular carcinoma without a p53 gene mutation ($P = 0.01$), indicating a possible interaction between *p53* gene mutation and 4q loss in the pathogenesis of hepatocellular carcinoma. Loss of 4q34-35 regions showed a significant association with alcohol intake ($P = 0.05$) and with high grade of differentiation ($P = 0.02$) in hepatocellular carcinoma[30]. Investigators[22] thought TSG on 4q play an important role in pathogenesis of malignant mesothelioma and small cell lung cancer, because non-small cell lung cancer had lower frequency at this region. Similarly, the result of primary bladder cancer correlated allelic loss with advanced tumor stage and grade of the lesions[14]. Piao *et al*[24] found five regions among seven common deleted regions was associated with tumor differentiation, suggesting that allelic loss on chromosome 4q is a late genetic event involved in the progression rather than in the initiation of hepatocarcinogenesis. Pershouse *et al*[35] reported that the allelic deletion involving 4q may represent an early event in head and neck squamous cell carcinoma oncogenesis.

A significant relationship was detected between LOH frequency on D4S3018 and tumor diameter. LOH frequency in tumors larger than 5 cm was significantly higher than tumors less than 5 cm in diameter ($P = 0.01$). On D4S1534 locus, LOH rate was significantly associated with liver metastasis ($P = 0.012$). It can be speculated that these events were associated with progression after tumorigenesis, indicating a late event in colorectal carcinoma.

No statistical significant correlation was found between the presence of LOH and clinicopathological data in oral squamous cell cancer[15], esophageal adenocarcinoma[17] and in Taiwanese hepatocellular carcinoma[28].

By scanning GeneMap' 99 database, and searching www.gdb.org, four genes should be studied further. PTPN13 (4q21.3) is a Fas-associated protein, tyrosine phosphate that binds to a negative regulatory domain in the FAS protein and inhibits Fas-induced apoptosis[38]. Caspase3 (4q34) and caspase6 (4q24-35), mammalian homologues of Ced-3 gene, are responsible for cleavage and inactivation of key homeostatic protein during apoptosis[39]. T1A12/mac25 (4q12-13) expression is abrogated during breast cancer progression concomitant with LOH on chromosome 4q, indicating its tumor suppressor function[40]. All of these three genes are putative TSGs.

In summary, this is the first detailed LOH analysis on chromosome 4q in CRC by using high resolution microsatellite markers. We detected two non-overlapping deletion region (4q12-21.1 and 4q25-31.1), which may contain TSGs. Additional attempts will aide in the discovery of the genes inactivated by these deletion events. The elucidation of the biochemical property of these gene products will be an important step in understanding the biology of this highly lethal and common cancer.

COMMENTS

Background

Cancer arises from the accumulation of inherited polymorphism (i.e. SNPs) and mutation and/or sporadic somatic polymorphism (i.e. non-germline polymorphism) in cell cycle, DNA repair, and growth signaling genes. Neoplastic progression is generally characterized by the accumulation of multiple somatic-cell genetic alterations as the tumor progresses to advanced stages. The classic mechanism of tumor suppressor gene (TSG) inactivation is described by two-hit modes in which one allele is mutated (or promoter hypermethylation or a small intragenic deletion) and the other allele is lost through a number of possible mechanisms, resulting in loss of heterozygosity (LOH) at multiple loci. LOH is the most common molecular genetic alteration observed in human cancers. In the model of colorectal tumorigenesis, mutational inactivation of TSGs predominates.

Research frontiers

Most genome-wide scans for LOH have been conducted at low resolution with a relatively small number of polymorphic markers. For example, an average of 120 microsatellites have been used to determine the allelotype of multiple different human neoplasms in a series of studies since 1995, and the highest density microsatellite allelotype was approximately 280 polymorphic markers before the year 2000. SNPs are the most common form of sequence variation in human genome, occurring approximately every 1200 bp. High density mapping of genetic losses reveals potential tumor suppressor loci and might be useful for clinical classification of individual tumors. SNP array has been introduced recently for genome-wide screening of chromosome imbalance. Higher density SNP array can effectively detect small regions of chromosomal changes and provide more information regarding the boundaries of loss regions.

Innovations and breakthroughs

A great deal of evidence supported the presence of TSGs in the short arm of chromosome 4. Fewer studies have been reported in colorectal cancer. Previous allelotyping analysis of cancer by many groups was used with a relatively low density of markers. By deletion dense markers mapping, authors detected two obvious high frequency LOH regions spanning D4S3013 and D4S405 locus in colorectal cancer (CRC). Candidate TSG might be located between D4S3017 and D4S2933 (about 1.7 cm).

Applications

This method could be used to detect some major allelic loss regions in genome-wide scans of LOH in patients with CRC.

Terminology

LOH is caused by a variety of genetic mechanisms, including physical deletion of chromosome non-disjunction and mitotic non-disjunction followed by republication of the remaining chromosomes, mitotic recombination and gene conversion. The mechanisms of LOH are remarkably chromosome-specific. Some chromosomes display a complete loss. However, more than half of the losses are associated with only a partial loss of a chromosome rather than a whole chromosome. LOH is also a common form of allelic imbalance and the detection of LOH has been used to identify genomic regions that harbor TSGs and to characterize different tumor types, pathological stages and progression.

Peer review

This is a report that describes the LOH events on 4q in sporadic CRC in Chinese patients, further studies will benefit from this paper. The data presented is clear and concise in the text.

REFERENCES

1 **Jemal A**, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, Feuer EJ, Thun MJ. Cancer statistics, 2005. *CA Cancer J Clin* 2005; **55**: 10-30

2 **Heitman SJ**, Manns BJ, Hilsden RJ, Fong A, Dean S, Romagnuolo J. Cost-effectiveness of computerized tomographic colonography versus colonoscopy for colorectal cancer screening. *CMAJ* 2005; **173**: 877-881

3 **Fearon ER**, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; **61**: 759-767

4 **Hardy RG**, Meltzer SJ, Jankowski JA. ABC of colorectal cancer. Molecular basis for risk factors. *BMJ* 2000; **321**: 886-889

5 **Kataoka M**, Okabayashi T, Johira H, Nakatani S, Nakashima A, Takeda A, Nishizaki M, Orita K, Tanaka N. Aberration of p53 and DCC in gastric and colorectal cancer. *Oncol Rep* 2000; **7**: 99-103

6 **Lengauer C**, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. *Nature* 1998; **396**: 643-649

7 **Baker SJ**, Fearon ER, Nigro JM, Hamilton SR, Preisinger AC, Jessup JM, vanTuinen P, Ledbetter DH, Barker DF, Nakamura Y, White R, Vogelstein B. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science* 1989; **244**: 217-221

8 **Kinzler KW**, Nilbert MC, Vogelstein B, Bryan TM, Levy DB, Smith KJ, Preisinger AC, Hamilton SR, Hedge P, Markham A. Identification of a gene located at chromosome 5q21 that is mutated in colorectal cancers. *Science* 1991; **251**: 1366-1370

9 **Xu SF**, Peng ZH, Li DP, Qiu GQ, Zhang F. Refinement of heterozygosity loss on chromosome 5p15 in sporadic colorectal cancer. *World J Gastroenterol* 2003; **9**: 1713-1718

10 **Dib C**, Faure S, Fizames C, Samson D, Drouot N, Vignal A, Millasseau P, Marc S, Hazan J, Seboun E, Lathrop M, Gyapay G, Morissette J, Weissenbach J. A comprehensive genetic map of the human genome based on 5,264 microsatellites. *Nature* 1996; **380**: 152-154

11 **Hinoi T**, Akyol A, Theisen BK, Ferguson DO, Greenson JK, Williams BO, Cho KR, Fearon ER. Mouse model of colonic adenoma-carcinoma progression based on somatic Apc inactivation. *Cancer Res* 2007; **67**: 9721-9730

12 **Komarova NL**, Lengauer C, Vogelstein B, Nowak MA. Dynamics of genetic instability in sporadic and familial colorectal cancer. *Cancer Biol Ther* 2002; **1**: 685-692

13 **Schwendel A**, Richard F, Langreck H, Kaufmann O, Lage H, Winzer KJ, Petersen I, Dietel M. Chromosome alterations in breast carcinomas: frequent involvement of DNA losses including chromosomes 4q and 21q. *Br J Cancer* 1998; **78**: 806-811

14 **Mitra AB**, Murty VV, Li RG, Pratap M, Luthra UK, Chaganti RS. Allelotype analysis of cervical carcinoma. *Cancer Res* 1994; **54**: 4481-4487

15 **Wang XL**, Uzawa K, Imai FL, Tanzawa H. Localization of a novel tumor suppressor gene associated with human oral cancer on chromosome 4q25. *Oncogene* 1999; **18**: 823-825

16 **Takeuchi S**, Seriu T, van Dongen JJ, Szczepanski T, Tsukasaki K, Takeuchi N, Fermin AC, Seo H, Bartram CR, Koeffler HP. Allelotype analysis in relapsed childhood acute lymphoblastic leukemia. *Oncogene* 2003; **22**: 6970-6976

17 **Hammoud ZT**, Kaleem Z, Cooper JD, Sundaresan RS, Patterson GA, Goodfellow PJ. Allelotype analysis of esophageal adenocarcinomas: evidence for the involvement of sequences on the long arm of chromosome 4. *Cancer Res* 1996; **56**: 4499-4502

18 **Rumpel CA**, Powell SM, Moskaluk CA. Mapping of genetic deletions on the long arm of chromosome 4 in human esophageal adenocarcinomas. *Am J Pathol* 1999; **154**: 1329-1334

19 **Hurst CD**, Fiegler H, Carr P, Williams S, Carter NP, Knowles MA. High-resolution analysis of genomic copy number alterations in bladder cancer by microarray-based comparative genomic hybridization. *Oncogene* 2004; **23**: 2250-2263

20 **Polascik TJ**, Cairns P, Chang WY, Schoenberg MP, Sidransky D. Distinct regions of allelic loss on chromosome 4 in human primary bladder carcinoma. *Cancer Res* 1995; **55**: 5396-5399

21 **Rosin MP**, Cairns P, Epstein JI, Schoenberg MP, Sidransky D. Partial allelotype of carcinoma in situ of the human bladder. *Cancer Res* 1995; **55**: 5213-5216

22 **Shivapurkar N**, Virmani AK, Wistuba II, Milchgrub S, Mackay B, Minna JD, Gazdar AF. Deletions of chromosome 4 at multiple sites are frequent in malignant mesothelioma and small cell lung carcinoma. *Clin Cancer Res* 1999; **5**: 17-23

23 **Cho ES**, Chang J, Chung KY, Shin DH, Kim YS, Kim SK, Kim SK. Identification of tumor suppressor loci on the long arm of chromosome 4 in primary small cell lung cancers. *Yonsei Med J* 2002; **43**: 145-151

24 **Piao Z**, Park C, Park JH, Kim H. Deletion mapping of chromosome 4q in hepatocellular carcinoma. *Int J Cancer* 1998; **79**: 356-360

25 **Fujimori M**, Tokino T, Hino O, Kitagawa T, Imamura T, Okamoto E, Mitsunobu M, Ishikawa T, Nakagama H, Harada H. Allelotype study of primary hepatocellular carcinoma. *Cancer Res* 1991; **51**: 89-93

26 **Nagai H**, Pineau P, Tiollais P, Buendia MA, Dejean A. Comprehensive allelotyping of human hepatocellular carcinoma. *Oncogene* 1997; **14**: 2927-2933

27 **Tsuda H**, Zhang WD, Shimosato Y, Yokota J, Terada M, Sugimura T, Miyamura T, Hirohashi S. Allele loss on chromosome 16 associated with progression of human hepatocellular carcinoma. *Proc Natl Acad Sci USA* 1990; **87**: 6791-6794

28 **Chou YH**, Chung KC, Jeng LB, Chen TC, Liaw YF. Frequent allelic loss on chromosomes 4q and 16q associated with human hepatocellular carcinoma in Taiwan. *Cancer Lett* 1998; **123**: 1-6

29 **Rashid A**, Wang JS, Qian GS, Lu BX, Hamilton SR, Groopman JD. Genetic alterations in hepatocellular carcinomas: association between loss of chromosome 4q and p53 gene mutations. *Br J Cancer* 1999; **80**: 59-66

30 **Bluteau O**, Beaudoin JC, Pasturaud P, Belghiti J, Franco D, Bioulac-Sage P, Laurent-Puig P, Zucman-Rossi J. Specific association between alcohol intake, high grade of differentiation and 4q34-q35 deletions in hepatocellular carcinomas identified by high resolution allelotyping.

Oncogene 2002; **21**: 1225-1232
31 **Vogelstein B**, Fearon ER, Kern SE, Hamilton SR, Preisinger AC, Nakamura Y, White R. Allelotype of colorectal carcinomas. *Science* 1989; **244**: 207-211
32 **Thorstensen L**, Qvist H, Nesland JM, Giercksky KE, Lothe RA. Allelotype profiles of local recurrences and distant metastases from colorectal-cancer patients. *Int J Cancer* 1996; **69**: 452-456
33 **Ried T**, Knutzen R, Steinbeck R, Blegen H, Schrock E, Heselmeyer K, du Manoir S, Auer G. Comparative genomic hybridization reveals a specific pattern of chromosomal gains and losses during the genesis of colorectal tumors. *Genes Chromosomes Cancer* 1996; **15**: 234-245
34 **Arribas R**, Risques RA, Gonzalez-Garcia I, Masramon L, Aiza G, Ribas M, Capella G, Peinado MA. Tracking recurrent quantitative genomic alterations in colorectal cancer: allelic losses in chromosome 4 correlate with tumor aggressiveness. *Lab Invest* 1999; **79**: 111-122
35 **Pershouse MA**, El-Naggar AK, Hurr K, Lin H, Yung WK, Steck PA. Deletion mapping of chromosome 4 in head and neck squamous cell carcinoma. *Oncogene* 1997; **14**: 369-373
36 **Okabe H**, Ikai I, Matsuo K, Satoh S, Momoi H, Kamikawa T, Katsura N, Nishitai R, Takeyama O, Fukumoto M, Yamaoka Y. Comprehensive allelotype study of hepatocellular carcinoma: potential differences in pathways to hepatocellular carcinoma between hepatitis B virus-positive and -negative tumors. *Hepatology* 2000; **31**: 1073-1079
37 **Konishi M**, Kikuchi-Yanoshita R, Tanaka K, Sato C, Tsuruta K, Maeda Y, Koike M, Tanaka S, Nakamura Y, Hattori N. Genetic changes and histopathological grades in human hepatocellular carcinomas. *Jpn J Cancer Res* 1993; **84**: 893-899
38 **Inazawa J**, Ariyama T, Abe T, Druck T, Ohta M, Huebner K, Yanagisawa J, Reed JC, Sato T. PTPN13, a fas-associated protein tyrosine phosphatase, is located on the long arm of chromosome 4 at band q21.3. *Genomics* 1996; **31**: 240-242
39 **Nasir J**, Theilmann JL, Chopra V, Jones AM, Walker D, Rasper DM, Vaillancourt JP, Hewitt JE, Nicholson DW, Hayden MR. Localization of the cell death genes CPP32 and Mch-2 to human chromosome 4q. *Mamm Genome* 1997; **8**: 56-59
40 **Burger AM**, Zhang X, Li H, Ostrowski JL, Beatty B, Venanzoni M, Papas T, Seth A. Down-regulation of T1A12/mac25, a novel insulin-like growth factor binding protein related gene, is associated with disease progression in breast carcinomas. *Oncogene* 1998; **16**: 2459-2467

S- Editor Li DL **L- Editor** Alpini GD **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2008 September 28; 14(36): 5612
World Journal of Gastroenterology ISSN 1007-9327

ACKNOWLEDGMENTS

Acknowledgments to Reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

David Adams, Professor
Liver Research Laboratories, Institute for Biomedical Research, Queen Elizabeth Hospital, University of Birmingham, Birmingham B15 2TT, United Kingdom

Marc Basson, MD, PhD, MBA, Chief of Surgery
John D. Dingell VA Medical Center, 4646 John R. Street, Detroit, MI 48301, United States

Luigi Bonavina, Professor
Department of Surgery, Policlinico San Donato, University of Milano, via Morandi 30, Milano 20097, Italy

Ana J Coito, Associate Professor of Surgery
Department of Surgery, The Dumont, UCLA Transplant Center, 77-120 CHS, Box 957054, Los Angeles, CA 90095-7054, United States

Giovanni D De Palma, Professor
Department of Surgery and Advanced Technologies, University of Naples Federico II, School of Medicine, Naples 80131, Italy

Dr. Olivier Detry
Department of Abdominal Surgery and Transplantation, University of Liège, CHU Sart Tilman B35, B-4000 Liège, Belgium

William Dickey
Altnagelvin Hospital, Londonderry, BT47 6SB, Northern Ireland, United Kingdom

Isabel Fabregat, PhD, Associate Professor
Laboratori d'Oncologia Molecular, Institut d'Investigación Biomèdica de Bellvitge, Gran Via, Km 2,7, L'Hospitalet, 08907 Barcelona, Spain

Kazuma Fujimoto, Professor
Department of Internal Medicine, Saga Medical School, Nabeshima, Saga, Saga 849-8501, Japan

Kazuhiro Hanazaki, MD, Professor and Chairman
Department of Surgery, Kochi Medical School, Kochi University, Kohasu, Okohcho, Nankoku, Kochi 783-8505, Japan

Per M Hellström, MD, Professor
Gastrocentre Medicine, Karolinska University Hospital Solna, SE-17176 Stockholm, Sweden

Peter R Holt, Professor, MD
Senior Research Associate, The Rockefeller University, 1230 York Avenue, New York, NY 10065, United States

Kenichi Ikejima, MD, PhD
Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1, Hongo, Bunkyo-ku, 113-8421, Japan

Hartmut Jaeschke, Professor
Liver Research Institute, University of Arizona, College of Medicine, 1501 N Campbell Ave, Room 6309, Tucson, Arizona 85724, United States

Peter L Lakatos, MD, PhD, Assistant Professor
First Department of Medicine, Semmelweis University, Koranyi S 2A, Budapest H1083, Hungary

Rene Lambert, Professor
International Agency for Research on Cancer, 150 Cours Albert Thomas, Lyon 69372 cedex 8, France

Syed MW Jafri, Professor
Medicine/Gastroenterology, Aga Khan University, POB 3500, Karachi 74800, Pakistan

Christian Maaser, MD
Department of Medicine B, University of Muenster, Albert Schweitzer Strasse 33, 48129 Muenster, Germany

Reza Malekzadeh, Professor, Director
Digestive Disease Research Center, Tehran University of Medical Sciences, Shariati Hospital, Kargar Shomali Avenue, 19119 Tehran, Iran

Mercedes Susan Mandell, MD, PhD
Department of Anesthesiology, University of Colorado Health Sciences Ctr., 12401 E. 17th Ave, B113 Aurora, CO 80045, United States

William G Paterson, Professor of Medicine, Chair
Division of Gastroenterology, Hotel Dieu Hospital, 166 Brock St., Kingston, Ontario, K7L 5G2, Canada

Oliviero Riggio, Professor
Dipartimento di Medicina Clinica, Sapienza Università di Roma,Viale dell'Università 37, 00185 Roma, Italy

Francis Seow-Choen, Professor
Seow-Choen Colorectal Centre, Mt Elizabeth Medical Centre, Singapore, 3 Mt Elizabeth Medical Centre #09-10, 228510, Singapore

Shinji Shimoda, MD, PhD
Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-Ku, Fukuoka 812-8582, Japan

Shanthi V Sitaraman, MD
Division of Digestive Diseases, Room 201-F, 615, Michael Street, Whitehead Research Building, Emory University, Atlanta, Georgia 30322, United States

Reinhold W Stockbrugger, Professor
Department of Gastroenterology and Hepatology, University Hospital Maastricht, Debyelaan 25, Maastricht NL-6206 AZ, The Netherlands

Koji Takeuchi, Professor
Department of Pharmacology & Experimental Therapeutics, Kyoto Pharmaceutical University, Misasagi, Yamashina, Kyoto 607-8414, Japan

Dr. Shinji Tanaka, Director
Department of Endoscopy, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan

Simon D Taylor-Robinson, MD
Department of Medicine A, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 0HS, United Kingdom

Adriana M Torres, Professor of Pharmacology
Suipacha 531, Rosario 2000, Argentina

Michael F Vaezi, Professor
Department of Gastroenterology and Hepatology, Vanderbilt University Medical Center, 1501 TVC, Nashville, TN 37232-5280, United States

Saúl Villa-Trevio, MD, PhD
Departamento de Biología Celular, Centro de Investigación y de Estudios Avanzados del IPN (Cinvestav), Ave. IPN No. 2508. Col. San Pedro, Zacatenco, CP 07360, México, DF, México

Kentaro Yoshika, Associate Professor
Division of Gastroenterology, Department of I, Fujita Health University School of Medicine, 1-98 Dengakugakubo, Kutsukade, Toyoake 470-1190, Japan

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2008 September 28; 14(36): 5613
World Journal of Gastroenterology ISSN 1007-9327

Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
January 24-25, Frankfurt, Germany
Falk Workshop: Perspectives in Liver
Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
E-mail: education@asge.org

June 4-7, Helsinki, Finland
The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
ESGAR 2008 19th Annual Meeting and Postgraduate Course
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden
16th International Congress of the European Association for Endoscopic Surgery
E-mail: info@eaes-eur.org

June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
10th World Congress on Gastrointestinal Cancer
Imedex and ESMO
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland
Joint Meeting of the European Pancreatic Club (EPC)
and the International Association of Pancreatology (IAP)
E-mail: office@epc-iap2008.org
www.e-p-c.org
www.pancreatology.org

June 26-28, Bratislava, Slovakia
5th Central European Gastroenterology Meeting
www.ceurgem2008.cz

July 9-12, Paris, France
ILTS 14th Annual International Congress
www.ilts.org

September 10-13, Budapest, Hungary
11th World Congress of the International Society for Diseases of the Esophagus
E-mail: isde@isde.net

September 13-16, New Delhi, India
Asia Pacific Digestive Week
E-mail: apdw@apdw2008.net

APDW 2008
September 13-16, New Delhi, Indian
Organized: Indian Society of Gastroenterology

III FALK GASTRO-CONFERENCE
September 17, Mainz, Germany
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
Falk Symposium 166:
GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
Falk Symposium 167:
Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
Third Annual Meeting
European Society of Coloproctology
www.escp.eu.com

October 8-11, Istanbul, Turkey
18th World Congress of the International Association of Surgeons,
Gastroenterologists and Oncologists
E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria
16th United European Gastroenterology Week
www.negf.org
www.acv.at

October 22-25, Minnesota, USA
Anstralian Gastroenterology Week 2008
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia
71st Annual Colon and Rectal Surgery Conference
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA
59th AASLD Annual Meeting and Postgraduate Course
The Liver Meeting
Information: www.aasld.org

November 6-9, Lucerne, Switzerland
Neurogastroenterology & Motility Joint International Meeting 2008
E-mail: ngm2008@mci-group.com
www.ngm2008.com

November 12, Santiago de Chile, Chile
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt
1st Hepatology and Gastroenterology Post Graduate Course
www.egyptgastrohep.com

December 7-9, Seoul, Korea
6th International Meeting
Hepatocellular Carcinoma: Eastern and Western Experiences
E-mail: sglee@amc.seoul.kr

INFORMATION FOR ALL
FALK FOUNDATION e.V.
E-mail: symposia@falkfoundation.de
www.falkfoundation.de

Advanced Courses - European Institute of Telesurgery EITS - 2008
Strasbourg, France
January 18-19, March 28-29, June 6-7, October 3-4

N.O.T.E.S
April 3-5, November 27-29
Laparoscopic Digestive Surgery

June 27-28, November 7-8
Laparoscopic Colorectal Surgery

July 3-5
Interventional GI Endoscopy Techniques
Contact address for all courses:
E-mail: info@eits.fr

International Gastroenterological Congresses 2009
March 23-26, Glasgow, Scotland
Meeting of the British Society of Gastroenterology (BSG)
E-mail: bsg@mailbox.ulcc.ac.uk

May 17-20, Denver, Colorado, USA
Digestive Disease Week 2009

November 21-25, London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2008 September 28; 14(36): 5614-5616
World Journal of Gastroenterology ISSN 1007-9327

Instructions to authors

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol* ISSN 1007-9327 CN 14-1219/R) is a weekly open access peer-reviewed journal supported by an editorial board consisting of 1208 experts in gastroenterology and hepatology from 60 countries. The aim of the journal is to deliver the most clinically relevant original and commentary articles to readers, and to make the full text publicly available to all clinicians, scientists, patients and biomedical students on an unrestricted platform, so that they can access and learn about the most recent key advances in the field.

In addition to the open access nature, another key characteristic of *WJG* is its reading guidance for each article which includes background, research frontier, related reports, breakthroughs, applications, terminology, and comments of peer reviewers for the general readers.

WJG publishes articles on esophageal, gastrointestinal, hepatobiliary and pancreatic tumors, and other esophageal, gastrointestinal, hepatic-biliary and pancreatic diseases in relation to epidermiology, immunology, microbiology, motility & nerve-gut interaction, endocrinology, nutrition & obesity, endoscopy, imaging and advanced hi-technology.

The main goal of *WJG* is to publish high quality commentary articles contributed by leading experts in gastroenterology and hepatology and original articles that combine the clinical practice and advanced basic research, to provide an interactive platform for clinicians and researchers in internal medicine, surgery, infectious diseases, traditional Chinese medicine, oncology, integrated Chinese and Western medicine, imaging, endoscopy, interventional therapy, pathology and other basic medical specialities, and thus eventually improving the clinical practice and healthcare for patients.

Indexed and abstracted in

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, Index Medicus, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology* and *Hepatology*, CAB Abstracts and Global Health. ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

Published by

The WJG Press

SUBMISSION OF MANUSCRIPTS

Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of The WJG Press, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the International Committee of Medical Journal Editors to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now available, to our knowledge, is http://www. clinicaltrials.gov sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

Authors should retain one copy of the text, tables, photographs and illustrations because rejected manuscripts will not be returned to the author(s) and the editors will not be responsible for loss or damage to photographs and illustrations sustained during mailing.

Online submissions

Manuscripts should be submitted through the Online Submission System at: http://wjg.wjgnet.com/wjg. Authors are highly recommended to consult the ONLINE INSTRUCTIONS TO AUTHORS (http://www.wjgnet.com/wjg/help/instructions.jsp) before attempting to submit online. For assistance, authors encountering problems with the Online Submission System may send an email describing the problem to submission@wjgnet.com, or by telephone: +86-10-85381892. If you submit your manuscript online, do not make a postal contribution. Repeated online submission for the same manuscript is strictly prohibited.

MANUSCRIPT PREPARATION

All contributions should be written in English. All articles must be submitted using word-processing software. All submissions must be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Style should conform to our house format. Required information for each of the manuscript sections is as follows:

Title page

Full manuscript title, running title, all author(s) name(s), affiliations, institution(s) and/or department(s) where the work was carried out; author contributions; disclosure of any financial support for the research; and the name, full address, telephone and fax numbers and email address of the corresponding author should be included. Titles should be concise and informative (remove all unnecessary words), emphasize what is new, and avoid abbreviations. A short running title of less than 40 letters should be provided. List the author(s)' name(s) as follows: initial and/or first name, middle name or initial(s), and full family name.

Author controbutions: The format of this section should be like this: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed research; Wang CL, Zou CC, Hong F and Wu XM performed research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed data; and Wang CL, Liang L and Fu JF wrote the paper.

Peer reviewers: All articles received are subject to peer review. Normally, three experts are invited for each article. Decision for acceptance is made only when at least two experts recommend an article for publication. Reviewers for accepted manuscripts are acknowledged in each manuscript, and reviewers of articles which were not accepted will be acknowledged at the end of each issue. To ensure the quality of the articles published in *WJG*, reviewers of accepted manuscripts will be announced by publishing the name, title/position and institution of the reviewer in the footnote accompanying the printed article. For example, reviewers: Professor Jing-Yuan Fang, Shanghai Institute of Digestive Disease, Shanghai, Affiliated Renji Hospital, Medical Faculty, Shanghai Jiaotong University, Shanghai, China; Professor Xin-Wei Han, Department of Radiology, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan Province, China; and Professor Anren Kuang, Department of Nuclear Medicine, Huaxi Hospital, Sichuan University, Chengdu, Sichuan Province, China.

Abstract

An informative, structured abstract of no more than 350 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections: AIM: Only the purpose should be included. METHODS: The materials, techniques, instruments and equipment, and the experimental procedures should be included. RESULTS: The observed and experimental results, including data, effects, outcome, *etc.* should be included. Authors should present *P* value where necessary, and also include any significant data. CONCLUSION: Accurate view and the value of the results should be included.

The format for structured abstracts can be found at: http://www.wjgnet.com/wjg/help/11.doc.

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles, rapid communication

and case reports, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the body text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, should be found at: http://www.wjgnet.com/wjg/help/instructions.jsp.

Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: …*etc.* It is our principle to publish high resolution-figures for the printed and E-versions.

Tables

Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of P values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscripts and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

Coding system

The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability[1,2]". If references are cited directly in the text, they should be put together within the text, for example, "From references[19,22-24], we know that..."

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

PMID requirement

PMID roots in the abstract serial number indexed by PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed). The author should supply the PMID for journal citation. For those references that have not been indexed by PubMed, a printed copy of the first page of the full reference should be submitted.

The accuracy of the information for journal citations is very important. Using the reference testing system, the authors and editor should check the authors name, title, journal title, publication date, volume number, start page, and end page. We will interlink all references with PubMed in an ASP file so that the readers can immediately access the abstract of the citations online.

DOI requirement

A CrossRef DOI® (Digital Object Identifier) name is a unique string created to identify a piece of scholarly content in the online environment. The author should supply the DOIs for journal citation(doi:10.3748/wjg.13.6458). This link (http://www.crossref.org/SimpleTextQuery/) allows you to retrieve Digital Object Identifiers (DOIs) for journal articles, books, and chapters by simply cutting and pasting the reference list into the box. You may use the form with any reference style, although the tool works most reliably if references are formatted in a standard style such as shown in this example: Assimakopoulos SF, Scopa CD, Vagianos CE. Pathophysiology of increased intestinal permeability in obstructive jaundice. *World J Gastroenterol* 2007; 13(48): 6458-6464

The accuracy of the information of journal citations is very important. We will interlink all references with DOI in ASP file so that readers can access the abstracts of cited articles online immediately.

Style for journal references

Authors: the name of the first author should be typed in bold-faced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

Style for book references

Authors: the name of the first author should be typed in bold-faced letters. The surname of all authors should be typed with the initial letter capitalized, followed by their abbreviated middle and first initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR) Book title. Publication number. Publication place: Publication press, Year: start page and end page.

Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Inappropriate references

Authors should always cite references that are relevant to their article, and avoid any inappropriate references. Inappropriate references include those linked with a hyphen when the difference between the two numbers is greater than five. For example, [1-6], [2-14] and [1, 3, 4-10, 22] are all considered inappropriate references. Authors should not cite their own unrelated published articles.

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/wjg/help/15.doc.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H pylori*, *E coli*, *etc.*

SUBMISSION OF THE REVISED MANUSCRIPTS AFTER ACCEPTED

Please revise your article according to the revision policies of *WJG*. The revised version including manuscript and high-resolution image figures (if any) should be copied on a floppy or compact disk. The author should send the revised manuscript, along with printed high-resolution color or black and white photos, copyright transfer letter, and responses to the reviewers by courier (such as EMS/DHL).

Editorial Office

World Journal of Gastroenterology
Editorial Department: Room 903
Ocean International Center, Building D
No. 62 Dongsihuan Zhonglu
Chaoyang District, Beijing 100025, China
E-mail: wjg@wjgnet.com
http://www.wjgnet.com
Telephone: +86-10-59080039
Fax: +86-10-85381893

Language evaluation

The language of a manuscript will be graded before it is sent for revision. (1) Grade A: priority publishing; (2) Grade B: minor language polishing; (3) Grade C: a great deal of language polishing needed; (4) Grade D: rejected. Revised articles should reach Grade A or B.

Copyright assignment form

Please download a Copyright assignment form from http://www.wjgnet.com/wjg/help/9.doc.

Responses to reviewers

Please revise your article according to the comments/suggestions provided by the reviewers. The format for responses to the reviewers' comments can be found at: http://www.wjgnet.com/wjg/help/10.doc.

Proof of financial support

For paper supported by a foundation, authors should provide a copy of the document and serial number of the foundation.

Links to documents related to the manuscript

WJG will be initiating a platform to promote dynamic interactions between the editors, peer reviewers, readers and authors. After a manuscript is published online, links to the PDF version of the submitted manuscript, the peer-reviewers' report and the revised manuscript will be put on-line. Readers can make comments on the peer reviewer's report, authors' responses to peer reviewers, and the revised manuscript. We hope that authors will benefit from this feedback and be able to revise the manuscript accordingly in a timely manner.

Science news releases

Authors of accepted manuscripts are suggested to write a science news item to promote their articles. The news will be released rapidly at EurekAlert/AAAS (http://www.eurekalert.org). The title for news items should be less than 90 characters; the summary should be less than 75 words; and main body less than 500 words. Science news items should be lawful, ethical, and strictly based on your original content with an attractive title and interesting pictures.

Publication fee

Authors of accepted articles must pay a publication fee.
EDITORIAL, TOPIC HIGHLIGHTS, BOOK REVIEWS and LETTERS TO THE EDITOR are published free of charge.

ISSN 1007-9327
CN 14-1219/R

World Journal of Gastroenterology®

Indexed and Abstracted in:

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, *Index Medicus*, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology and Hepatology*, CAB Abstracts and Global Health.
ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

Volume 14 Number 37
October 7, 2008
World J Gastroenterol
2008 October 7; 14(37): 5617-5780

Online Submissions
wjg.wjgnet.com
www.wjgnet.com
Printed on Acid-free Paper
世界胃肠病学杂志

A Weekly Journal of Gastroenterology and Hepatology

World Journal of Gastroenterology®

Editorial Board

2007-2009

Published by The WJG Press and Baishideng
Room 903, Ocean International Center, Building D
No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Fax: +86-10-8538-1893 E-mail: wjg@wjgnet.com http://www.wjgnet.com

The World Journal of Gastroenterology Editorial Board consists of 1208 members, representing a team of worldwide experts in gastroenterology and hepatology. They are from 60 countries, including Albania (1), Argentina (4), Australia (39), Austria (10), Belarus (1), Belgium (15), Brazil (2), Bulgaria (1), Canada (28), Chile (1), China (60), Croatia (2), Cuba (1), Czech (2), Denmark (7), Egypt (4), Estonia (1), Finland (4), France (44), Germany (108), Greece (9), Hungary (2), Iceland (1), India (12), Iran (3), Ireland (4), Israel (8), Italy (96), Japan (176), Lebanon (3), Lithuania (1), Macedonia (1), Malaysia (3), Mexico (6), Monaco (1), Morocco (1), The Netherlands (26), New Zealand (1), Nigeria (1), Norway (3), Pakistan (2), Peru (1), Poland (6), Portugal (1), Russia (3), Saudi Arabia (2), Serbia (1), Singapore (4), Slovakia (2), Slovenia (1), South Africa (2), South Korea (14), Spain (38), Sweden (15), Switzerland (13), Turkey (8), United Arab Emirates (1), United Kingdom (83), United States (316) and Uruguay (2).

Andrew V Biankin, *Sydney*
Filip Braet, *Sydney*
Andrew D Clouston, *Sydney*
Graham Cooksley, *Queensland*
Darrell HG Crawford, *Brisbane*
Adrian G Cummins, *Woodville South*
Guy D Eslick, *Sydney*
Michael A Fink, *Melbourne*
Robert JL Fraser, *Daw Park*
Peter Raymond Gibson, *Victoria*
Jacob George, *Westmead*
Mark D Gorrell, *Sydney*
Yik-Hong Ho, *Townsville*
Gerald J Holtmann, *Adelaide*
Michael Horowitz, *Adelaide*
John E Kellow, *Sydney*
Rupert Leong, *Concord*
Geoffrey W McCaughan, *Sydney*
Finlay A Macrae, *Victoria*
Daniel Markovich, *Brisbane*
Phillip S Oates, *Perth*
Jacqui Richmond, *Victoria*
Stephen M Riordan, *Sydney*
Ian C Roberts-Thomson, *Adelaide*
Devanshi Seth, *Camperdown*
Arthur Shulkes, *Melbourne*
Ross C Smith, *Sydney*
Kevin J Spring, *Brisbane*
Huy A Tran, *New South Wales*
Debbie Trinder, *Fremantle*
Martin J Veysey, *Gosford*
Daniel L Worthley, *Bedford*

Austria

Peter Ferenci, *Vienna*
Valentin Fuhrmann, *Vienna*
Alfred Gangl, *Vienna*
Christoph Gasche, *Vienna*
Kurt Lenz, *Linz*
Markus Peck-Radosavljevic, *Vienna*
Rudolf E Stauber, *Auenbruggerplatz*
Herbert Tilg, *Innsbruck*
Michael Trauner, *Graz*
Harald Vogelsang, *Vienna*
Guenter Weiss, *Innsbruck*

Belarus

Yury K Marakhouski, *Minsk*

Belgium

Rudi Beyaert, *Gent*
Bart Rik De Geest, *Leuven*
Inge I Depoortere, *Leuven*
Olivier Detry, *Liège*
Benedicte Y De Winter, *Antwerp*
Karel Geboes, *Leuven*
Thierry Gustot, *Brussels*
Yves J Horsmans, *Brussels*
Geert G Leroux-Roels, *Ghent*
Louis Libbrecht, *Leuven*
Etienne M Sokal, *Brussels*
Marc Peeters, *De Pintelaan*
Gert A Van Assche, *Leuven*
Yvan Vandenplas, *Brussels*
Eddie Wisse, *Keerbergen*

Brazil

Heitor Rosa, *Goiania*
Ana Cristina Simões e Silva, *Belo Horizonte*

Bulgaria

Zahariy Krastev, *Sofia*

Canada

Fernando Alvarez, *Québec*
David Armstrong, *Ontario*
Jeffrey P Baker, *Toronto*
Olivier Barbier, *Québec*
Nancy Baxter, *Toronto*
Matthew Bjerknes, *Toronto*
Frank J Burczynski, *Manitoba*
Michael F Byrne, *Vancouver*
Wang-Xue Chen, *Ottawa*
Chantal Guillemette, *Québec*
Samuel S Lee, *Calgary*
Gary A Levy, *Toronto*
Andrew L Mason, *Alberta*
John K Marshall, *Ontario*
Donna-Marie McCafferty, *Calgary*
Thomas I Michalak, *St. John's*
Gerald Y Minuk, *Manitoba*
Paul Moayyedi, *Hamilton*
Kostas Pantopoulos, *Québec*
William G Paterson, *Kingston*
Eldon Shaffer, *Calgary*
Morris Sherman, *Toronto*
Martin Storr, *Calgary*
Elena F Verdu, *Ontario*
John L Wallace, *Calgary*
Eric M Yoshida, *Vancouver*

Chile

Silvana Zanlungo, *Santiago*

China

Henry LY Chan, *Hongkong*
Xiao-Ping Chen, *Wuhan*
Zong-Jie Cui, *Beijing*
Da-Jun Deng, *Beijing*
Er-Dan Dong, *Beijing*
Sheung-Tat Fan, *Hong Kong*
Jin Gu, *Beijing*
Xin-Yuan Guan, *Pokfulam*
De-Wu Han, *Taiyuan*
Ming-Liang He, *Hong Kong*
Wayne HC Hu, *Hong Kong*
Chee-Kin Hui, *Hong Kong*
Ching-Lung Lai, *Hong Kong*
Kam Chuen Lai, *Hong Kong*
James YW Lau, *Hong Kong*
Yuk-Tong Lee, *Hong Kong*
Suet-Yi Leung, *Hong Kong*
Wai-Keung Leung, *Hong Kong*
John M Luk, *Pokfulam*
Chung-Mau Lo, *Hong Kong*
Jing-Yun Ma, *Beijing*
Ronnie Tung Ping Poon, *Hong Kong*
Lun-Xiu Qin, *Shanghai*
Yu-Gang Song, *Guangzhou*
Qin Su, *Beijing*
Wai-Man Wong, *Hong Kong*
Hong Xiao, *Shanghai*
Dong-Liang Yang, *Wuhan*
Winnie Yeo, *Hong Kong*
Yuan Yuan, *Shenyang*
Man-Fung Yuen, *Hong Kong*
Jian-Zhong Zhang, *Beijing*
Xin-Xin Zhang, *Shanghai*
Bo-Jian Zheng, *Hong Kong*
Shu Zheng, *Hangzhou*

Croatia

Tamara Cacev, *Zagreb*
Marko Duvnjak, *Zagreb*

Cuba

Damian C Rodriguez, *Havana*

Czech

Milan Jirsa, *Praha*
Pavel Trunečka, *Prague*

Denmark

Peter Bytzer, *Copenhagen*
Asbjørn M Drewes, *Aalborg*
Hans Gregersen, *Aalborg*
Jens H Henriksen, *Hvidovre*
Claus P Hovendal, *Odense*
Fin S Larsen, *Copenhagen*
Søren Møller, *Hvidovre*

Egypt

Abdel-Rahman El-Zayadi, *Giza*
Amr M Helmy, *Cairo*
Ayman Yosry, *Cairo*

Estonia

Riina Salupere, *Tartu*

Finland

Irma E Jarvela, *Helsinki*
Katri M Kaukinen, *Tampere*
Minna Nyström, *Helsinki*
Pentti Sipponen, *Espoo*

France

Bettaieb Ali, *Dijon*
Corlu Anne, *Rennes*
Denis Ardid, *Clermont-Ferrand*
Charles P Balabaud, *Bordeaux*
Soumeya Bekri, *Rouen*
Jacques Belghiti, *Clichy*
Jacques Bernuau, *Clichy Cedex*
Pierre Brissot, *Rennes*
Patrice P Cacoub, *Paris*
Franck Carbonnel, *Besancon*
Laurent Castera, *Pessac*
Bruno Clément, *Rennes*
Benoit Coffin, *Colombes*
Jacques Cosnes, *Paris*
Thomas Decaens, *Cedex*

Francoise L Fabiani, *Angers*
Gérard Feldmann, *Paris*
Jean Fioramonti, *Toulouse*
Jean-Noël Freund, *Strasbourg*
Jean-Paul Galmiche, *Nantes*
Catherine Guettier, *Villejuif*
Chantal Housset, *Paris*
Juan L Iovanna, *Marseille*
Rene Lambert, *Lyon*
Patrick Marcellin, *Paris*
Philippe Mathurin, *Lille*
Tamara Matysiak–Budnik, *Paris*
Francis Mégraud, *Bordeaux*
Richard Moreau, *Clichy*
Thierry Piche, *Nice*
Raoul Poupon, *Paris*
Jean Rosenbaum, *Bordeaux*
Dominique Marie Roulot, *Bobigny*
Thierry Poynard, *Paris*
Jean-Philippe Salier, *Rouen*
Didier Samuel, *Villejuif*
Jean-Yves Scoazec, *Lyon*
Khalid A Tazi, *Clichy*
Emmanuel Tiret, *Paris*
Baumert F Thomas, *Strasbourg*
Marie-Catherine Vozenin-brotons, *Villejuif*
Jean-Pierre H Zarski, *Grenoble*
Jessica Zucman-Rossi, *Paris*

Germany

Hans-Dieter Allescher, *G-Partenkirchen*
Martin Anlauf, *Kiel*
Rudolf Arnold, *Marburg*
Max G Bachem, *Ulm*
Thomas F Baumert, *Freiburg*
Daniel C Baumgart, *Berlin*
Hubert Blum, *Freiburg*
Thomas Bock, *Tuebingen*
Katja Breitkopf, *Mannheim*
Dunja Bruder, *Braunschweig*
Markus W Büchler, *Heidelberg*
Christa Buechler, *Regensburg*
Reinhard Buettner, *Bonn*
Elke Cario, *Essen*
Uta Dahmen, *Essen*
Christoph F Dietrich, *Bad Mergentheim*
Arno J Dormann, *Koeln*
Rainer J Duchmann, *Berlin*
Volker F Eckardt, *Wiesbaden*
Paul Enck, *Tuebingen*
Fred Fändrich, *Kiel*
Ulrich R Fölsch, *Kiel*
Helmut Friess, *Heidelberg*
Peter R Galle, *Mainz*
Nikolaus Gassler, *Aachen*
Andreas Geier, *Aachen*
Markus Gerhard, *Munich*
Wolfram H Gerlich, *Giessen*
Dieter Glebe, *Giessen*
Burkhard Göke, *Munich*
Florian Graepler, *Tuebingen*
Axel M Gressner, *Aachen*
Veit Gülberg, *Munich*
Rainer Haas, *Munich*
Eckhart G Hahn, *Erlangen*
Stephan Hellmig, *Kiel*
Martin Hennenberg, *Bonn*
Johannes Herkel, *Hamburg*
Klaus R Herrlinger, *Stuttgart*
Eva Herrmann, *Homburg/Saar*
Eberhard Hildt, *Berlin*
Joerg C Hoffmann, *Berlin*
Ferdinand Hofstaedter, *Regensburg*
Werner Hohenberger, *Erlangen*
Jörg C Kalff, *Bonn*
Ralf Jakobs, *Ludwigshafen*
Jutta Keller, *Hamburg*
Andrej Khandoga, *Munich*
Sibylle Koletzko, *München*
Stefan Kubicka, *Hannover*
Joachim Labenz, *Siegen*
Frank Lammert, *Bonn*
Thomas Langmann, *Regensburg*
Christian Liedtke, *Aachen*
Matthias Löhr, *Mannheim*
Christian Maaser, *Muenster*
Ahmed Madisch, *Dresden*
Peter Malfertheiner, *Magdeburg*
Michael P Manns, *Hannover*
Helmut Messmann, *Augsburg*
Stephan Miehlke, *Dresden*
Sabine Mihm, *Göttingen*
Silvio Nadalin, *Essen*
Markus F Neurath, *Mainz*
Johann Ockenga, *Berlin*
Florian Obermeier, *Regensburg*
Gustav Paumgartner, *Munich*
Ulrich KS Peitz, *Magdeburg*
Markus Reiser, *Bochum*
Emil C Reisinger, *Rostock*
Steffen Rickes, *Magdeburg*
Tilman Sauerbruch, *Bonn*
Dieter Saur, *Munich*
Hans Scherubl, *Berlin*
Joerg Schirra, *Munich*
Roland M Schmid, *München*
Volker Schmitz, *Bonn*
Andreas G Schreyer, *Regensburg*
Tobias Schroeder, *Essen*
Henning Schulze-Bergkamen, *Mainz*
Hans Seifert, *Oldenburg*
Norbert Senninger, *Muenster*
Manfred V Singer, *Mannheim*
Gisela Sparmann, *Rostock*
Christian J Steib, *München*
Jurgen M Stein, *Frankfurt*
Ulrike S Stein, *Berlin*
Manfred Stolte, *Bayreuth*
Christian P Strassburg, *Hannover*
Wolfgang R Stremmel, *Heidelberg*
Harald F Teutsch, *Ulm*
Robert Thimme, *Freiburg*
Hans L Tillmann, *Leipzig*
Tung-Yu Tsui, *Regensburg*
Axel Ulsenheimer, *Munich*
Patrick Veit-Haibach, *Essen*
Claudia Veltkamp, *Heidelberg*
Siegfried Wagner, *Deggendorf*
Henning Walczak, *Heidelberg*
Heiner Wedemeyer, *Hannover*
Fritz von Weizsacker, *Berlin*
Jens Werner, *Heidelberg*
Bertram Wiedenmann, *Berlin*
Reiner Wiest, *Regensburg*
Stefan Wirth, *Wuppertal*
Stefan JP Zeuzem, *Homburg*

Greece

Alexandra A Alexopoulou, *Athens*
George N Dalekos, *Larissa*
Christos Dervenis, *Athens*
Melanie Maria Deutsch, *Athens*
Tsianos Epameinondas, *Ioannina*
Elias A Kouroumalis, *Heraklion*
George Papatheodoridis, *Athens*
Spiros Sgouros, *Athens*

Hungary

Peter L Lakatos, *Budapest*
Zsuzsa Szondy, *Debrecen*

Iceland

Hallgrimur Gudjonsson, *Reykjavik*

India

Philip Abraham, *Mumbai*
Rakesh Aggarwal, *Lucknow*
Kunissery A Balasubramanian, *Vellore*
Deepak Kumar Bhasin, *Chandigarh*
Sujit K Bhattacharya, *Kolkata*
Yogesh K Chawla, *Chandigarh*
Radha K Dhiman, *Chandigarh*
Sri Prakash Misra, *Allahabad*
Ramesh Roop Rai, *Jaipur*
Nageshwar D Reddy, *Hyderabad*
Rakesh Kumar Tandon, *New Delhi*

Iran

Seyed-Moayed Alavian, *Tehran*
Reza Malekzadeh, *Tehran*
Seyed A Taghavi, *Shiraz*

Ireland

Billy Bourke, *Dublin*
Ronan A Cahill, *Cork*
Anthony P Moran, *Galway*

Israel

Simon Bar-Meir, *Hashomer*
Abraham R Eliakim, *Haifa*
Zvi Fireman, *Hadera*
Yaron Ilan, *Jerusalem*
Avidan U Neumann, *Ramat-Gan*
Yaron Niv, *Pardesia*
Ran Oren, *Tel Aviv*
Ami D Sperber, *Beer-Sheva*

Italy

Giovanni Addolorato, *Roma*
Luigi E Adinolfi, *Naples*
Domenico Alvaro, *Rome*
Mario Angelico, *Rome*
Vito Annese, *San Giovanni Rotond*
Filippo Ansaldi, *Genoa*
Adolfo F Attili, *Roma*
Giovanni Barbara, *Bologna*
Claudio Bassi, *Verona*
Gabrio Bassotti, *Perugia*
Pier M Battezzati, *Milan*
Stefano Bellentani, *Carpi*
Antomio Benedetti, *Ancona*
Mauro Bernardi, *Bologna*
Livia Biancone, *Rome*
Luigi Bonavina, *Milano*
Flavia Bortolotti, *Padova*
Giuseppe Brisinda, *Rome*
Elisabetta Buscarini, *Crema*
Giovanni Cammarota, *Roma*

Antonino Cavallari, *Bologna*
Giuseppe Chiarioni, *Valeggio*
Michele Cicala, *Rome*
Massimo Colombo, *Milan*
Amedeo Columbano, *Cagliari*
Massimo Conio, *Sanremo*
Dario Conte, *Milano*
Gino R Corazza, *Pavia*
Francesco Costa, *Pisa*
Antonio Craxi, *Palermo*
Silvio Danese, *Milan*
Roberto de Franchis, *Milano*
Roberto De Giorgio, *Bologna*
Maria Stella De Mitri, *Bologna*
Giovanni D De Palma, *Naples*
Fabio Farinati, *Padua*
Giammarco Fava, *Ancona*
Francesco Feo, *Sassari*
Fiorucci Stefano, *Perugia*
Andrea Galli, *Firenze*
Valeria Ghisett, *Turin*
Gianluigi Giannelli, *Bari*
Edoardo G Giannini, *Genoa*
Paolo Gionchetti, *Bologna*
Fabio Grizzi, *Milan*
Salvatore Gruttadauria, *Palermo*
Mario Guslandi, *Milano*
Pietro Invernizzi, *Milan*
Ezio Laconi, *Cagliari*
Giacomo Laffi, *Firenze*
Giovanni Maconi, *Milan*
Lucia Malaguarnera, *Catania*
Emanuele D Mangoni, *Napoli*
Paolo Manzoni, *Torino*
Giulio Marchesini, *Bologna*
Fabio Marra, *Florence*
Marco Marzioni, *Ancona*
Giuseppe Mazzella, *Bologna*
Mario U Mondelli, *Pavia*
Giuseppe Montalto, *Palermo*
Giovanni Monteleone, *Rome*
Giovanni Musso, *Torino*
Gerardo Nardone, *Napoli*
Valerio Nobili, *Rome*
Fabio Pace, *Milano*
Luisi Pagliaro, *Palermo*
Francesco Pallone, *Rome*
Fabrizio R Parente, *Milan*
Maurizio Parola, *Torino*
Francesco Perri, *San Giovanni Rotondo*
Raffaele Pezzilli, *Bologna*
Alberto Pilotto, *San Giovanni Rotondo*
Alberto Piperno, *Monza*
Mario Pirisi, *Novara*
Anna C Piscaglia, *Roma*
Paolo Del Poggio, *Treviglio*
Gabriele B Porro, *Milano*
Piero Portincasa, *Bari*
Cosimo Prantera, *Roma*
Bernardino Rampone, *Siena*
Oliviero Riggio, *Rome*
Claudio Romano, *Messina*
Marco Romano, *Napoli*
Gerardo Rosati, *Potenza*
Mario Del Tacca, *Pisa*
Gloria Taliani, *Rome*
Pier A Testoni, *Milan*
Enrico Roda, *Bologna*
Domenico Sansonno, *Bari*
Vincenzo Savarino, *Genova*
Vincenzo Stanghellini, *Bologna*
Giovanni Tarantino, *Naples*
Roberto Testa, *Genoa*
Dino Vaira, *Bologna*
Anna Linda Zignego, *Florence*

Japan

Kyoichi Adachi, *Izumo*
Yasushi Adachi, *Sapporo*
Taiji Akamatsu, *Matsumoto*
Sk Md Fazle Akbar, *Ehime*
Takafumi Ando, *Nagoya*
Akira Andoh, *Otsu*
Taku Aoki, *Tokyo*
Masahiro Arai, *Tokyo*
Tetsuo Arakawa, *Osaka*
Yasuji Arase, *Tokyo*
Masahiro Asaka, *Sapporo*
Hitoshi Asakura, *Tokyo*
Takeshi Azuma, *Fukui*
Yoichi Chida, *Fukuoka*
Takahiro Fujimori, *Tochigi*
Jiro Fujimoto, *Hyogo*
Kazuma Fujimoto, *Saga*
Mitsuhiro Fujishiro, *Tokyo*
Yoshihide Fujiyama, *Otsu*
Hiroyuki Fukui, *Tochigi*
Hiroyuki Hanai, *Hamamatsu*
Naohiko Harada, *Fukuoka*
Makoto Hashizume, *Fukuoka*
Tetsuo Hayakawa, *Nagoya*
Toru Hiyama, *Higashihiroshima*
Kazuhide Higuchi, *Osaka*
Keisuke Hino, *Ube*
Keiji Hirata, *Kitakyushu*
Yuji Iimuro, *Nishinomiya*
Kenji Ikeda, *Tokyo*
Toru Ikegami, *Fukuoka*
Kenichi Ikejima, *Bunkyo-ku*
Fumio Imazeki, *Chiba*
Yutaka Inagaki, *Kanagawa*
Yasuhiro Inokuchi, *Yokohama*
Haruhiro Inoue, *Yokohama*
Masayasu Inoue, *Osaka*
Hiromi Ishibashi, *Nagasaki*
Shunji Ishihara, *Izumo*
Toru Ishikawa, *Niigata*
Kei Ito, *Sendai*
Masayoshi Ito, *Tokyo*
Hiroaki Itoh, *Akita*
Ryuichi Iwakiri, *Saga*
Yoshiaki Iwasaki, *Okayama*
Terumi Kamisawa, *Tokyo*
Hiroshi Kaneko, *Aichi-Gun*
Shuichi Kaneko, *Kanazawa*
Takashi Kanematsu, *Nagasaki*
Mitsuo Katano, *Fukuoka*
Junji Kato, *Sapporo*
Mototsugu Kato, *Sapporo*
Shinzo Kato, *Tokyo*
Norifumi Kawada, *Osaka*
Sunao Kawano, *Osaka*
Mitsuhiro Kida, *Kanagawa*
Yoshikazu Kinoshita, *Izumo*
Tsuneo Kitamura, *Chiba*
Seigo Kitano, *Oita*
Kazuhiko Koike, *Tokyo*
Norihiro Kokudo, *Tokyo*
Satoshi Kondo, *Sapporo*
Shoji Kubo, *Osaka*
Shigeki Kuriyama, *Kagawa*[2]
Katsunori Iijima, *Sendai*
Masato Kusunoki, *Tsu Mie*
Shin Maeda, *Tokyo*
Shigeru Marubashi, *Suita*
Masatoshi Makuuchi, *Tokyo*
Osamu Matsui, *Kanazawa*
Yasuhiro Matsumura, *Chiba*
Yasushi Matsuzaki, *Tsukuba*
Kiyoshi Migita, *Omura*
Kenji Miki, *Tokyo*
Tetsuya Mine, *Kanagawa*
Hiroto Miwa, *Hyogo*
Masashi Mizokami, *Nagoya*
Yoshiaki Mizuguchi, *Tokyo*
Motowo Mizuno, *Hiroshima*
Morito Monden, *Suita*
Hisataka S Moriwaki, *Gifu*
Yasuaki Motomura, *Iizuka*
Yoshiharu Motoo, *Kanazawa*
Naofumi Mukaida, *Kanazawa*
Kazunari Murakami, *Oita*
Kunihiko Murase, *Tusima*
Hiroaki Nagano, *Suita*
Masahito Nagaki, *Gifu*
Masaki Nagaya, *Kawasaki*
Yujl Naito, *Kyoto*
Atsushi Nakajima, *Yokohama*
Hisato Nakajima, *Tokyo*
Hiroki Nakamura, *Yamaguchi*
Shotaro Nakamura, *Fukuoka*
Mikio Nishioka, *Niihama*
Shuji Nomoto, *Nagoya*
Susumu Ohmada, *Maebashi*
Hirohide Ohnishi, *Akita*
Masayuki Ohta, *Oita*
Tetsuo Ohta, *Kanazawa*
Kazuichi Okazaki, *Osaka*
Katsuhisa Omagari, *Nagasaki*
Saburo Onishi, *Nankoku*
Morikazu Onji, *Ehime*
Satoshi Osawa, *Hamamatsu*
Masanobu Oshima, *Kanazawa*
Hiromitsu Saisho, *Chiba*
Hidetsugu Saito, *Tokyo*
Yutaka Saito, *Tokyo*
Isao Sakaida, *Yamaguchi*
Michiie Sakamoto, *Tokyo*
Yasushi Sano, *Chiba*
Hiroki Sasaki, *Tokyo*
Iwao Sasaki, *Sendai*
Motoko Sasaki, *Kanazawa*
Chifumi Sato, *Tokyo*
Shuichi Seki, *Osaka*
Hiroshi Shimada, *Yokohama*
Mitsuo Shimada, *Tokushima*
Tomohiko Shimatan, *Hiroshima*
Hiroaki Shimizu, *Chiba*
Ichiro Shimizu, *Tokushima*
Yukihiro Shimizu, *Kyoto*
Shinji Shimoda, *Fukuoka*
Tooru Shimosegawa, *Sendai*
Tadashi Shimoyama, *Hirosaki*
Ken Shirabe, *Iizuka City*
Yoshio Shirai, *Niigata*
Katsuya Shiraki, *Mie*
Yasushi Shiratori, *Okayama*
Masayuki Sho, *Nara*
Yasuhiko Sugawara, *Tokyo*
Hidekazu Suzuki, *Tokyo*
Minoru Tada, *Tokyo*
Tadatoshi Takayama, *Tokyo*
Tadashi Takeda, *Osaka*
Koji Takeuchi, *Kyoto*
Kiichi Tamada, *Tochigi*
Akira Tanaka, *Kyoto*
Eiji Tanaka, *Matsumoto*
Noriaki Tanaka, *Okayama*
Shinji Tanaka, *Hiroshima*
Hideki Taniguchi, *Yokohama*
Kyuichi Tanikawa, *Kurume*
Akira Terano, *Shimotsugagun*
Hitoshi Togash, *Yamagata*
Shinji Togo, *Yokohama*
Kazunari Tominaga, *Osaka*
Takuji Torimura, *Fukuoka*

Minoru Toyota, *Sapporo*
Akihito Tsubota, *Chiba*
Takato Ueno, *Kurume*
Naomi Uemura, *Tokyo*
Shinichi Wada, *Tochigi*
Hiroyuki Watanabe, *Kanazawa*
Toshio Watanabe, *Osaka*
Yuji Watanabe, *Ehime*
Toshiaki Watanabe, *Tokyo*
Chun-Yang Wen, *Nagasaki*
Satoshi Yamagiwa, *Niigata*
Koji Yamaguchi, *Fukuoka*
Takayuki Yamamoto, *Yokkaichi*
Takashi Yao, *Fukuoka*
Masashi Yoneda, *Tochigi*
Hiroshi Yoshida, *Tokyo*
Masashi Yoshida, *Tokyo*
Norimasa Yoshida, *Kyoto*
Hitoshi Yoshiji, *Nara*
Kentaro Yoshika, *Toyoake*
Yasunobu Yoshikai, *Fukuoka*
Masahide Yoshikawa, *Kashihara*
Katsutoshi Yoshizato, *Higashihiroshima*

Lebanon

Bassam N Abboud, *Beirut*
Ala I Sharara, *Beirut*
Joseph D Boujaoude, *Beirut*

Lithuania

Limas Kupcinskas, *Kaunas*

Macedonia

Vladimir C Serafimoski, *Skopje*

Malaysia

Andrew Seng Boon Chua, *Ipoh*
Khean-Lee Goh, Kuala *Lumpur*
Jayaram Menon, *Sabah*

Mexico

Diego Garcia-Compean, *Monterrey*
Eduardo R Marin-Lopez, *Jesús García*
Nahum Méndez-Sánchez, *Mexico*
Saúl Villa-Treviño, *México*

Monaco

Patrick Rampal, *Monaco*

Morocco

Abdellah Essaid, *Rabat*

The Netherlands

Ulrich Beuers, *Amsterdam*
Gerd Bouma, *Amsterdam*
Lee Bouwman, *Leiden*
J Bart A Crusius, *Amsterdam*
NKH de Boer, *Amsterdam*
Koert P de Jong, *Groningen*
Henrike Hamer, *Maastricht*
Frank Hoentjen, *Haarlem*
Janine K Kruit, *Groningen*
Ernst J Kuipers, *Rotterdam*
CBHW Lamers, *Leiden*
Ton Lisman, *Utrecht*
Yi Liu, *Amsterdam*
Jeroen Maljaars, *Maastricht*
Servaas Morré, *Amsterdam*
Chris JJ Mulder, *Amsterdam*
Michael Müller, *Wageningen*
Amado S Peña, *Amsterdam*
Robert J Porte, *Groningen*
Ingrid B Renes, *Rotterdam*
Andreas Smout, *Utrecht*
Paul E Sijens, *Groningen*
Reinhold W Stockbrugger, *Maastricht*
Luc JW van der Laan, *Rotterdam*
Karel van Erpecum, *Utrecht*
Gerard P VanBerge-Henegouwen,*Utrecht*

New Zealand

Ian D Wallace, *Auckland*

Nigeria

Samuel B Olaleye, *Ibadan*

Norway

Trond Berg, *Oslo*
Tom H Karlsen, *Oslo*
Helge L Waldum, *Trondheim*

Pakistan

Muhammad S Khokhar, *Lahore*
Syed MW Jafri, *Karachi*

Peru

Hector H Garcia, *Lima*

Poland

Tomasz Brzozowski, *Cracow*
Robert Flisiak, *Bialystok*
Hanna Gregorek, *Warsaw*
Dariusz M Lebensztejn, *Bialystok*
Wojciech G Polak, *Wroclaw*
Marek Hartleb, *Katowice*

Portugal

Miguel C De Moura, *Lisbon*

Russia

Vladimir T Ivashkin, *Moscow*
Leonid Lazebnik, *Moscow*
Vasiliy I Reshetnyak, *Moscow*

Saudi Arabia

Ibrahim A Al Mofleh, *Riyadh*
Ahmed Helmy, *Riyadh*

Serbia

Dusan M Jovanovic, *Sremska Kamenica*

Singapore

Bow Ho, *Singapore*
Khek-Yu Ho, *Singapore*
Fock Kwong Ming, *Singapore*
Francis Seow-Choen, *Singapore*

Slovakia

Silvia Pastorekova, *Bratislava*
Anton Vavrecka, *Bratislava*

Slovenia

Sasa Markovic, *Ljubljana*

South Africa

Rosemar Joyce Burnett, *Pretoria*
Michael C Kew, *Parktown*

South Korea

Byung Ihn Choi, *Seoul*
Ho Soon Choi, *Seoul*
Marie Yeo, *Suwon*
Sun Pyo Hong, *Gyeonggi-do*
Jae J Kim, *Seoul*
Jin-Hong Kim, *Suwon*
Myung-Hwan Kim, *Seoul*
Chang Hong Lee, *Seoul*
Jong Kyun Lee, *Seoul*
Eun-Yi Moon, *Seoul*
Jae-Gahb Park, *Seoul*
Dong Wan Seo, *Seoul*
Dong Jin Suh, *Seoul*
Byung Chul Yoo, *Seoul*

Spain

Juan G Abraldes, *Barcelona*
Agustin Albillos, *Madrid*
Raul J Andrade, *Málaga*
Luis Aparisi, *Valencia*
Fernando Azpiroz, *Barcelona*
Ramon Bataller, *Barcelona*
Josep M Bordas, *Barcelona*
Xavier Calvet, *Sabadell*
Jordi Camps, *Catalunya*
Andres Cardenas, *Barcelona*
Vicente Carreño, *Madrid*
Jose Castellote, *Barcelona*
Antoni Castells, *Barcelona*
Vicente Felipo, *Valencia*
Juan C Garcia-Pagán, *Barcelona*
Jaime B Genover, *Barcelona*
Javier P Gisbert, *Madrid*
Jaime Guardia, *Barcelona*
Isabel Fabregat, *Barcelona*
Mercedes Fernandez, *Barcelona*
Angel Lanas, *Zaragoza*
Juan-Ramón Larrubia, *Guadalajara*
Laura Lladóa, *Barcelona*
María IT López, *Jaén*
Juan R Malagelada, *Barcelona*
José M Mato, *Derio*
Juan F Medina, *Pamplona*
Miguel A Muñoz-Navas, *Pamplona*
Julian Panes, *Barcelona*
Miguel M Perez, *Valencia*
Miguel Perez-Mateo, *Alicante*

Josep M Pique, *Barcelona*
Jesús M Prieto, *Pamplona*
Sabino Riestra, *Pola De Siero*
Luis Rodrigo, *Oviedo*
Manuel Romero-Gómez, *Sevilla*
Joan Roselló-Catafau, *Barcelona*

Sweden

Einar S Björnsson, *Gothenburg*
Curt Einarsson, *Huddinge*
Per M Hellström, *Stockholm*
Ulf Hindorf, *Lund*
Elisabeth Hultgren-Hörnquist, *Örebro*
Anders E Lehmann, *Mölndal*
Hanns-Ulrich Marschall, *Stockholm*
Lars C Olbe, *Molndal*
Lars A Pahlman, *Uppsala*
Matti Sallberg, *Stockholm*
Magnus Simrén, *Göteborg*
Xiao-Feng Sun, *Linköping*
Ervin Tóth, *Malmö*
Weimin Ye, *Stockholm*
Christer S von Holstein, *Lund*

Switzerland

Chrish Beglinger, *Basel*
Pierre A Clavien, *Zurich*
Jean-Francois Dufour, *Bern*
Franco Fortunato, *Zürich*
Jean L Frossard, *Geneva*
Gerd A Kullak-Ublick, *Zurich*
Pierre Michetti, *Lausanne*
Francesco Negro, *Genève*
Bruno Stieger, *Zurich*
Radu Tutuian, *Zurich*
Stephan R Vavricka, *Zurich*
Gerhard Rogler, *Zurich*
Arthur Zimmermann, *Berne*

Turkey

Yusuf Bayraktar, *Ankara*
Figen Gurakan, *Ankara*
Aydin Karabacakoglu, *Konya*
Serdar Karakose, *Konya*
Hizir Kurtel, *Istanbul*
Osman C Ozdogan, *Istanbul*
Özlem Yilmaz, *Izmir*
Cihan Yurdaydin, *Ankara*

United Arab Emirates

Sherif M Karam, *Al-Ain*

United Kingdom

David H Adams, *Birmingham*
Simon Afford, *Birmingham*
Navneet K Ahluwalia, *Stockport*
Ahmed Alzaraa, *Manchester*
Lesley A Anderson, *Belfast*
Charalambos G Antoniades, *London*
Anthony TR Axon, *Leeds*
Qasim Aziz, *Manchester*
Nicholas M Barnes, *Birmingham*
Jim D Bell, *London*
Mairi Brittan, *London*
Alastair D Burt, *Newcastle*
Simon S Campbell, *Manchester*
Simon R Carding, *Leeds*
Paul J Ciclitira, *London*
Eithne Costello, *Liverpool*
Tatjana Crnogorac-Jurcevic, *London*
Harry Dalton, *Truro*
Amar P Dhillon, *London*
William Dickey, *Londonderry*
James E East, *London*
Emad M El-Omar, *Aberdeen*
Ahmed M Elsharkawy, *Newcastle Upon Tyne*
Annette Fristscher-Ravens, *London*
Elizabeth Furrie, *Dundee*
Daniel R Gaya, *Edinburgh*
Subrata Ghosh, *London*
William Greenhalf, *Liverpool*
Indra N Guha, *Southampton*
Peter C Hayes, *Edinburgh*
Gwo-Tzer Ho, *Edinburgh*
Anthony R Hobson, *Salford*
Lesley A Houghton, *Manchester*
Stefan G Hübscher, *Birmingham*
Robin Hughes, *London*
Pali Hungin, *Stockton*
David P Hurlstone, *Sheffield*
Rajiv Jalan, *London*
Janusz AZ Jankowski, *Oxford*
Brian T Johnston, *Belfast*
David EJ Jones, *Newcastle*
Roger Jones, *London*
Michael A Kamm, *Harrow*
Peter Karayiannis, *London*
Laurens Kruidenier, *Harlow*
Patricia F Lalor, *Birmingham*
Chee Hooi Lim, *Midlands*
Hong-Xiang Liu, *Cambridge*
Yun Ma, *London*
Kenneth E L McColl, *Glasgow*
Stuart AC McDonald, *London*
Dermot P Mcgovern, *Oxford*
Giorgina Mieli-Vergani, *London*
Nikolai V Naoumov, *London*
John P Neoptolemos, *Liverpool*
James Neuberger, *Birmingham*
Philip Noel Newsome, *Birmingham*
Mark S Pearce, *Newcastle Upon Tyne*
Stephen P Pereira, *London*
D Mark Pritchard, *Liverpool*
Sakhawat Rahman, *London*
Stephen E Roberts, *Swansea*
Marco Senzolo, *Padova*
Soraya Shirazi-Beechey, *Liverpool*
Robert Sutton, *Liverpool*
Simon D Taylor-Robinson, *London*
Paris P Tekkis, *London*
Ulrich Thalheimer, *London*
David G Thompson, *Salford*
Nick P Thompson, *Newcastle*
David Tosh, *Bath*
Frank I Tovey, *London*
Chris Tselepis, *Birmingham*
Diego Vergani, *London*
Geoffrey Warhurst, *Salford*
Alastair John Watson, *Liverpool*
Peter J Whorwell, *Manchester*
Roger Williams, *London*
Karen L Wright, *Bath*
Min Zhao, *Foresterhill*

United States

Manal F Abdelmalek, *Durham*
Gary A Abrams, *Birmingham*
Maria T Abreu, *New York*
Reid B Adams, *Virginia*
Golo Ahlenstiel, *Bethesda*
BS Anand, *Houston*
Frank A Anania, *Atlanta*
M Ananthanarayanan, *New York*
Gavin E Arteel, *Louisville*
Jasmohan S Bajaj, *Milwaukee*
Subhas Banerjee, *Palo Alto*
Peter A Banks, *Boston*
Jamie S Barkin, *Miami Beach*
Kim E Barrett, *San Diego*
Marc D Basson, *Detroit*
Anthony J Bauer, *Pittsburgh*
Wallace F Berman, *Durham*
Timothy R Billiar, *Pittsburgh*
Edmund J Bini, *New York*
David G Binion, *Milwaukee*
Jennifer D Black, *Buffalo*
Herbert L Bonkovsky, *Charlotte*
Carla W Brady, *Durham*
Andrea D Branch, *New York*
Robert S Bresalier, *Houston*
Alan L Buchman, *Chicago*
Ronald W Busuttil, *Los Angeles*
Alan Cahill, *Philadelphia*
John M Carethers, *San Diego*
David L Carr-Locke, *Boston*
Maurice A Cerulli, *New York*
Ravi S Chari, *Nashville*
Jiande Chen, *Galveston*
Xian-Ming Chen, *Omaha*
Xin Chen, *San Francisco*
Ramsey Chi-man Cheung, *Palo Alto*
William D Chey, *Ann Arbor*
John Y Chiang, *Rootstown*
Parimal Chowdhury, *Arkansas*
Raymond T Chung, *Boston*
James M Church, *Cleveland*
Ram Chuttani, *Boston*
Mark G Clemens, *Charlotte*
Ana J Coito, *Los Angeles*
Vincent Coghlan, *Beaverton*
David Cronin II, *New Haven*
John Cuppoletti, *Cincinnati*
Mark J Czaja, *New York*
Peter V Danenberg, *Los Angeles*
Kiron M Das, New *Brunswick*
Conor P Delaney, *Cleveland*
Jose L del Pozo, *Rochester*
Sharon DeMorrow, *Temple*
Deborah L Diamond, *Seattle*
Douglas A Drossman, *Chapel Hill*
Katerina Dvorak, *Tucson*
Bijan Eghtesad, *Cleveland*
Hala El-Zimaity, *Houston*
Michelle Embree-Ku, *Providence*
Sukru Emre, New *Haven*
Douglas G Farmer, *Los Angeles*
Alessio Fasano, *Baltimore*
Mark A Feitelson, *Philadelphia*
Ariel E Feldstein, *Cleveland*
Alessandro Fichera, *Chicago*
Robert L Fine, *New York*
Magali Fontaine, *Stanford*
Chris E Forsmark, *Gainesville*
Glenn T Furuta, *Aurora*
Chandrashekhar R Gandhi, *Pittsburgh*
Susan L Gearhart, *Baltimore*
Xupeng Ge, *Boston*
Xin Geng, *New Brunswick*
M Eric Gershwin, *Suite*
Jean-Francois Geschwind, *Baltimore*
Ignacio Gil-Bazo, *New York*
Shannon S Glaser, *Temple*
Ajay Goel, *Dallas*
Richard M Green, *Chicago*
Julia B Greer, *Pittsburgh*

James H Grendell, *New York*
David R Gretch, *Seattle*
Stefano Guandalini, *Chicago*
Anna S Gukovskaya, *Los Angeles*
Sanjeev Gupta, *Bronx*
David J Hackam, *Pittsburgh*
Stephen B Hanauer, *Chicago*
Gavin Harewood, *Rochester*
Margaret M Heitkemper, *Washington*
Alan W Hemming, *Gainesville*
Samuel B Ho, *San Diego*
Peter R Holt, *New York*
Colin W Howden, *Chicago*
Hongjin Huang, *Alameda*
Jamal A Ibdah, *Columbia*
Atif Iqbal, *Omaha*
Hajime Isomoto, *Rochester*
Hartmut Jaeschke, *Tucson*
Dennis M Jensen, *Los Angeles*
Cheng Ji, *Los Angeles*
Leonard R Johnson, *Memphis*
Michael P Jones, *Chicago*
Peter J Kahrilas, *Chicago*
Anthony N Kalloo, *Baltimore*
Marshall M Kaplan, *Boston*
Neil Kaplowitz, *Los Angeles*
Serhan Karvar, *Los Angeles*
Rashmi Kaul, *Tulsa*
Jonathan D Kaunitz, *Los Angeles*
Ali Keshavarzian, *Chicago*
Miran Kim, *Providence*
Joseph B Kirsner, *Chicago*
Leonidas G Koniaris, *Miami*
Burton I Korelitz, *New York*
Robert J Korst, *New York*
Richard A Kozarek, *Seattle*
Alyssa M Krasinskas, *Pittsburgh*
Michael Kremer, *Chapel Hill*
Shiu-Ming Kuo, *Buffalo*
Paul Y Kwo, *Indianapolis*
Daryl Tan Yeung Lau, *Galvesto*
Stephen J Lanspa, *Omaha*
Joel E Lavine, *San Diego*
Bret Lashner, *Cleveland*
Dirk J van Leeuwen, *Lebanon*
Glen A Lehman, *Indianapolis*
Alex B Lentsch, *Cincinnati*
Andreas Leodolter, *La Jolla*
Gene LeSage, *Houston*
Josh Levitsky, *Chicago*
Cynthia Levy, *Gainesville*
Ming Li, *New Orleans*
Zhiping Li, *Baltimore*
Zhe-Xiong Lian, *Davis*
Lenard M Lichtenberger, *Houston*
Gary R Lichtenstein, *Philadelphia*
Otto Schiueh-Tzang Lin, *Seattle*
Martin Lipkin, *New York*
Chen Liu, *Gainesville*
Edward V Loftus, *Rocheste*
Robin G Lorenz, *Birmingham*
Michael R Lucey, *Madison*
James D Luketich, *Pittsburgh*
Guangbin Luo, *Cheveland*
Henry T Lynch, *Omaha*
Patrick M Lynch, *Houston*
John S Macdonald, *New York*
Bruce V MacFadyen, *Augusta*
Willis C Maddrey, *Dallas*
Ashok Malani, *Los Angeles*
Mercedes Susan Mandell, *Aurora*
Peter J Mannon, *Bethesda*
Charles M Mansbach, *Tennessee*
John F Di Mari, *Texas*
John M Mariadason, *Bronx*
Jorge A Marrero, *Ann Arbor*
Paul Martin, *New York*
Paulo Ney Aguiar Martins, *Boston*
Wendy M Mars, *Pittsburgh*
Laura E Matarese, *Pittsburgh*
Richard W McCallum, *Kansas*
Beth A McCormick, *Charlestown*
Lynne V McFarland, *Washington*
Kevin McGrath, *Pittsburgh*
Harihara Mehendale, *Monroe*
Ali Mencin, *New York*
Fanyin Meng, *Ohio*
Stephan Menne, *New York*
Didier Merlin, *Atlanta*
Howard Mertz, *Nashville*
George W Meyer, *Sacramento*
George Michalopoulos, *Pittsburgh*
James M Millis, *Chicago*
Fabrizio Michelassi, *New York*
Albert D Min, *New York*
Pramod K Mistry, *New Haven*
Emiko Mizoguchi, *Boston*
Smruti R Mohanty, *Chicago*
Satdarshan S Monga, *Pittsburgh*
Timothy H Moran, *Baltimore*
Peter L Moses, *Burlington*
Steven F Moss, *Providence*
Andrew J Muir, *Durham*
Milton G Mutchnick, *Detroit*
Masaki Nagaya, *Boston*
Victor Navarro, *Philadelphia*
Laura E Nagy, *Cleveland*
Hiroshi Nakagawa, *Philadelphia*
Douglas B Nelson, *Minneapolis*
Justin H Nguyen, *Florida*
Patrick G Northup, *Charlottesville*
Christopher O'Brien, *Miami*
Robert D Odze, *Boston*
Brant K Oelschlager, *Washington*
Curtis T Okamoto, *Los Angeles*
Stephen JD O'Keefe, *Pittsburgh*
Dimitry Oleynikov, *Omaha*
Stephen J Pandol, *Los Angeles*
Georgios Papachristou, *Pittsburgh*
Pankaj J Pasricha, *Galveston*
Zhiheng Pei, *New York*
Michael A Pezzone, *Pittsburgh*
CS Pitchumoni, *New Brunswiuc*
Paul J Pockros, *La Jolla*
Jay Pravda, *Gainesville*
Massimo Raimondo, *Jacksonville*
GS Raju, *Galveston*
Raymund R Razonable, *Minnesota*
Murray B Resnick, *Providence*
Adrian Reuben, *Charleston*
Douglas K Rex, *Indianapolis*
Victor E Reyes, *Galveston*
Basil Rigas, *New York*
Yehuda Ringel, *Chapel Hill*
Richard A Rippe, *Chapel Hill*
Maribel Rodriguez-Torres, *Santurce*
Marcos Rojkind, *Washington*
Philip Rosenthal, *San Francisco*
Barry Rosser, *Jacksonville Florida*
Hemant K Roy, *Evanston*
Sammy Saab, *Los Angeles*
Shawn D Safford, *Norfolk*
Dushyant V Sahani, *Boston*
Bruce E Sands, *Boston*
James M Scheiman, *Ann Arbor*
Eugene R Schiff, *Miami*
Nicholas J Shaheen, *Chapel Hill*
Vanessa M Shami, *Charlottesville*
Prateek Sharma, *Kansas City*
Harvey L Sharp, *Minneapolis*
Stuart Sherman, *Indianapolis*
Shivendra Shukla, *Columbia*
Alphonse E Sirica, *Virginia*
Shanthi V Sitaraman, *Atlanta*
Stuart J Spechler, *Dallas*
Shanthi Srinivasan, *Atlanta*
Michael Steer, *Boston*
Peter D Stevens, *New York*
Charmaine A Stewart, *Rochester*
Christian D Stone, *Saint Louis*
Gary D Stoner, *Columbus*
R Todd Stravitz, *Richmond*
Liping Su, *Chicago*
Christina Surawicz, *Seattle*
Robert W Summers, *Iowa City*
Wing-Kin Syn, *Durham*
Gyongyi Szabo, *Worcester*
Yvette Taché, *Los Angeles*
Seng-Lai Tan, *Seattle*
Andrzej S Tarnawski, *Orange*
K-M Tchou-Wong, *New York*
Jonathan P Terdiman, *San Francisco*
Neil D Theise, *New York*
Christopher C Thompson, *Boston*
Swan N Thung, *New York*
Michael Torbenson, *Baltimore*
Natalie J Torok, *Sacramento*
RA Travagli, *Baton Rouge*
George Triadafilopoulos, *Stanford*
Chung-Jyi Tsai, *Lexington*
Janet Elizabeth Tuttle-Newhall, *Durham*
Andrew Ukleja, *Florida*
Michael F Vaezi, *Nashville*
Hugo E Vargas, *Scottsdale*
Arnold Wald, *Wisconsin*
Scott A Waldman, *Philadelphia*
Jian-Ying Wang, *Baltimore*
Timothy C Wang, *New York*
Irving Waxman, *Chicago*
Steven A Weinman, *Galveston*
Steven D Wexner, *Weston*
Keith T Wilson, *Baltimore*
Jacqueline L Wolf, *Boston*
Jackie Wood, *Ohio*
George Y Wu, *Farmington*
Jian Wu, *Sacramento*
Samuel Wyllie, *Houston*
Wen Xie, *Pittsburgh*
Vijay Yajnik, *Boston*
Vincent W Yang, *Atlanta*
Francis Y Yao, *San Francisco*
Hal F Yee, *San Francisco*
Xiao-Ming Yin, *Pittsburgh*
Min You, *Tampa*
Zobair M Younossi, *Virginia*
Liqing Yu, *Winston-Salem*
David Yule, *Rochester*
Ruben Zamora, *Pittsburgh*
Michael E Zenilman, *New York*
Zhi Zhong, *Chapel Hill*
Michael A Zimmerman, *Colorado*
Stephen D Zucker, *Cincinnati*

Uruguay

Henry Cohen, *Montevideo*

[1]Passed away on October 20, 2007
[2]Passed away on June 11, 2007

National Journal Award
2005

World Journal of Gastroenterology®

Weekly Established in October 1995

Volume 14 Number 37
October 7, 2008

Contents

Contents

World Journal of Gastroenterology
Volume 14 **Number** 37 **October** 7, 2008

World Journal of Gastroenterology
Volume 14 **Number** 37 **October** 7, 2008

Contents

NAME OF JOURNAL
World Journal of Gastroenterology

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

EDITING
Editorial Board of *World Journal of Gastroenterology,* Room 903, Ocean International Center, Building D, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

PUBLISHING
The WJG Press and Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Ocean International Center, Building D, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

PRINTING
Beijing Kexin Printing House

OVERSEAS DISTRIBUTOR
Beijing Bureau for Distribution of Newspapers and Journals (Code No. 82-261)
China International Book Trading Corporation PO Box 399, Beijing, China (Code No. M4481)

PUBLICATION DATE
October 7, 2008

EDITOR-IN-CHIEF
Lian-Sheng Ma, *Beijing*

SUBSCRIPTION
RMB 50 Yuan for each issue, RMB 2400 Yuan for one year

CSSN
ISSN 1007-9327
CN 14-1219/R

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5617

World J Gastroenterol 2008 October 7; 14(37): 5617-5619
World Journal of Gastroenterology ISSN 1007-9327

EDITORIAL

How we have learned about the complexity of physiology, pathobiology and pharmacology of bile acids and biliary secretion

Jose JG Marin

Jose JG Marin, Laboratory of Experimental Hepatology and Drug Targeting, CIBERehd, University of Salamanca, Salamanca 37007, Spain
Author contributions: Marin JJG is the sole contributor to this paper.
Correspondence to: Jose JG Marin, Professor, Department of Physiology and Pharmacology, Campus Miguel de Unamuno E.I.D. S-09, Salamanca 37007, Spain. jjgmarin@usal.es
Telephone: +34-923-294674 Fax: +34-923-294669
Received: July 24, 2008 Revised: September 16, 2008
Accepted: September 23, 2008
Published online: October 7, 2008

Abstract

During the last decades the concept of bile secretion as merely a way to add detergent components to the intestinal mixture to facilitate fat digestion/absorption and to eliminate side products of heme metabolism has evolved considerably. In the series of mini-reviews that the *World Journal of Gastroenterology* is to publish in its section of "Highlight Topics", we will intend to give a brief but updated overview of our knowledge in this field. This introductory letter is intended to thank all scientists who have contributed to the development of this area of knowledge in gastroenterology.

Key words: Bile flow; Cholestasis; Hepatocyte; Liver; Transport

Marin JJG. How we have learned about the complexity of physiology, pathobiology and pharmacology of bile acids and biliary secretion. *World J Gastroenterol* 2008; 14(37): 5617-5619 Available from: URL: http://www.wjgnet.com/1007-9327/14/5617.asp DOI: http://dx.doi.org/10.3748/wjg.14.5617

INTRODUCTION

During the last decades the concept of bile secretion as merely a way to add detergent components to the intestinal mixture to facilitate fat digestion/absorption and to eliminate side products of heme metabolism has evolved considerably. In the series of mini-reviews that the *World Journal of Gastroenterology* is to publish in its section of "Highlight Topics", we intend to give a brief but updated overview of our knowledge in this field. The first opening title by Esteller[1], in addition to review the mechanisms of bile formation, will serve as an introduction to the rest of mini-reviews. Therefore, I will skip this task here and devote this letter to thank all scientists who have contributed to the development of this area of knowledge in gastroenterology. First of all, I wish to apology because only a limited number of them will be cited here and also for the fact that, in most cases, I will cite them by the main investigator of the group. I assume that we all know, in some cases personally, what excellent scientists have participated in the contributions published by these teams. Nevertheless, I hope that every important contribution and research group will be cited in the appropriate mini-review of the series. Let me start this brief historical overview by reminding you the important initial steps in understanding bile acid physiology given by Schiff[2] and Wheeler[3], which permitted the establishment of the bases for further investigations by Boyer[4], Erlinger[5], Anwer[6] and others on the role of osmotic mechanisms accounting for bile acid-dependent and-independent fractions of bile flow. The elucidation of the physical-chemical characteristics of bile acids has been determinant in understanding the role of different bile acid species in bile formation, bile acid-induced injury of liver tissue and cholestasis or, in contrast, hepatoprotection, as well as the process of gallstone formation or prevention, *etc.* This has been possible thank to the investigations carried out by many groups, including those of Hofmann[7], Carey[8], Small[7,8], Paumgartner[9], Reichen[9], Danielsson[10], Sjovall[10], Reyes[11], Setchell[12], Poupon[13] and others. It has been with the help of molecular biology techniques that Meier[14,15], Stieger[14], Hagenbuch[15,16], Keppler[17], Thompson[18] and others have been able to identify transport proteins involved in the efficient uptake and secretion of bile acids by the liver. Similar studies by Dawson[16] and others have also contributed to our understanding of the role of intestinal transporters in the so-called enterohepatic circulation of bile acids. Advances in the research of substrate-transporter interactions by many important groups, including several mentioned above in addition to those of Sugiyama[19], Klaassen[20] and Petzinger[21], has lead to Kramer[22] and others, including

our own laboratory[23,24] to undertake the development of promising new drugs based on the substrate selectivity of plasma membrane transporters and the possibility of either blocking their function or targeting bile acid derivatives toward healthy liver tissue or toward tumours located in the enterohepatic circuit. Over the last few years the novel concept of bile acids as signalling molecules in several cell types has emerged. Thus, the possibility that bile acids, as well as other oxysterols, may activate nuclear receptors and regulate the expression of enzymes and transporters was suggested from results obtained at the same time by three different groups[25-27], and developed by these groups, and others, such as those of Karpen[28], Kullak-Ublick[29], Chiang[30] and Houten[31]. These findings are helping us to understand how liver cells may respond to endocrine signals (e.g. during pregnancy) or to the accumulation of bile acids occurring in cholestasis, which is the subject of research currently carried out by several groups, including those of Trauner[32,33], Suchy[33,34], Ananthanarayanan[33,34], Lammert[35], Williamson[35], Accatino[33], Arrese[33,36] and others. Moreover, bile acids can also interact with plasma membrane elements and therefore participate in autocrine and paracrine functions, interacting with several signalling pathways as it is being brilliantly investigated by Haussinger, Kubitz, Keitel and the rest of this group[37], as well as by Dent[38], Fujino[39], Beuers[40], Dufour[41] and others. A complete view of biliary physiology also needs to consider the participation of cholangiocytes in bile formation and the knowledge of mechanisms of bile secretion of other important endogenous compounds, such as cholesterol, bilirubin, glutathione and xenobiotics, such as drugs and toxins. We owe many important contributions in these fields to LaRusso[42], Ballatori[43], Arias[44], Keppler[17], Sugiyama[19], Wolkoff[45], Oude Elferink[46], Meijer[46], Kuipers[46], Jansen[46], Groen[46], Groothuis[46], Ostrow[47], Fevery[48], Coleman[49], Berenson[50], Vore[51] and many others. As I said this editorial letter of gratitude is highly incomplete, but it would be even more so without mentioning the appreciation of many hepatologists to the supporting role of Dr. Falk and the Falk Foundation e.V. to the research in this field.

REFERENCES

1 **Esteller A**, Lopez MA. The effect of secretin and cholecystokinin-pancreozymin on the secretion of bile in the anaesthetized rabbit. *Q J Exp Physiol Cogn Med Sci* 1977; **62**: 353-359

2 **Schiff MI**. Gallenbildung, abhangig von der Aufsangung der Gallenstoffe. *Arch Ges Physiol Menschen Thiere* 1870; **3**: 598-613

3 **Wheeler HO**. Secretion of bile acids by the liver and their role in the formation of hepatic bile. *Arch Intern Med* 1972; **130**: 533-541

4 **Boyer JL**. New concepts of mechanisms of hepatocyte bile formation. *Physiol Rev* 1980; **60**: 303-326

5 **Erlinger S**. Physiology of bile flow. *Prog Liver Dis* 1972; **4**: 63-82

6 **Anwer MS**, Hegner D. Importance of solvent drag and diffusion in bile acid-dependent bile formation: ion substitution studies in isolated perfused rat liver. *Hepatology* 1982; **2**: 580-586

7 **Hofmann AF**, Small DM. Detergent properties of bile salts: correlation with physiological function. *Annu Rev Med* 1967; **18**: 333-376

8 **Carey MC**, Small DM. The characteristics of mixed micellar solutions with particular reference to bile. *Am J Med* 1970; **49**: 590-608

9 **Paumgartner G**, Reichen J, von Bergmann K, Preisig R. Elaboration of hepatocytic bile. *Bull N Y Acad Med* 1975; **51**: 455-471

10 **Danielsson H**, Sjovall J. Bile acid metabolism. *Annu Rev Biochem* 1975; **44**: 233-253

11 **Reyes H**. Review: intrahepatic cholestasis. A puzzling disorder of pregnancy. *J Gastroenterol Hepatol* 1997; **12**: 211-216

12 **Setchell KD**, Street JM. Inborn errors of bile acid synthesis. *Semin Liver Dis* 1987; **7**: 85-99

13 **Poupon RE**, Balkau B, Eschwege E, Poupon R. A multicenter, controlled trial of ursodiol for the treatment of primary biliary cirrhosis. UDCA-PBC Study Group. *N Engl J Med* 1991; **324**: 1548-1554

14 **Meier PJ**, Stieger B. Bile salt transporters. *Annu Rev Physiol* 2002; **64**: 635-661

15 **Hagenbuch B**, Meier PJ. The superfamily of organic anion transporting polypeptides. *Biochim Biophys Acta* 2003; **1609**: 1-18

16 **Hagenbuch B**, Dawson P. The sodium bile salt cotransport family SLC10. *Pflugers Arch* 2004; **447**: 566-570

17 **Keppler D**, Konig J. Hepatic secretion of conjugated drugs and endogenous substances. *Semin Liver Dis* 2000; **20**: 265-272

18 **Strautnieks SS**, Bull LN, Knisely AS, Kocoshis SA, Dahl N, Arnell H, Sokal E, Dahan K, Childs S, Ling V, Tanner MS, Kagalwalla AF, Nemeth A, Pawlowska J, Baker A, Mieli-Vergani G, Freimer NB, Gardiner RM, Thompson RJ. A gene encoding a liver-specific ABC transporter is mutated in progressive familial intrahepatic cholestasis. *Nat Genet* 1998; **20**: 233-238

19 **Shitara Y**, Sato H, Sugiyama Y. Evaluation of drug-drug interaction in the hepatobiliary and renal transport of drugs. *Annu Rev Pharmacol Toxicol* 2005; **45**: 689-723

20 **Klaassen CD**. Biliary excretion of xenobiotics. *CRC Crit Rev Toxicol* 1975; **4**: 1-30

21 **Petzinger E**, Geyer J. Drug transporters in pharmacokinetics. *Naunyn Schmiedebergs Arch Pharmacol* 2006; **372**: 465-475

22 **Kramer W**, Wess G. Bile acid transport systems as pharmaceutical targets. *Eur J Clin Invest* 1996; **26**: 715-732

23 **Dominguez MF**, Macias RI, Izco-Basurko I, de La Fuente A, Pascual MJ, Criado JM, Monte MJ, Yajeya J, Marin JJ. Low in vivo toxicity of a novel cisplatin-ursodeoxycholic derivative (Bamet-UD2) with enhanced cytostatic activity versus liver tumors. *J Pharmacol Exp Ther* 2001; **297**: 1106-1112

24 **Briz O**, Macias RI, Vallejo M, Silva A, Serrano MA, Marin JJ. Usefulness of liposomes loaded with cytostatic bile acid derivatives to circumvent chemotherapy resistance of enterohepatic tumors. *Mol Pharmacol* 2003; **63**: 742-750

25 **Makishima M**, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ, Shan B. Identification of a nuclear receptor for bile acids. *Science* 1999; **284**: 1362-1365

26 **Parks DJ**, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, Stimmel JB, Willson TM, Zavacki AM, Moore DD, Lehmann JM. Bile acids: natural ligands for an orphan nuclear receptor. *Science* 1999; **284**: 1365-1368

27 **Wang H**, Chen J, Hollister K, Sowers LC, Forman BM. Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. *Mol Cell* 1999; **3**: 543-553

28 **Karpen SJ**. Nuclear receptor regulation of hepatic function. *J Hepatol* 2002; **36**: 832-850

29 **Eloranta JJ**, Kullak-Ublick GA. Coordinate transcriptional regulation of bile acid homeostasis and drug metabolism. *Arch Biochem Biophys* 2005; **433**: 397-412

30 **Chiang JY**. Bile acid regulation of gene expression: roles of nuclear hormone receptors. *Endocr Rev* 2002; **23**: 443-463

31 **Houten SM**, Watanabe M, Auwerx J. Endocrine functions of bile acids. *EMBO J* 2006; **25**: 1419-1425
32 **Zollner G**, Marschall HU, Wagner M, Trauner M. Role of nuclear receptors in the adaptive response to bile acids and cholestasis: pathogenetic and therapeutic considerations. *Mol Pharm* 2006; **3**: 231-251
33 **Arrese M**, Trauner M, Ananthanarayanan M, Pizarro M, Solis N, Accatino L, Soroka C, Boyer JL, Karpen SJ, Miquel JF, Suchy FJ. Down-regulation of the Na+/taurocholate cotransporting polypeptide during pregnancy in the rat. *J Hepatol* 2003; **38**: 148-55
34 **Suchy FJ**, Ananthanarayanan M. Bile salt excretory pump: biology and pathobiology. *J Pediatr Gastroenterol Nutr* 2006; **43** Suppl 1: S10-S16
35 **Van Mil SW**, Milona A, Dixon PH, Mullenbach R, Geenes VL, Chambers J, Shevchuk V, Moore GE, Lammert F, Glantz AG, Mattsson LA, Whittaker J, Parker MG, White R, Williamson C. Functional variants of the central bile acid sensor FXR identified in intrahepatic cholestasis of pregnancy. *Gastroenterology* 2007; **133**: 507-516
36 **Arrese M**, Macias RI, Briz O, Perez MJ, Marin JJ. Molecular pathogenesis of intrahepatic cholestasis of pregnancy. *Expert Rev Mol Med* 2008; **10**: e9
37 **Keitel V**, Reinehr R, Gatsios P, Rupprecht C, Gorg B, Selbach O, Haussinger D, Kubitz R. The G-protein coupled bile salt receptor TGR5 is expressed in liver sinusoidal endothelial cells. *Hepatology* 2007; **45**: 695-704
38 **Dent P**, Fang Y, Gupta S, Studer E, Mitchell C, Spiegel S, Hylemon PB. Conjugated bile acids promote ERK1/2 and AKT activation via a pertussis toxin-sensitive mechanism in murine and human hepatocytes. *Hepatology* 2005; **42**: 1291-1299
39 **Kawamata Y**, Fujii R, Hosoya M, Harada M, Yoshida H, Miwa M, Fukusumi S, Habata Y, Itoh T, Shintani Y, Hinuma S, Fujisawa Y, Fujino M. A G protein-coupled receptor responsive to bile acids. *J Biol Chem* 2003; **278**: 9435-9440
40 **Wimmer R**, Hohenester S, Pusl T, Denk GU, Rust C, Beuers U. Tauroursodeoxycholic acid exerts anticholestatic effects by a cooperative cPKC{alpha}-/PKA-dependent mechanism in rat liver. *Gut* 2008; **57**: 1448-1454
41 **Wang L**, Piguet AC, Schmidt K, Tordjmann T, Dufour JF. Activation of CREB by tauroursodeoxycholic acid protects cholangiocytes from apoptosis induced by mTOR inhibition. *Hepatology* 2005; **41**: 1241-1251
42 **Bogert PT**, LaRusso NF. Cholangiocyte biology. *Curr Opin Gastroenterol* 2007; **23**: 299-305
43 **Ballatori N**, Hammond CL, Cunningham JB, Krance SM, Marchan R. Molecular mechanisms of reduced glutathione transport: role of the MRP/CFTR/ABCC and OATP/SLC21A families of membrane proteins. *Toxicol Appl Pharmacol* 2005; **204**: 238-255
44 **Arias IM**. Hepatic aspects of bilirubin metabolism. *Annu Rev Med* 1966; **17**: 257-274
45 **Wolkoff AW**. Hepatocellular sinusoidal membrane organic anion transport and transporters. *Semin Liver Dis* 1996; **16**: 121-127
46 **Oude Elferink RP**, Meijer DK, Kuipers F, Jansen PL, Groen AK, Groothuis GM. Hepatobiliary secretion of organic compounds; molecular mechanisms of membrane transport. *Biochim Biophys Acta* 1995; **1241**: 215-268
47 **Ostrow JD**. Photochemical and biochemical basis of the treatment of neonatal jaundice. *Prog Liver Dis* 1972; **4**: 447-462
48 **Fevery J**, Blanckaert N, Heirwegh KP, De Groote J. Bilirubin conjugates: formation and detection. *Prog Liver Dis* 1976; **5**: 183-214
49 **Coleman R**. Bile salts and biliary lipids. *Biochem Soc Trans* 1987; **15** Suppl: 68S-80S
50 **Berenson MM**, Marin JJ, Larsen R, Avner D. Effect of bile acids on hepatic protoporphyrin metabolism in perfused rat liver. *Gastroenterology* 1987; **93**: 1086-1093
51 **Vore M**. Canalicular transport: discovery of ATP-dependent mechanisms. *Toxicol Appl Pharmacol* 1993; **118**: 2-7

S- Editor Li DL **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5620

World J Gastroenterol 2008 October 7; 14(37): 5620-5629
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Jose JG Marin, Professor, *Series Editor*

Endocrine and paracrine role of bile acids

Verena Keitel, Ralf Kubitz, Dieter Häussinger

Verena Keitel, Ralf Kubitz, Dieter Häussinger, Clinic of Gastroenterology, Hepatology and Infectiology, Heinrich-Heine-University, Moorenstrasse 5, Düsseldorf D-40225, Germany
Author contributions: Keitel V, Kubitz R and Häussinger D contributed equally in writing the paper.
Correspondence to: Dr. Dieter Häussinger, Professor, Clinic of Gastroenterology, Hepatology and Infectiology, Heinrich-Heine-University, Moorenstrasse 5, Düsseldorf D-40225, Germany. haeussin@uni-duesseldorf.de
Telephone: +49-211-8116330 Fax: +49-211-818752
Received: July 24, 2008 Revised: September 16, 2008
Accepted: September 23, 2008
Published online: October 7, 2008

Abstract

Bile acids are not only important for the absorption of dietary lipids and fat soluble vitamins but are signalling molecules with diverse endocrine and paracrine functions. Bile acids regulate bile acid, lipid and glucose metabolism and modulate temperature and energy homeostasis. Furthermore, bile acids can not only promote cell proliferation and liver regeneration but can also induce programmed cell death. Bile acid functions are mediated through different pathways which comprise the activation of nuclear hormone receptors, of intracellular kinases and of the plasma membrane-bound, G-protein coupled bile acid receptor TGR5/Gp-bar-1.

Key words: Bile acids; Farnesoid X receptor; TGR5; Glucose metabolism; Lipid metabolism

Peer reviewer: Tom H Karlsen, MD, Institute of Immunology, Rikshospitalet University Hospital, Oslo N-0027, Norway

Keitel V, Kubitz R, Häussinger D. Endocrine and paracrine role of bile acids. *World J Gastroenterol* 2008; 14(37): 5620-5629 Available from: URL: http://www.wjgnet.com/1007-9327/14/5620.asp DOI: http://dx.doi.org/10.3748/wjg.14.5620

INTRODUCTION

Bile acids are synthesized from cholesterol in the liver and secreted into bile and the small intestine, where they enable the absorption of dietary lipids and fat soluble vitamins. Most of the bile acids are reabsorbed in the terminal ileum, carried through the enterocytes into the portal blood and returned to the liver, where uptake into the hepatocytes and subsequent transport into bile completes their enterohepatic circulation[1,2]. However, bile acids are not only important for the absorption of dietary lipids but are also signalling molecules, which regulate bile acid synthesis, conjugation and transport and modulate lipid, glucose and energy homeostasis. Bile acids can activate nuclear hormone receptors, such as the farnesoid X receptor (FXR)[3,4], which is a ligand-activated transcription factor[5]. FXR is highly expressed in liver, intestine and kidney[6], and regulates the expression of proteins involved in bile acid synthesis, detoxification and secretion in these organs thereby maintaining bile acid homeostasis[7-9].

Bile acid effects can also be mediated by transcription-independent pathways through the activation of intracellular protein kinases, such as mitogen-activated protein kinases and protein kinase C[10-15]. Furthermore, bile acids can modulate ion fluxes leading to an increase in intracellular ceramide levels, activation of NADPH oxidase, cell shrinkage and apoptosis[16,17]. Recently, the first plasma membrane bound, G-protein coupled bile acid receptor TGR5 (Gpbar-1, M-Bar) has been described[18,19]. *TGR5* mRNA was detected in many tissues, the highest expression being present in macrophages/monocytes, placenta, gallbladder, liver and intestine[18-20]. While FXR has been identified as important regulator of bile acid, lipid and glucose homeostasis, the role of TGR5 in bile acid mediated signalling is largely unclear. However, it has been suggested that bile acids *via* TGR5 induce energy expenditure in brown adipose tissue and skeletal muscle[21], modulate hepatic microcirculation[22], and suppress cytokine release in macrophages[18].

BILE ACIDS REGULATE BILE ACID HOMEOSTASIS

Sustained elevation of bile acid levels leads to severe liver damage and may promote the development of liver tumors. Patients with progressive familial intrahepatic cholestasis type 2 (PFIC-2), which is caused by mutations in the bile salt export pump (BSEP, ABCB11)[23], as well as FXR knockout mice[24] have chronically elevated

serum bile acid levels and spontaneously develop hepatocellular carcinomas[25-27]. In order to prevent bile acid-dependent liver damage and carcinogenesis the bile acid pool size needs to be tightly regulated.

It is well established that bile acids regulate their own biosynthesis, detoxification and transport both in the liver and the intestine. The identification of bile acids as natural ligands for the FXR[3,4] led to the discovery that FXR is essential for the maintenance of bile acid homeostasis, and protects the organism from the accumulation of potentially toxic bile acids (for reviews see[7,28-30]).

In the liver bile acids suppress the transcription of the CYP7A1, the rate-controlling enzyme of bile acid synthesis in the neutral pathway, and of the CYP8B1, the enzyme controlling the production of cholic acid[30]. This repression is mediated through several distinct, mainly FXR-dependent pathways. Activation of FXR by bile acids leads to an upregulation of the small heterodimer partner-1 (SHP)[31,32], which in turn interacts with several transcription factors, including hepatocyte nuclear factor-4α (HNF-4α) and liver receptor homolog-1 (LRH-1), which both bind to the bile acid-response elements (BAREs) in the promoters of the *CYP7A1* and *CYP8B1* genes[31-35]. Using SHP knockout mice, it has become apparent that the bile acid-dependent repression of CYP7A1 is mediated through several redundant pathways, since loss of SHP impaired but did not abolish bile acid-dependent feedback repression of CYP7A1 in these mice[36,37]. Activation of FXR by bile acids leads to the increased expression of fibroblast growth factor 19 (FGF-19) in hepatocytes, which is subsequently secreted and binds its receptor FGFR4 on adjacent hepatocytes. Stimulation of FGFR4 by FGF-19 leads to receptor dimerization, autophosphorylation and activation of the c-Jun N-terminal kinase (JNK) pathway resulting in the repression of CYP7A1 transcription[38]. Therefore FXR can inhibit bile acid synthesis through tow independent pathways. The importance of the FXR-dependent feedback mechanisms on bile acid synthesis is underscored by the finding, that FXR knockout mice failed to downregulate CYP7A and CYP8B in response to bile acid feeding, resulting in an elevation of serum bile acid levels and death of 30% of knockout mice by day 7[24]. Besides the FXR-dependent feedback regulation, bile acid signalling through the G-protein coupled bile acid receptor TGR5 (Gpbar-1) may also contribute to the regulation of bile acid synthesis and bile acid homeostasis. TGR5 knockout mice showed a significantly reduced bile acid pool size[39] and feeding of cholic acid to these mice did not repress the expression of CYP7A1 as observed in wild type littermates[20], indicating that TGR5 may play a role in the regulation of bile acid synthesis and bile acid pool size.

Conjugation of bile acids with taurine and glycine is mediated by the enzymes bile acid coenzyme A (CoA) synthetase and bile acid-CoA amino acid N-acetyltransferase and controlled by FXR[40]. Bile acids are secreted from the hepatocyte into bile across the canalicular membrane by the BSEP. Bile acids induce transcription of BSEP through FXR thereby promoting their own excretion[41,42]. Besides BSEP, two other canalicular transport proteins MDR3 (ABCB4), which is a phospholipid flippase, and MRP2 (ABCC2), which excretes bilirubin and organic anions, are also positively regulated by FXR[43,44]. Thus FXR facilitates bile flow and excretion of cholephilic compounds from hepatocytes. Short-term regulation of canalicular bile secretion occurs through exocytic insertion and endocytic retrieval of transport proteins into and from the plasma membrane and is differentially regulated by hydrophobic and hydrophilic bile acids (for reviews see[45-49]). Hydrophobic bile acids such as taurolithocholylsulfate (TLCS), glycochenodeoxycholate (GCDC) and taurochenodeoxycholate (TCDC) induce NADPH oxidase-dependent hepatocyte shrinkage[16]. Hepatocyte shrinkage is known to lead to the rapid retrieval of MRP2 and BSEP from the canalicular membrane and thus impairs bile formation[50-54]. On the contrary, taurocholate induces hepatocyte swelling and subsequent choleresis, which in the presence of increased hepatocyte bile acid load may serve as a feed-forward regulation leading to the insertion of BSEP into the canalicular membrane and enhanced bile acid excretion[52,54,55]. The choleretic effect of tauroursodeoxycholate, which is widely used for the treatment of cholestatic liver diseases, is mediated through integrins and Src, which trigger the downstream activation of mitogen-activated protein kinases resulting in the recruitment of BSEP to the canalicular membrane[12,13]. Therefore bile acids can alter bile flow both on the long-term scale on the transcriptional level *via* activation of FXR as well as on the short-term scale at the posttranscriptional level through alteration in hepatocyte hydration and subsequent translocation of transporter proteins.

In the intestine bile acid uptake is mediated by the apical sodium dependent bile salt transporter (ASBT, SLC10A2)[56]. While marked interspecies differences in the regulation of ASBT exist[7], human ASBT expression is controlled by FXR[57,58]. The ileal bile acid binding protein (IBABP) binds bile acids within the enterocytes and transfers them to the basolateral membrane for secretion into the portal blood. Despite the regulation of IBABP by FXR[3,59] and the downregulation of IBABP in FXR knockout mice[24], the enterohepatic circulation of bile acids in these mice is increased[60], suggesting the existence of further, FXR-independent pathways for the regulation of bile acid absorption in the ileum.

In the liver bile acids are taken up from the portal blood into hepatocytes across the sinusoidal membrane by several transport proteins. The sodium-dependent sodium taurocholate cotransporting peptide (NTCP, SLC10A1) accounts for the uptake of more than 80% of conjugated bile acids into hepatocytes. Similar to CYP7A1 and ASBT, bile acids repress the expression of NTCP *via* FXR-SHP-dependent mechanisms[61,62]. However, further SHP-independent mechanisms for the regulation of NTCP expression must exist, since *NTCP* mRNA levels in SHP knockout mice were unchanged[36]. Bile acid uptake by NTCP may also be regulated posttranslationally at the protein level, in an FXR-

independent way, since livers from patients with PFIC showed a significant downregulation of the NTCP protein, while *NTCP* mRNA levels were similar to control livers[63]. The other bile acid uptake transporters in the liver belong to the family of organic anion transporters (OATPs). While OATP1B1 expression is downregulated by FXR[64,65], OATP1B3 expression is enhanced[66]. The repression of bile acid uptake transporters (NTCP, OATP1B1) may protect hepatocytes from accumulation of toxic bile acids, while the upregulation of OATP1B3 may help to maintain excretion of xenobiotics under cholestatic conditions[66]. Upregulation of the basolateral bile acid export pumps multidrug resistance protein 4 (MRP4, ABCC4) and the organic solute transporter α/β (OSTα/β) is also observed under cholestatic conditions in humans and rodents[63,67,68]. While the expression of Ostα/β is induced by bile acids *via* FXR[69], the upregulation of MRP4 by bile acids is independent of FXR and is observed both on the translational and posttranslational level[63,67,69]. Bile acids increase the expression of different detoxification enzymes through FXR, such as the UDP-glucuronosyltranserase UGT2B4 and the sulfotransferase SULT2A1[40,70,71]. In hepatocytes, bile acids activate FXR, which in turn induces suppression of de novo synthesis, enhances conjugation and detoxification and increases efflux both across the canalicular as well as the basolateral membrane thereby preventing hepatic accumulation of bile acids and liver damage. This is supported by the finding that administration of a synthetic FXR agonist (GW4064) reduced liver injury in rat models of cholestasis[72].

BILE ACIDS MODULATE GLUCOSE HOMEOSTASIS

A link between bile acids and glucose homeostasis was recognized when patients with type Ⅱ diabetes were treated for dyslipidemia with cholestyramine, a bile acid sequestrant[73]. Besides lowering total cholesterol and LDL cholesterol, cholestyramine also improved glycemia and reduced blood glucose levels by 13%[73]. Treatment of isolated rat hepatocytes with glucose increased *FXR* mRNA levels and target gene expression through activation of the pentose phosphate pathway[74] and thus may interfere with bile acid metabolism. Insulin counteracted the glucose effects in these experiments[74]. Vice versa, several recent studies indicate that bile acids modulate hepatic gluconeogenesis, however, the data are controversial. While the expression of the rate-limiting enzyme of gluconeogenesis phosphoenolpyruvate carboxykinase (PEPCK) was upregulated by bile acids *via* FXR in both primary hepatocytes and hepatoma cell lines in one recent study[75], bile acids repressed PEPCK expression in an FXR-dependent and FXR-independent, SHP-dependent mechanism in both HepG2 cells and mouse liver in several other studies[76-79]. Other enzymes involved in gluconeogenesis, such as the glucose-6 phosphatase (G6Pase) and the fructose 1,6-bisphosphatase (FBP1) were also downregulated by bile acids through SHP[77]. Furthermore, activation of FXR may not only reduce gluconeogenesis, but also increase glycogen synthesis the liver, thereby lowering blood glucose levels[80]. These findings are supported by studies with FXR knockout mice, which show increased gluconeogenesis in the liver and reduced glucose uptake in the skeletal muscle, resulting in elevated blood glucose levels[79]. Inhibition of gluconeogenesis by bile acids was ameliorated in FXR knockout mice but observed in wildtype mice, however, a synthetic FXR agonist (GW4064) failed to repress gluconeogenesis in wildtype animals[79]. The authors give two possible explanations for these observations. Either, the *in vivo* pharmacokinetics of the synthetic agonist prevented long term effects on glucose homeostasis, or bile acids alter gluconeogenesis not only through activation of FXR but also through additional signalling pathways, which together lead to the suppression of gluconeogenesis[79]. The later seems more likely and it has recently been described that bile acids stimulated secretion of glucagon-like peptide 1 through activation of the membrane bound bile acid receptor TGR5[81] and thus may lower serum glucose levels and improve insulin resistance in non-insulin dependent diabetes[82].

Furthermore, bile acids can modulate glucose homeostasis through alteration of the cellular hydration state. Hypo-osmotic hepatocyte swelling, which can be induced by taurocholate[52], stimulates within minutes glycogen synthesis[83] as well as flux through the pentose phosphate pathway[84] and inhibits glycolysis and glycogenolysis[85-87]. Besides these rapid mechanisms, cell swelling leads to an increase in *PEPCK* mRNA expression thus linking hepatocyte hydration to carbohydrate metabolism[88]. Hydrophobic bile acids, such as taurolithocholylsulfate (TLCS) and glycochenodeoxycholate (GCDC), can confer insulin resistance in hepatocytes through inhibition of insulin-dependent phosphorylation of the insulin receptor and impaired recruitment of phosphoinositide-3 (PI-3) kinase as well as protein kinase B activation[89]. Tauroursodeoxycholate (TUDCA) restored insulin signalling under these conditions[89]. Cell shrinkage induced by hydrophobic bile acids independently contributes to insulin resistance since hepatocyte shrinkage itself has insulin-antagonistic effects, stimulating glycogenolysis, proteolysis and inhibiting glycogen and protein synthesis[46,85,86,90,91]. However, hyper-osmolarity does not affect insulin-dependent activation of the insulin receptor but rather acts on the level of or downstream to the PI-3 kinase[92]. These data suggest that hydrophobic bile acids confer insulin resistance in the liver through several distinct pathways.

Taken together, these data demonstrate that bile acids through FXR regulate gluconeogenesis, glycogen synthesis and insulin sensitivity[93], therefore FXR agonists may be useful in the treatment of type Ⅱ diabetes. Apart from FXR, the bile acid receptor TGR5 may also influence glucose metabolism and insulin sensitivity *via* GLP-1 secretion, but additional *in vivo*

studies are required to elucidate the role of TGR5 for glucose homeostasis. Furthermore, hydrophobic bile acids confer insulin resistance in the liver, which may be ameliorated by TUDCA treatment.

BILE ACIDS AFFECT LIPID HOMEOSTASIS

The existence of an inverse relationship between bile acid and triglyceride levels has been recognized over three decades ago, when patients with cholesterol gallstones were treated with chenodeoxycholic acid (CDCA)[94]. The administration of CDCA was accompanied by a reduction in plasma triglyceride levels, which was more pronounced in patients with endogenous hypertriglyceridemia[94], therefore CDCA has been suggested for treatment of hypertriglyceridemia[94-96]. The opposite effect was observed in patients who took bile acid binding resins, which lead to an increased production of very low density lipoproteins (VLDL) and an elevation of serum triglyceride levels[97,98]. The mechanisms underlying this bile acid effect on lipid metabolism remained largely unknown, however, the recent findings that FXR knockout mice have elevated liver and plasma triglyceride levels[24,99] and that the expression of a variety of lipid homeostasis-modulating proteins is regulated by FXR (summarized in[7,100]) suggest, that bile acids modulate lipid homeostasis mainly through activation of FXR.

The triglyceride lowering effect of bile acids involves different pathways downstream of FXR. Activation of FXR increases the expression of SHP, which in turn inhibits the liver X receptor (LXR) mediated upregulation of the sterol regulatory element-binding protein-1c (SREBP-1c) and its target gene expression[100]. SREBP-1c serves as master regulator of fatty acid and triglyceride synthesis[101,102]. Both, basal and inducible expression of SREBP-1c is controlled by LXR[101]. Activation of SREBP-1c positively regulates the expression of genes involved in fatty acid synthesis, such as acetyl CoA synthetase, acetyl CoA carboxylase and fatty acid synthetase[102]. Bile acids inhibit SREBP-1c mediated lipogenesis in an FXR-SHP dependent manner, since the effect was also observed after administration of a synthetic FXR agonist and was attenuated in SHP knockout mice[100]. Besides the inhibition of fatty acid and triglyceride synthesis, bile acids can also stimulate the triglyceride clearance from serum through activation of lipoprotein lipase and subsequent hydrolysis of triglycerides in VLDL and chylomicrons. Bile acids activate FXR and subsequently induce the expression of the apolipoprotein CⅡ (ApoC Ⅱ)[103], which serves as an activator of lipoprotein lipase[104,105]. Furthermore, the expression of another apolipoprotein, ApoCⅢ, which is known to inhibit LPL activity[106], is repressed by activation of FXR[107], thus enhancing the hydrolysis of triglycerides from VLDL. The expression of angiopoetin-like protein 3 (Angptl3), which can also inhibit lipoprotein lipase function[108], was decreased by bile acids[100] and may contribute further to bile acid induced triglyceride hydrolysis. Bile acids, such as CDCA, repressed the expression of the microsomal triglyceride transfer protein (MTP) and ApoB[109], which are essential for the assembly of chylomicrons and VLDL particles[110]. A synthetic FXR agonist failed to lower MTP levels[109] and MTP expression in FXR knockout mice was reduced[100] suggesting a FXR-independent action of bile acids on MTP.

Bile acids may also influence lipid metabolism through cross-talk with peroxisome proliferator-activated receptor α (PPARα)-dependent pathways. PPARα is a nuclear receptor, which plays an important role in lipid and lipoprotein metabolism and controls several enzymes critical for fatty acid oxidation[111]. Expression of human PPARα is directly regulated by bile acids and a synthetic FXR agonist, however, murine PPARα expression is not responsive to bile acids[112]. Furthermore, expression of pyruvate dehydrogenase kinase-4 (PDK4) is upregulated by bile acids *via* FXR, which leads to inactivation of the pyruvate dehydrogenase complex with subsequent suppression of glycolysis and increased fatty acid oxidation[113]. Thus, bile acids may lower triglyceride levels *via* FXR-dependent activation of PPARα and PDK4 eventually leading to increased fatty acid oxidation.

Not only VLDL synthesis and turnover is affected by bile acids, but also HDL clearance is modulated through activation of FXR. FXR knockout mice have increased serum VLDL, LDL and HDL levels[24,99]. Reduced expression of the scavenger receptor BI (SRBI) and subsequent delay in hepatic uptake of HDL cholesterol esters account for the increase in plasma HDL in FXR knockout mice demonstrating that SRBI is a target gene of FXR[99].

In summary, bile acids reduce triglyceride levels through several mechanisms. Besides inhibition of triglyceride and VLDL synthesis *via* SREBP-1c-dependent mechanisms and FXR-independent repression of MTP and ApoB, bile acids also promote VLDL clearance *via* effects on ApoCⅡ, ApoCⅢ and Angptl3 and induce fatty acid oxidation *via* PPARα and PDK4. Furthermore, the close connection between bile acid synthesis and cholesterol elimination, underscores the important role of bile acids in the regulation of triglyceride and cholesterol homeostasis.

BILE ACIDS INCREASE ENERGY EXPENDITURE

Bile acids not only regulate lipid and glucose homeostasis but also modulate energy metabolism *via* the membrane-bound bile acid receptor TGR5 (Gpbar-1). TGR5 is coupled to stimulatory G-protein and activation of the receptor by bile acids increases intracellular cyclic AMP levels[18,19]. Administration of bile acids to mice increased energy expenditure in brown adipose tissue and prevented development of obesity and insulin resistance[21]. The stimulation of TGR5 induced an increased expression of the cAMP-dependent iodothyronine deiodinase type 2 (D2), which converts inactive thyroxine (T4) to active 3,5,3'-triiodothyronine and is crucial for adaptive

thermogenesis in brown adipose tissue. The observed metabolic effect of bile acids was abolished in D2 knockout mice. In isolated brown adipocytes and human skeletal myocytes bile acids stimulated TGR5, increased intracellular cAMP, activated deiodinase D2 and lead to an increase in oxygen consumption[21]. Tissues important for thermogenesis, such as brown adipose tissue in rodents and skeletal muscle in humans co-express TGR5 and deiodinase 2 and the TGR5-cAMP-D2 pathway may be activated in these tissues in response to plasma bile acid levels, which rise after food intake, and may therefore regulate diet induced thermogenesis[21]. The important role of TGR5 for energy homeostasis was confirmed by studies with TGR5 knockout mice. Administration of a high fat diet to TGR5 knockout mice led to a significant accumulation of adipose tissue and increase in body weight as compared to wild type littermates[39].

FURTHER ENDOCRINE EFFECTS OF BILE ACIDS

Bile acids have immunomodulatory functions as oral administration of bile acids successfully reduced endotoxin-related complications following surgery in patients with obstructive cholestasis[114-116]. This beneficial effect of bile acids was attributed to inhibition of endotoxin-induced TNF-α production[117].

The bile acid receptor TGR5 is highly expressed in CD14-positive monocytes, alveolar macrophages[18] and Kupffer cells[118], which are resident macrophages in the liver. Bile acids can alter macrophage function by affecting phagocytic activity as well as cytokine production[119-123]. Bile acids inhibited LPS-induced cytokine production in alveolar macrophages and Kupffer cells in a TGR5-cAMP-dependent manner[18,118], thus supporting the hypothesis that TGR5 plays an important role for macrophage function. Activation of TGR5 in Kupffer cells may prevent excessive cytokine production in sepsis-associated or obstructive cholestasis thereby alleviating liver injury.

Bile acids can prevent bacterial overgrowth and mucosal injury in the small intestine of mice. Subsequently, in mice elimination of bile acids from the intestine through bile duct ligation resulted in bacterial propagation, disruption of the epithelial barrier and translocation of bacteria across the mucosa into lymph nodes. These effects could be prevented by the administration of a synthetic FXR agonist, revealing a novel mechanism of bile acid dependent enteroprotection[124]. The antibacterial action of the FXR agonist (GW4064) were indirect since no bacteriostatic effects were observed when ileal contents were cultured in the presence of GW4064[124].

Bile acids may also interfere with interleukin-6 (IL-6) signalling in hepatocytes. IL-6 has hepatoprotective properties and can ameliorate liver injury induced by obstructive cholestasis[125,126]. It has been shown recently, that the hydrophobic bile acid glycochenodeoxycholate (GCDC) impairs IL-6 induced activation of the signal transducer and activator of transcription (STAT) 3 through caspase-dependent cleavage of the IL-6 receptor glycoprotein 130 as well as through MAP kinase-dependent inhibition of STAT3 phosphorylation, thus contributing to the bile acid-induced liver injury[127,128].

Taken together, bile acids may influence immune functions in the intestine and the liver through activation of FXR-, TGR5-, MAP-kinase- and caspase-dependent pathways.

A role for bile acids in regulation of hepatic microcirculation has also been recently suggested. TGR5 is localized in the plasma membrane of sinusoidal endothelial cells of rat liver and is responsive to bile acids[22]. Stimulation of TGR5 increased cAMP levels, activated protein kinase A and lead to a serine phosphorylation of endothelial NO synthetase and subsequent elevation of NO production[22]. Furthermore, activation of TGR5 resulted in an enhanced serine phosphorylation of the CD95 receptor[22], which may promote the internalization of the receptor from the plasma membrane thereby preventing CD95-induced apoptosis as it has been observed in hepatocytes[129].

A role for bile acids in liver regeneration has also recently been identified[130]. Increased bile acid levels after partial hepatectomy promoted liver regeneration which was attenuated in FXR knockout mice, suggesting that these effects are mediated by FXR[130]. Bile acids have been shown to affect cell proliferation, survival and cell death. These effects of bile acids are reviewed in another article in this issue.

Protein metabolism in the liver can also be affected by bile acids *via* alterations in cell hydration. Taurocholate can induce hepatocyte swelling, which is sensed by integrins and leads to the activation of Src-type kinases and mitogen-activated kinases and subsequent inhibition of autophagic proteolysis at the level of autophagosome formation[131-133] (for reviews on hydration-dependent pathways see[46,86,90,91]).

PARACRINE ROLE OF BILE ACIDS

After excretion into bile, bile acids are in close contact with cholangiocytes, the bile duct forming epithelial cells. Bile acids modulate cholangiocyte secretion, proliferation and survival in a paracrine manner (for a recent review see[134]). Taurocholic acid and taurolithocholic acid increased secretin stimulated intracellular cAMP levels and Cl^-/HCO_3^- exchanger activity thus promoting ductal secretion and bile flow[135,136]. These effects were dependent on bile acid uptake into the cholangiocytes and led to an activation of phosphatidylinositol 3-kinase (PI3-K), followed by an increase in cAMP[135,137]. Ursodeoxycholic acid induced ductal secretion through CFTR-dependent secretion of ATP into bile, which in turn activated apical purinergic P_{2Y} receptors and stimulated chloride efflux and fluid secretion[138]. While taurocholic and taurolithocholic acid stimulated proliferation of bile ducts and increased bile duct mass up to 3-fold[135,136], ursodeoxycholic acid has been shown

to inhibit cholangiocyte proliferation in bile duct ligated rats[139], and to prevent cholangiocyte apoptosis in vagotomized, bile-duct ligated rats[140].

Recently, TGR5 has been detected in cholangiocytes of rat liver[118]. Among the endogenous bile acids taurolithocholic acid represents the most potent TGR5 agonist with an EC_{50} of 0.29 μmol/L[141]. The finding that hydrophobic bile acids, such as taurolithocholic acid, increase cAMP in cholangiocytes[135] suggest, that TGR5 may play a role in cholangiocyte secretion and proliferation and prevention of apoptosis[22].

REFERENCES

1 **Hofmann AF**. Biliary secretion and excretion in health and disease: current concepts. *Ann Hepatol* 2007; **6**: 15-27
2 **Kullak-Ublick GA**, Stieger B, Meier PJ. Enterohepatic bile salt transporters in normal physiology and liver disease. *Gastroenterology* 2004; **126**: 322-342
3 **Makishima M**, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ, Shan B. Identification of a nuclear receptor for bile acids. *Science* 1999; **284**: 1362-1365
4 **Parks DJ**, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, Stimmel JB, Willson TM, Zavacki AM, Moore DD, Lehmann JM. Bile acids: natural ligands for an orphan nuclear receptor. *Science* 1999; **284**: 1365-1368
5 **Mangelsdorf DJ**, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM. The nuclear receptor superfamily: the second decade. *Cell* 1995; **83**: 835-839
6 **Forman BM**, Goode E, Chen J, Oro AE, Bradley DJ, Perlmann T, Noonan DJ, Burka LT, McMorris T, Lamph WW, Evans RM, Weinberger C. Identification of a nuclear receptor that is activated by farnesol metabolites. *Cell* 1995; **81**: 687-693
7 **Kalaany NY**, Mangelsdorf DJ. LXRS and FXR: the yin and yang of cholesterol and fat metabolism. *Annu Rev Physiol* 2006; **68**: 159-191
8 **Claudel T**, Staels B, Kuipers F. The Farnesoid X receptor: a molecular link between bile acid and lipid and glucose metabolism. *Arterioscler Thromb Vasc Biol* 2005; **25**: 2020-2030
9 **Scotti E**, Gilardi F, Godio C, Gers E, Krneta J, Mitro N, De Fabiani E, Caruso D, Crestani M. Bile acids and their signaling pathways: eclectic regulators of diverse cellular functions. *Cell Mol Life Sci* 2007; **64**: 2477-2491
10 **Beuers U**, Throckmorton DC, Anderson MS, Isales CM, Thasler W, Kullak-Ublick GA, Sauter G, Koebe HG, Paumgartner G, Boyer JL. Tauroursodeoxycholic acid activates protein kinase C in isolated rat hepatocytes. *Gastroenterology* 1996; **110**: 1553-1563
11 **Gupta S**, Stravitz RT, Dent P, Hylemon PB. Down-regulation of cholesterol 7alpha-hydroxylase (CYP7A1) gene expression by bile acids in primary rat hepatocytes is mediated by the c-Jun N-terminal kinase pathway. *J Biol Chem* 2001; **276**: 15816-15822
12 **Häussinger D**, Kurz AK, Wettstein M, Graf D, Vom Dahl S, Schliess F. Involvement of integrins and Src in tauroursodeoxycholate-induced and swelling-induced choleresis. *Gastroenterology* 2003; **124**: 1476-1487
13 **Kurz AK**, Graf D, Schmitt M, Vom Dahl S, Häussinger D. Tauroursodesoxycholate-induced choleresis involves p38(MAPK) activation and translocation of the bile salt export pump in rats. *Gastroenterology* 2001; **121**: 407-419
14 **Qiao D**, Chen W, Stratagoules ED, Martinez JD. Bile acid-induced activation of activator protein-1 requires both extracellular signal-regulated kinase and protein kinase C signaling. *J Biol Chem* 2000; **275**: 15090-15098
15 **Schliess F**, Kurz AK, vom Dahl S, Häussinger D. Mitogen-activated protein kinases mediate the stimulation of bile acid secretion by tauroursodeoxycholate in rat liver. *Gastroenterology* 1997; **113**: 1306-1314
16 **Becker S**, Reinehr R, Grether-Beck S, Eberle A, Häussinger D. Hydrophobic bile salts trigger ceramide formation through endosomal acidification. *Biol Chem* 2007; **388**: 185-196
17 **Reinehr R**, Becker S, Keitel V, Eberle A, Grether-Beck S, Häussinger D. Bile salt-induced apoptosis involves NADPH oxidase isoform activation. *Gastroenterology* 2005; **129**: 2009-2031
18 **Kawamata Y**, Fujii R, Hosoya M, Harada M, Yoshida H, Miwa M, Fukusumi S, Habata Y, Itoh T, Shintani Y, Hinuma S, Fujisawa Y, Fujino M. A G protein-coupled receptor responsive to bile acids. *J Biol Chem* 2003; **278**: 9435-9440
19 **Maruyama T**, Miyamoto Y, Nakamura T, Tamai Y, Okada H, Sugiyama E, Nakamura T, Itadani H, Tanaka K. Identification of membrane-type receptor for bile acids (M-BAR). *Biochem Biophys Res Commun* 2002; **298**: 714-719
20 **Vassileva G**, Golovko A, Markowitz L, Abbondanzo SJ, Zeng M, Yang S, Hoos L, Tetzloff G, Levitan D, Murgolo NJ, Keane K, Davis HR Jr, Hedrick J, Gustafson EL. Targeted deletion of Gpbar1 protects mice from cholesterol gallstone formation. *Biochem J* 2006; **398**: 423-430
21 **Watanabe M**, Houten SM, Mataki C, Christoffolete MA, Kim BW, Sato H, Messaddeq N, Harney JW, Ezaki O, Kodama T, Schoonjans K, Bianco AC, Auwerx J. Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. *Nature* 2006; **439**: 484-489
22 **Keitel V**, Reinehr R, Gatsios P, Rupprecht C, Gorg B, Selbach O, Häussinger D, Kubitz R. The G-protein coupled bile salt receptor TGR5 is expressed in liver sinusoidal endothelial cells. *Hepatology* 2007; **45**: 695-704
23 **Strautnieks SS**, Bull LN, Knisely AS, Kocoshis SA, Dahl N, Arnell H, Sokal E, Dahan K, Childs S, Ling V, Tanner MS, Kagalwalla AF, Nemeth A, Pawlowska J, Baker A, Mieli-Vergani G, Freimer NB, Gardiner RM, Thompson RJ. A gene encoding a liver-specific ABC transporter is mutated in progressive familial intrahepatic cholestasis. *Nat Genet* 1998; **20**: 233-238
24 **Sinal CJ**, Tohkin M, Miyata M, Ward JM, Lambert G, Gonzalez FJ. Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. *Cell* 2000; **102**: 731-744
25 **Knisely AS**, Strautnieks SS, Meier Y, Stieger B, Byrne JA, Portmann BC, Bull LN, Pawlikowska L, Bilezikci B, Ozcay F, Laszlo A, Tiszlavicz L, Moore L, Raftos J, Arnell H, Fischler B, Nemeth A, Papadogiannakis N, Cielecka-Kuszyk J, Jankowska I, Pawlowska J, Melin-Aldana H, Emerick KM, Whitington PF, Mieli-Vergani G, Thompson RJ. Hepatocellular carcinoma in ten children under five years of age with bile salt export pump deficiency. *Hepatology* 2006; **44**: 478-486
26 **Strautnieks SS**, Byrne JA, Pawlikowska L, Cebecauerova D, Rayner A, Dutton L, Meier Y, Antoniou A, Stieger B, Arnell H, Ozcay F, Al-Hussaini HF, Bassas AF, Verkade HJ, Fischler B, Nemeth A, Kotalova R, Shneider BL, Cielecka-Kuszyk J, McClean P, Whitington PF, Sokal E, Jirsa M, Wali SH, Jankowska I, Pawlowska J, Mieli-Vergani G, Knisely AS, Bull LN, Thompson RJ. Severe bile salt export pump deficiency: 82 different ABCB11 mutations in 109 families. *Gastroenterology* 2008; **134**: 1203-1214
27 **Yang F**, Huang X, Yi T, Yen Y, Moore DD, Huang W. Spontaneous development of liver tumors in the absence of the bile acid receptor farnesoid X receptor. *Cancer Res* 2007; **67**: 863-867
28 **Chiang JY**. Bile acid regulation of gene expression: roles of nuclear hormone receptors. *Endocr Rev* 2002; **23**: 443-463
29 **Chiang JY**. Bile acid regulation of hepatic physiology: III. Bile acids and nuclear receptors. *Am J Physiol Gastrointest Liver Physiol* 2003; **284**: G349-G356
30 **Russell DW**. The enzymes, regulation, and genetics of bile

acid synthesis. *Annu Rev Biochem* 2003; **72**: 137-174
31 **Goodwin B**, Jones SA, Price RR, Watson MA, McKee DD, Moore LB, Galardi C, Wilson JG, Lewis MC, Roth ME, Maloney PR, Willson TM, Kliewer SA. A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. *Mol Cell* 2000; **6**: 517-526
32 **Lu TT**, Makishima M, Repa JJ, Schoonjans K, Kerr TA, Auwerx J, Mangelsdorf DJ. Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. *Mol Cell* 2000; **6**: 507-515
33 **Stroup D**, Crestani M, Chiang JY. Identification of a bile acid response element in the cholesterol 7 alpha-hydroxylase gene CYP7A. *Am J Physiol* 1997; **273**: G508-G517
34 **Yang Y**, Zhang M, Eggertsen G, Chiang JY. On the mechanism of bile acid inhibition of rat sterol 12alpha-hydroxylase gene (CYP8B1) transcription: roles of alpha-fetoprotein transcription factor and hepatocyte nuclear factor 4alpha. *Biochim Biophys Acta* 2002; **1583**: 63-73
35 **Zhang M**, Chiang JY. Transcriptional regulation of the human sterol 12alpha-hydroxylase gene (CYP8B1): roles of heaptocyte nuclear factor 4alpha in mediating bile acid repression. *J Biol Chem* 2001; **276**: 41690-41699
36 **Wang L**, Lee YK, Bundman D, Han Y, Thevananther S, Kim CS, Chua SS, Wei P, Heyman RA, Karin M, Moore DD. Redundant pathways for negative feedback regulation of bile acid production. *Dev Cell* 2002; **2**: 721-731
37 **Kerr TA**, Saeki S, Schneider M, Schaefer K, Berdy S, Redder T, Shan B, Russell DW, Schwarz M. Loss of nuclear receptor SHP impairs but does not eliminate negative feedback regulation of bile acid synthesis. *Dev Cell* 2002; **2**: 713-720
38 **Holt JA**, Luo G, Billin AN, Bisi J, McNeill YY, Kozarsky KF, Donahee M, Wang DY, Mansfield TA, Kliewer SA, Goodwin B, Jones SA. Definition of a novel growth factor-dependent signal cascade for the suppression of bile acid biosynthesis. *Genes Dev* 2003; **17**: 1581-1591
39 **Maruyama T**, Tanaka K, Suzuki J, Miyoshi H, Harada N, Nakamura T, Miyamoto Y, Kanatani A, Tamai Y. Targeted disruption of G protein-coupled bile acid receptor 1 (Gpbar1/M-Bar) in mice. *J Endocrinol* 2006; **191**: 197-205
40 **Pircher PC**, Kitto JL, Petrowski ML, Tangirala RK, Bischoff ED, Schulman IG, Westin SK. Farnesoid X receptor regulates bile acid-amino acid conjugation. *J Biol Chem* 2003; **278**: 27703-27711
41 **Ananthanarayanan M**, Balasubramanian N, Makishima M, Mangelsdorf DJ, Suchy FJ. Human bile salt export pump promoter is transactivated by the farnesoid X receptor/bile acid receptor. *J Biol Chem* 2001; **276**: 28857-28865
42 **Plass JR**, Mol O, Heegsma J, Geuken M, Faber KN, Jansen PL, Muller M. Farnesoid X receptor and bile salts are involved in transcriptional regulation of the gene encoding the human bile salt export pump. *Hepatology* 2002; **35**: 589-596
43 **Huang L**, Zhao A, Lew JL, Zhang T, Hrywna Y, Thompson JR, de Pedro N, Royo I, Blevins RA, Pelaez F, Wright SD, Cui J. Farnesoid X receptor activates transcription of the phospholipid pump MDR3. *J Biol Chem* 2003; **278**: 51085-51090
44 **Kast HR**, Goodwin B, Tarr PT, Jones SA, Anisfeld AM, Stoltz CM, Tontonoz P, Kliewer S, Willson TM, Edwards PA. Regulation of multidrug resistance-associated protein 2 (ABCC2) by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor. *J Biol Chem* 2002; **277**: 2908-2915
45 **Häussinger D**, Schmitt M, Weiergraber O, Kubitz R. Short-term regulation of canalicular transport. *Semin Liver Dis* 2000; **20**: 307-321
46 **Häussinger D**, Kubitz R, Reinehr R, Bode JG, Schliess F. Molecular aspects of medicine: from experimental to clinical hepatology. *Mol Aspects Med* 2004; **25**: 221-360
47 **Kipp H**, Arias IM. Intracellular trafficking and regulation of canalicular ATP-binding cassette transporters. *Semin Liver Dis* 2000; **20**: 339-351
48 **Kubitz R**, Helmer A, Häussinger D. Biliary transport systems: short-term regulation. *Methods Enzymol* 2005; **400**: 542-557
49 **Kubitz R**, Häussinger D. Osmoregulation of bile formation. *Methods Enzymol* 2007; **428**: 313-324
50 **Dombrowski F**, Kubitz R, Chittattu A, Wettstein M, Saha N, Häussinger D. Electron-microscopic demonstration of multidrug resistance protein 2 (Mrp2) retrieval from the canalicular membrane in response to hyperosmolarity and lipopolysaccharide. *Biochem J* 2000; **348** (Pt 1): 183-188
51 **Hallbrucker C**, Lang F, Gerok W, Häussinger D. Cell swelling increases bile flow and taurocholate excretion into bile in isolated perfused rat liver. *Biochem J* 1992; **281** (Pt 3): 593-595
52 **Häussinger D**, Hallbrucker C, Saha N, Lang F, Gerok W. Cell volume and bile acid excretion. *Biochem J* 1992; **288** (Pt 2): 681-689
53 **Kubitz R**, D'urso D, Keppler D, Häussinger D. Osmode-pendent dynamic localization of the multidrug resistance protein 2 in the rat hepatocyte canalicular membrane. *Gastroenterology* 1997; **113**: 1438-1442
54 **Schmitt M**, Kubitz R, Lizun S, Wettstein M, Häussinger D. Regulation of the dynamic localization of the rat Bsep gene-encoded bile salt export pump by anisoosmolarity. *Hepatology* 2001; **33**: 509-518
55 **Noe B**, Schliess F, Wettstein M, Heinrich S, Häussinger D. Regulation of taurocholate excretion by a hypo-osmolarity-activated signal transduction pathway in rat liver. *Gastroenterology* 1996; **110**: 858-865
56 **Dawson PA**, Haywood J, Craddock AL, Wilson M, Tietjen M, Kluckman K, Maeda N, Parks JS. Targeted deletion of the ileal bile acid transporter eliminates enterohepatic cycling of bile acids in mice. *J Biol Chem* 2003; **278**: 33920-33927
57 **Oelkers P**, Kirby LC, Heubi JE, Dawson PA. Primary bile acid malabsorption caused by mutations in the ileal sodium-dependent bile acid transporter gene (SLC10A2). *J Clin Invest* 1997; **99**: 1880-1887
58 **Neimark E**, Chen F, Li X, Shneider BL. Bile acid-induced negative feedback regulation of the human ileal bile acid transporter. *Hepatology* 2004; **40**: 149-156
59 **Grober J**, Zaghini I, Fujii H, Jones SA, Kliewer SA, Willson TM, Ono T, Besnard P. Identification of a bile acid-responsive element in the human ileal bile acid-binding protein gene. Involvement of the farnesoid X receptor/9-cis-retinoic acid receptor heterodimer. *J Biol Chem* 1999; **274**: 29749-29754
60 **Kok T**, Hulzebos CV, Wolters H, Havinga R, Agellon LB, Stellaard F, Shan B, Schwarz M, Kuipers F. Enterohepatic circulation of bile salts in farnesoid X receptor-deficient mice: efficient intestinal bile salt absorption in the absence of ileal bile acid-binding protein. *J Biol Chem* 2003; **278**: 41930-41937
61 **Denson LA**, Sturm E, Echevarria W, Zimmerman TL, Makishima M, Mangelsdorf DJ, Karpen SJ. The orphan nuclear receptor, shp, mediates bile acid-induced inhibition of the rat bile acid transporter, ntcp. *Gastroenterology* 2001; **121**: 140-147
62 **Jung D**, Hagenbuch B, Fried M, Meier PJ, Kullak-Ublick GA. Role of liver-enriched transcription factors and nuclear receptors in regulating the human, mouse, and rat NTCP gene. *Am J Physiol Gastrointest Liver Physiol* 2004; **286**: G752-G761
63 **Keitel V**, Burdelski M, Warskulat U, Kuhlkamp T, Keppler D, Häussinger D, Kubitz R. Expression and localization of hepatobiliary transport proteins in progressive familial intrahepatic cholestasis. *Hepatology* 2005; **41**: 1160-1172
64 **Jung D**, Hagenbuch B, Gresh L, Pontoglio M, Meier PJ, Kullak-Ublick GA. Characterization of the human OATP-C (SLC21A6) gene promoter and regulation of liver-specific OATP genes by hepatocyte nuclear factor 1 alpha. *J Biol*

Chem 2001; **276**: 37206-37214
65 **Jung D**, Kullak-Ublick GA. Hepatocyte nuclear factor 1 alpha: a key mediator of the effect of bile acids on gene expression. *Hepatology* 2003; **37**: 622-631
66 **Jung D**, Podvinec M, Meyer UA, Mangelsdorf DJ, Fried M, Meier PJ, Kullak-Ublick GA. Human organic anion transporting polypeptide 8 promoter is transactivated by the farnesoid X receptor/bile acid receptor. *Gastroenterology* 2002; **122**: 1954-1966
67 **Denk GU**, Soroka CJ, Takeyama Y, Chen WS, Schuetz JD, Boyer JL. Multidrug resistance-associated protein 4 is up-regulated in liver but down-regulated in kidney in obstructive cholestasis in the rat. *J Hepatol* 2004; **40**: 585-591
68 **Schuetz EG**, Strom S, Yasuda K, Lecureur V, Assem M, Brimer C, Lamba J, Kim RB, Ramachandran V, Komoroski BJ, Venkataramanan R, Cai H, Sinal CJ, Gonzalez FJ, Schuetz JD. Disrupted bile acid homeostasis reveals an unexpected interaction among nuclear hormone receptors, transporters, and cytochrome P450. *J Biol Chem* 2001; **276**: 39411-39418
69 **Wagner M**, Fickert P, Zollner G, Fuchsbichler A, Silbert D, Tsybrovskyy O, Zatloukal K, Guo GL, Schuetz JD, Gonzalez FJ, Marschall HU, Denk H, Trauner M. Role of farnesoid X receptor in determining hepatic ABC transporter expression and liver injury in bile duct-ligated mice. *Gastroenterology* 2003; **125**: 825-838
70 **Song CS**, Echchgadda I, Baek BS, Ahn SC, Oh T, Roy AK, Chatterjee B. Dehydroepiandrosterone sulfotransferase gene induction by bile acid activated farnesoid X receptor. *J Biol Chem* 2001; **276**: 42549-42556
71 **Barbier O**, Torra IP, Sirvent A, Claudel T, Blanquart C, Duran-Sandoval D, Kuipers F, Kosykh V, Fruchart JC, Staels B. FXR induces the UGT2B4 enzyme in hepatocytes: a potential mechanism of negative feedback control of FXR activity. *Gastroenterology* 2003; **124**: 1926-1940
72 **Liu Y**, Binz J, Numerick MJ, Dennis S, Luo G, Desai B, MacKenzie KI, Mansfield TA, Kliewer SA, Goodwin B, Jones SA. Hepatoprotection by the farnesoid X receptor agonist GW4064 in rat models of intra- and extrahepatic cholestasis. *J Clin Invest* 2003; **112**: 1678-1687
73 **Garg A**, Grundy SM. Cholestyramine therapy for dyslipidemia in non-insulin-dependent diabetes mellitus. A short-term, double-blind, crossover trial. *Ann Intern Med* 1994; **121**: 416-422
74 **Duran-Sandoval D**, Mautino G, Martin G, Percevault F, Barbier O, Fruchart JC, Kuipers F, Staels B. Glucose regulates the expression of the farnesoid X receptor in liver. *Diabetes* 2004; **53**: 890-898
75 **Stayrook KR**, Bramlett KS, Savkur RS, Ficorilli J, Cook T, Christe ME, Michael LF, Burris TP. Regulation of carbohydrate metabolism by the farnesoid X receptor. *Endocrinology* 2005; **146**: 984-991
76 **De Fabiani E**, Mitro N, Gilardi F, Caruso D, Galli G, Crestani M. Coordinated control of cholesterol catabolism to bile acids and of gluconeogenesis via a novel mechanism of transcription regulation linked to the fasted-to-fed cycle. *J Biol Chem* 2003; **278**: 39124-39132
77 **Yamagata K**, Daitoku H, Shimamoto Y, Matsuzaki H, Hirota K, Ishida J, Fukamizu A. Bile acids regulate gluconeogenic gene expression via small heterodimer partner-mediated repression of hepatocyte nuclear factor 4 and Foxo1. *J Biol Chem* 2004; **279**: 23158-23165
78 **Yamagata K**, Yoshimochi K, Daitoku H, Hirota K, Fukamizu A. Bile acid represses the peroxisome proliferator-activated receptor-gamma coactivator-1 promoter activity in a small heterodimer partner-dependent manner. *Int J Mol Med* 2007; **19**: 751-756
79 **Ma K**, Saha PK, Chan L, Moore DD. Farnesoid X receptor is essential for normal glucose homeostasis. *J Clin Invest* 2006; **116**: 1102-1109
80 **Zhang Y**, Lee FY, Barrera G, Lee H, Vales C, Gonzalez FJ, Willson TM, Edwards PA. Activation of the nuclear receptor FXR improves hyperglycemia and hyperlipidemia in diabetic mice. *Proc Natl Acad Sci USA* 2006; **103**: 1006-1011
81 **Katsuma S**, Hirasawa A, Tsujimoto G. Bile acids promote glucagon-like peptide-1 secretion through TGR5 in a murine enteroendocrine cell line STC-1. *Biochem Biophys Res Commun* 2005; **329**: 386-390
82 **Houten SM**, Watanabe M, Auwerx J. Endocrine functions of bile acids. *EMBO J* 2006; **25**: 1419-1425
83 **Baquet A**, Hue L, Meijer AJ, van Woerkom GM, Plomp PJ. Swelling of rat hepatocytes stimulates glycogen synthesis. *J Biol Chem* 1990; **265**: 955-959
84 **Saha N**, Stoll B, Lang F, Häussinger D. Effect of anisotonic cell-volume modulation on glutathione-S-conjugate release, t-butylhydroperoxide metabolism and the pentose-phosphate shunt in perfused rat liver. *Eur J Biochem* 1992; **209**: 437-444
85 **Graf J**, Haddad P, Haeussinger D, Lang F. Cell volume regulation in liver. *Ren Physiol Biochem* 1988; **11**: 202-220
86 **Häussinger D**, Lang F. Cell volume in the regulation of hepatic function: a mechanism for metabolic control. *Biochim Biophys Acta* 1991; **1071**: 331-350
87 **Häussinger D**. The role of cellular hydration in the regulation of cell function. *Biochem J* 1996; **313** (Pt 3): 697-710
88 **Newsome WP**, Warskulat U, Noe B, Wettstein M, Stoll B, Gerok W, Häussinger D. Modulation of phosphoenolpyruvate carboxykinase mRNA levels by the hepatocellular hydration state. *Biochem J* 1994; **304** (Pt 2): 555-560
89 **Mannack G**, Graf D, Donner MM, Richter L, Gorg B, Vom Dahl S, Häussinger D, Schliess F. Taurolithocholic acid-3 sulfate impairs insulin signaling in cultured rat hepatocytes and perfused rat liver. *Cell Physiol Biochem* 2008; **21**: 137-150
90 **Häussinger D**, Hallbrucker C, vom Dahl S, Decker S, Schweizer U, Lang F, Gerok W. Cell volume is a major determinant of proteolysis control in liver. *FEBS Lett* 1991; **283**: 70-72
91 **Häussinger D**. The role of cellular hydration in the regulation of cell function. *Biochem J* 1996; **313** (Pt 3): 697-710
92 **Schliess F**, von Dahl S, Häussinger D. Insulin resistance induced by loop diuretics and hyperosmolarity in perfused rat liver. *Biol Chem* 2001; **382**: 1063-1069
93 **Cariou B**, van Harmelen K, Duran-Sandoval D, van Dijk TH, Grefhorst A, Abdelkarim M, Caron S, Torpier G, Fruchart JC, Gonzalez FJ, Kuipers F, Staels B. The farnesoid X receptor modulates adiposity and peripheral insulin sensitivity in mice. *J Biol Chem* 2006; **281**: 11039-11049
94 **Fromm H**, Eschler A, Tollner D, Canzler H, Schmidt FW. [In vivo dissolving of gall-stones: the effect of chenodeoxycholic acid. (author's transl)] *Dtsch Med Wochenschr* 1975; **100**: 1619-1624
95 **Camarri E**, Fici F, Marcolongo R. Influence of chenodeoxycholic acid on serum triglycerides in patients with primary hypertriglyceridemia. *Int J Clin Pharmacol Biopharm* 1978; **16**: 523-526
96 **Carulli N**, Ponz de Leon M, Podda M, Zuin M, Strata A, Frigerio G, Digrisolo A. Chenodeoxycholic acid and ursodeoxycholic acid effects in endogenous hypertriglyceridemias. A controlled double-blind trial. *J Clin Pharmacol* 1981; **21**: 436-442
97 **Crouse JR 3rd**. Hypertriglyceridemia: a contraindication to the use of bile acid binding resins. *Am J Med* 1987; **83**: 243-248
98 **Beil U**, Crouse JR, Einarsson K, Grundy SM. Effects of interruption of the enterohepatic circulation of bile acids on the transport of very low density-lipoprotein triglycerides. *Metabolism* 1982; **31**: 438-444
99 **Lambert G**, Amar MJ, Guo G, Brewer HB Jr, Gonzalez FJ, Sinal CJ. The farnesoid X-receptor is an essential regulator of cholesterol homeostasis. *J Biol Chem* 2003; **278**: 2563-2570
100 **Watanabe M**, Houten SM, Wang L, Moschetta A, Mangelsdorf DJ, Heyman RA, Moore DD, Auwerx J. Bile acids lower triglyceride levels via a pathway involving FXR,

SHP, and SREBP-1c. *J Clin Invest* 2004; **113**: 1408-1418
101 **Repa JJ**, Liang G, Ou J, Bashmakov Y, Lobaccaro JM, Shimomura I, Shan B, Brown MS, Goldstein JL, Mangelsdorf DJ. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes Dev* 2000; **14**: 2819-2830
102 **Horton JD**, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* 2002; **109**: 1125-1131
103 **Kast HR**, Nguyen CM, Sinal CJ, Jones SA, Laffitte BA, Reue K, Gonzalez FJ, Willson TM, Edwards PA. Farnesoid X-activated receptor induces apolipoprotein C-II transcription: a molecular mechanism linking plasma triglyceride levels to bile acids. *Mol Endocrinol* 2001; **15**: 1720-1728
104 **Breckenridge WC**, Little JA, Steiner G, Chow A, Poapst M. Hypertriglyceridemia associated with deficiency of apolipoprotein C-II. *N Engl J Med* 1978; **298**: 1265-1273
105 **LaRosa JC**, Levy RI, Herbert P, Lux SE, Fredrickson DS. A specific apoprotein activator for lipoprotein lipase. *Biochem Biophys Res Commun* 1970; **41**: 57-62
106 **Ginsberg HN**, Le NA, Goldberg IJ, Gibson JC, Rubinstein A, Wang-Iverson P, Norum R, Brown WV. Apolipoprotein B metabolism in subjects with deficiency of apolipoproteins CIII and AI. Evidence that apolipoprotein CIII inhibits catabolism of triglyceride-rich lipoproteins by lipoprotein lipase in vivo. *J Clin Invest* 1986; **78**: 1287-1295
107 **Claudel T**, Inoue Y, Barbier O, Duran-Sandoval D, Kosykh V, Fruchart J, Fruchart JC, Gonzalez FJ, Staels B. Farnesoid X receptor agonists suppress hepatic apolipoprotein CIII expression. *Gastroenterology* 2003; **125**: 544-555
108 **Inaba T**, Matsuda M, Shimamura M, Takei N, Terasaka N, Ando Y, Yasumo H, Koishi R, Makishima M, Shimomura I. Angiopoietin-like protein 3 mediates hypertriglyceridemia induced by the liver X receptor. *J Biol Chem* 2003; **278**: 21344-21351
109 **Hirokane H**, Nakahara M, Tachibana S, Shimizu M, Sato R. Bile acid reduces the secretion of very low density lipoprotein by repressing microsomal triglyceride transfer protein gene expression mediated by hepatocyte nuclear factor-4. *J Biol Chem* 2004; **279**: 45685-45692
110 **Hussain MM**, Shi J, Dreizen P. Microsomal triglyceride transfer protein and its role in apoB-lipoprotein assembly. *J Lipid Res* 2003; **44**: 22-32
111 **Evans RM**, Barish GD, Wang YX. PPARs and the complex journey to obesity. *Nat Med* 2004; **10**: 355-361
112 **Pineda Torra I**, Claudel T, Duval C, Kosykh V, Fruchart JC, Staels B. Bile acids induce the expression of the human peroxisome proliferator-activated receptor alpha gene via activation of the farnesoid X receptor. *Mol Endocrinol* 2003; **17**: 259-272
113 **Savkur RS**, Bramlett KS, Michael LF, Burris TP. Regulation of pyruvate dehydrogenase kinase expression by the farnesoid X receptor. *Biochem Biophys Res Commun* 2005; **329**: 391-396
114 **Cahill CJ**. Prevention of postoperative renal failure in patients with obstructive jaundice--the role of bile salts. *Br J Surg* 1983; **70**: 590-595
115 **Pain JA**, Bailey ME. Prevention of endotoxaemia in obstructive jaundice--a comparative study of bile salts. *HPB Surg* 1988; **1**: 21-27
116 **Pain JA**, Cahill CJ, Gilbert JM, Johnson CD, Trapnell JE, Bailey ME. Prevention of postoperative renal dysfunction in patients with obstructive jaundice: a multicentre study of bile salts and lactulose. *Br J Surg* 1991; **78**: 467-469
117 **Greve JW**, Gouma DJ, Buurman WA. Bile acids inhibit endotoxin-induced release of tumor necrosis factor by monocytes: an in vitro study. *Hepatology* 1989; **10**: 454-458
118 **Keitel V**, Donner M, Winandy S, Kubitz R, Häussinger D. Expression and function of the bile acid receptor TGR5 in Kupffer cells. *Biochem Biophys Res Commun* 2008; **372**: 78-84
119 **Calmus Y**, Guechot J, Podevin P, Bonnefis MT, Giboudeau J, Poupon R. Differential effects of chenodeoxycholic and ursodeoxycholic acids on interleukin 1, interleukin 6 and tumor necrosis factor-alpha production by monocytes. *Hepatology* 1992; **16**: 719-723
120 **Funaoka M**, Komatsu M, Toyoshima I, Mikami K, Ono T, Hoshino T, Kato J, Kuramitsu T, Ishii T, Masamune O. Tauroursodeoxycholic acid enhances phagocytosis of the cultured rat Kupffer cell. *J Gastroenterol Hepatol* 1999; **14**: 652-658
121 **Minter RM**, Fan MH, Sun J, Niederbichler A, Ipaktchi K, Arbabi S, Hemmila MR, Remick DG, Wang SC, Su GL. Altered Kupffer cell function in biliary obstruction. *Surgery* 2005; **138**: 236-245
122 **Scott-Conner CE**, Grogan JB. The pathophysiology of biliary obstruction and its effect on phagocytic and immune function. *J Surg Res* 1994; **57**: 316-336
123 **Sung JJ**, Go MY. Reversible Kupffer cell suppression in biliary obstruction is caused by hydrophobic bile acids. *J Hepatol* 1999; **30**: 413-418
124 **Inagaki T**, Moschetta A, Lee YK, Peng L, Zhao G, Downes M, Yu RT, Shelton JM, Richardson JA, Repa JJ, Mangelsdorf DJ, Kliewer SA. Regulation of antibacterial defense in the small intestine by the nuclear bile acid receptor. *Proc Natl Acad Sci USA* 2006; **103**: 3920-3925
125 **Sewnath ME**, van der Poll T, van Noorden CJ, ten Kate FJ, Gouma DJ. Cholestatic interleukin-6-deficient mice succumb to endotoxin-induced liver injury and pulmonary inflammation. *Am J Respir Crit Care Med* 2004; **169**: 413-420
126 **Ezure T**, Sakamoto T, Tsuji H, Lunz JG 3rd, Murase N, Fung JJ, Demetris AJ. The development and compensation of biliary cirrhosis in interleukin-6-deficient mice. *Am J Pathol* 2000; **156**: 1627-1639
127 **Graf D**, Kohlmann C, Haselow K, Gehrmann T, Bode JG, Häussinger D. Bile acids inhibit interleukin-6 signaling via gp130 receptor-dependent and -independent pathways in rat liver. *Hepatology* 2006; **44**: 1206-1217
128 **Graf D**, Haselow K, Munks I, Bode JG, Häussinger D. Caspase-mediated cleavage of the signal-transducing IL-6 receptor subunit gp130. *Arch Biochem Biophys* 2008; **477**: 330-338
129 **Reinehr R**, Häussinger D. Inhibition of bile salt-induced apoptosis by cyclic AMP involves serine/threonine phosphorylation of CD95. *Gastroenterology* 2004; **126**: 249-262
130 **Huang W**, Ma K, Zhang J, Qatanani M, Cuvillier J, Liu J, Dong B, Huang X, Moore DD. Nuclear receptor-dependent bile acid signaling is required for normal liver regeneration. *Science* 2006; **312**: 233-236
131 **vom Dahl S**, Dombrowski F, Schmitt M, Schliess F, Pfeifer U, Häussinger D. Cell hydration controls autophagosome formation in rat liver in a microtubule-dependent way downstream from p38MAPK activation. *Biochem J* 2001; **354**: 31-36
132 **Häussinger D**, Schliess F, Dombrowski F, Vom Dahl S. Involvement of p38MAPK in the regulation of proteolysis by liver cell hydration. *Gastroenterology* 1999; **116**: 921-935
133 **vom Dahl S**, Schliess F, Reissmann R, Gorg B, Weiergraber O, Kocalkova M, Dombrowski F, Häussinger D. Involvement of integrins in osmosensing and signaling toward autophagic proteolysis in rat liver. *J Biol Chem* 2003; **278**: 27088-27095
134 **Xia X**, Francis H, Glaser S, Alpini G, LeSage G. Bile acid interactions with cholangiocytes. *World J Gastroenterol* 2006; **12**: 3553-3563
135 **Alpini G**, Glaser S, Robertson W, Phinizy JL, Rodgers RE, Caligiuri A, LeSage G. Bile acids stimulate proliferative and secretory events in large but not small cholangiocytes. *Am J Physiol* 1997; **273**: G518-G529
136 **Alpini G**, Glaser SS, Ueno Y, Rodgers R, Phinizy JL, Francis H, Baiocchi L, Holcomb LA, Caligiuri A, LeSage GD. Bile acid feeding induces cholangiocyte proliferation and secretion: evidence for bile acid-regulated ductal secretion.

Gastroenterology 1999; **116**: 179-186
137 **Alpini G**, Glaser S, Alvaro D, Ueno Y, Marzioni M, Francis H, Baiocchi L, Stati T, Barbaro B, Phinizy JL, Mauldin J, Lesage G. Bile acid depletion and repletion regulate cholangiocyte growth and secretion by a phosphatidylinositol 3-kinase-dependent pathway in rats. *Gastroenterology* 2002; **123**: 1226-1237
138 **Fiorotto R**, Spirli C, Fabris L, Cadamuro M, Okolicsanyi L, Strazzabosco M. Ursodeoxycholic acid stimulates cholangiocyte fluid secretion in mice via CFTR-dependent ATP secretion. *Gastroenterology* 2007; **133**: 1603-1613
139 **Alpini G**, Baiocchi L, Glaser S, Ueno Y, Marzioni M, Francis H, Phinizy JL, Angelico M, Lesage G. Ursodeoxycholate and tauroursodeoxycholate inhibit cholangiocyte growth and secretion of BDL rats through activation of PKC alpha. *Hepatology* 2002; **35**: 1041-1052
140 **Marzioni M**, Francis H, Benedetti A, Ueno Y, Fava G, Venter J, Reichenbach R, Mancino MG, Summers R, Alpini G, Glaser S. Ca2+-dependent cytoprotective effects of ursodeoxycholic and tauroursodeoxycholic acid on the biliary epithelium in a rat model of cholestasis and loss of bile ducts. *Am J Pathol* 2006; **168**: 398-409
141 **Sato H**, Macchiarulo A, Thomas C, Gioiello A, Une M, Hofmann AF, Saladin R, Schoonjans K, Pellicciari R, Auwerx J. Novel potent and selective bile acid derivatives as TGR5 agonists: biological screening, structure-activity relationships, and molecular modeling studies. *J Med Chem* 2008; **51**: 1831-1841

S- Editor Li DL **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5630

World J Gastroenterol 2008 October 7; 14(37): 5630-5640
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Jose JG Marin, Professor, *Series Editor*

Intestinal bile acid physiology and pathophysiology

Olga Martínez-Augustin, Fermín Sánchez de Medina

Olga Martínez-Augustin, Department of Biochemistry and Molecular Biology II, School of Pharmacy, University of Granada, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas, Granada 18071, Spain
Fermín Sánchez de Medina, Department of Pharmacology, School of Pharmacy, University of Granada, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas, Granada 18071, Spain
Author contributions: Both authors contributed equally to this work.
Supported by Grants from the Instituto de Salud Carlos III (PI051625 and PI051651). The group is member of the Network for Cooperative Research on Membrane Transport Proteins (REIT), co-funded by the Ministerio de Educación y Ciencia, Spain and the European Regional Development Fund (Grant BFU2005-24983-E/BFI). The CIBER-EHD is funded by the Instituto de Salud Carlos III
Correspondence to: Fermín Sánchez de Medina, Department of Pharmacology, Facultad de Farmacia, University of Granada, Campus de Cartuja s/n, Granada 18071, Spain. fsanchez@ugr.es
Telephone: +34-958-243889 Fax: +34-958-248964
Received: July 24, 2008 Revised: September 16, 2008
Accepted: September 23, 2008
Published online: October 7, 2008

Abstract

Bile acids (BAs) have a long established role in fat digestion in the intestine by acting as tensioactives, due to their amphipathic characteristics. BAs are reabsorbed very efficiently by the intestinal epithelium and recycled back to the liver *via* transport mechanisms that have been largely elucidated. The transport and synthesis of BAs are tightly regulated in part by specific plasma membrane receptors and nuclear receptors. In addition to their primary effect, BAs have been claimed to play a role in gastrointestinal cancer, intestinal inflammation and intestinal ionic transport. BAs are not equivalent in any of these biological activities, and structural requirements have been generally identified. In particular, some BAs may be useful for cancer chemoprevention and perhaps in inflammatory bowel disease, although further research is necessary in this field. This review covers the most recent developments in these aspects of BA intestinal biology.

Key words: Colitis; Bile acid absorption; Colon cancer

Peer reviewer: Serhan Karvar, MD, Assistant Professor of Medicine, University of Southern California, Keck School of Medicine, Division of Gastrointestinal & Liver Diseases, 2011 Zonal Avenue, HMR 101, Los Angeles, CA 90089, United States

Martínez-Augustin O, Sánchez de Medina F. Intestinal bile acid physiology and pathophysiology. *World J Gastroenterol* 2008; 14(37): 5630-5640 Available from: URL: http://www.wjgnet.com/1007-9327/14/5630.asp DOI: http://dx.doi.org/10.3748/wjg.14.5630

INTRODUCTION

Separate chapters in this series of minireviews are devoted to cover various aspects of bile acids (BAs) chemistry, physiology and pathophysiology, including the hepatic synthesis and handling of BAs and their implications in health and disease. Here we will deal with the normal and pathological roles of BAs in one of the traditionally known natural sites of action, i.e. the intestine. It is well known that BAs are secreted into the duodenal lumen after meals in order to act as tensioactives and facilitate fat digestion. This is possible because of the amphipathic characteristics of BAs, which are molecules with a highly hydrophobic core and a number of hydroxyl groups attached. Because these groups may extend to one of the two sides of the basically planar structure formed by the hydrocarbon rings of the BA molecule, its polarity is maximal when all hydroxyl groups are set out in the same side. Thus cholic acid (CA), deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA), all of which have alpha hydroxyl groups, are more efficient tensioactives than ursodeoxycholic acid (UDCA), which has hydroxyls with both alpha and beta conformation. In order to act efficiently as tensioactives BAs must work in coordination with other amphipathic compounds, namely phospholipids, which are another essential component of bile.

Once their physiological function is accomplished, most BA molecules are efficiently reabsorbed in the distal part of the small intestine and reach the liver *via* portal blood, where they are avidly taken up by hepatocytes. Thus BAs are not wasted but recycled with an almost perfect yield (approximately 95%). The molecular details of this enterohepatic cycle have been elucidated (Figure 1). The type and amount of BAs thus

reabsorbed, as well as of those that pass into the colon, are not constant but subject to variation as a result of diet, transit time, drugs, disease, *etc*. This in turn has an impact on the effects of BAs because they are not equivalent in terms of bioactivity. Certain BAs have been involved in colon cancer, but also in other conditions such as intestinal inflammation, diarrhea, *etc*.

The dynamics of BAs transport and metabolism is tightly regulated. This is necessary because intracellular accumulation of BAs, as happens in cholestasis, may result in cytotoxicity. Because BAs are synthesized in the liver, an important part of the control of their homeostasis takes place in hepatocytes, the details of these processes are covered in other chapters of this series. However, in part of the regulatory system the intestine plays important direct (transport/metabolism) and indirect (endocrine response of intestinal epithelium to BAs) roles.

BA IN THE INTESTINE

As mentioned above, the liver synthesizes BAs at the expense of cholesterol and also retrieves reabsorbed BAs from the blood. From hepatocytes they are secreted against steep concentration gradients into bile, together with cholesterol and phospholipids. Thus, between meals, most of the pool of BAs resides in the gallbladder ready to be used at short notice. The mechanisms whereby hepatocytes take up BAs from the bloodstream and synthetize and secrete them into bile have been reviewed in other chapters of this series. When food is ingested (more precisely if the meal is rich in fat) the gallbladder contracts in response to cholecystokinin. It has been proposed that intraduodenal BAs exert a negative feedback control on postprandial cholecystokinin release and the resulting gallbladder contraction. Thus the acute (but not chronic) intraduodenal bile salt depletion with cholestyramine affects gallbladder and also antroduodenal motility, possibly by enhanced motilin release[1].

The mix of BAs contained in bile represents a balance of primary and secondary compounds. Primary BAs are those synthesized as such by the liver, and comprise predominantly CA and CDCA. These are secreted to bile mainly conjugated with glycine and taurine, thus having enhanced water solubility. Secondary BAs are derived from primary BAs by modifications carried out by intestinal bacteria. The main modifications are deconjugation, oxidation of hydroxyl groups in 3, 7 and 12 positions, and 7-dehydroxylation[2]. The main secondary BAs are lithocholic acid (LCA) and DCA. The overall result is an increase in the hydrophobicity of BA pool. The transformation of BAs by bacterial enzymes has several important consequences. First, it favors passive absorption in the colon of those BAs escaping the active uptake that takes place in the ileum. If both mechanisms operate normally, only 1%-3% of the amount of BA that is secreted by the liver is ultimately excreted in faeces (deconjugated and otherwise transformed). Second, it increases the potentiality of BAs to cause noxious effect, like carcinogenicity and cholesterol gallstone disease[3]. Third, the composition

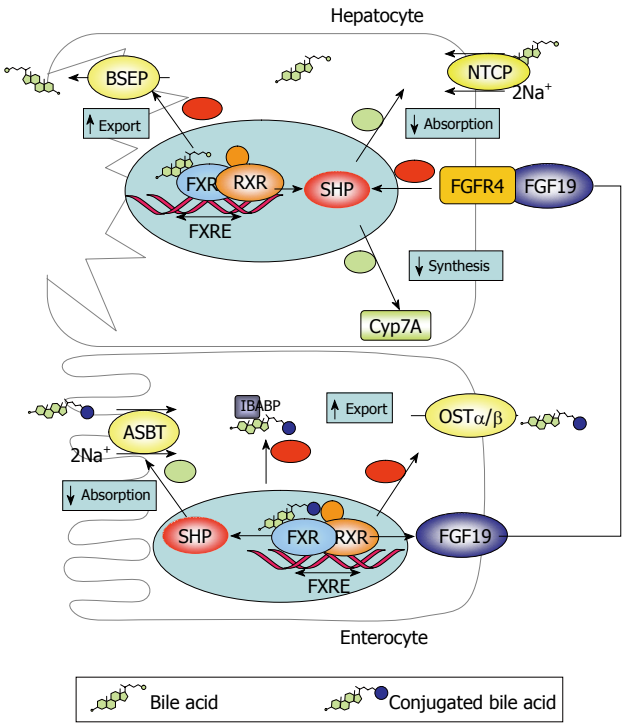


Figure 1 Diagram showing the mechanisms of BA transport and regulation at the intestinal and hepatic level.

of the BA pool will vary when the conditions of biotransformation are altered, for instance by changes in transit time or alterations in the microbiota brought about by drugs, diet, *etc*.[3,4]. For instance, a high fat diet and a long (slowed) transit time favor DCA generation from CA and absorption, which in turn is associated with higher risk of cholesterol gallstones and cancer (see below). In addition, diets rich in fat and poor in fiber can increase more than 10-fold the amount of taurine conjugated BAs reaching the colon, due to higher conjugation and production (higher conjugation reduces ileal absorption)[5,6]. Taurine dietary intake (meat, seafood) also contributes to this result. Overweight and accelerated intestinal transit reduce BA absorption and may cause idiopathic BA malabsorption[7]. Drugs such as cholestiramine bind BAs and reduce absorption.

Owing to the fact that 7-dehydroxylation cannot be reversed by the host enzymatic machinery, LCA and DCA tend to accumulate in the BA pool. However, LCA is 3-sulfated and conjugated at C-24 by the liver, resulting in a derivative that is poorly absorbed from the colonic mucosa, and consequently LCA is not present in significant amounts in the bile[8]. Thus major BAs in human bile are CA, CDCA and DCA, which are accompanied by minor amounts of UDCA, LCA and other BAs, whereas faeces contain mainly DCA, LCA, minor amounts of CDCA, CA and UDCA and a variety of bacteria transformed derivatives[2]. Concentrations of BAs in the intestinal lumen are variable but usually high, estimated in the medium millimolar range. This is consistent with their critical micellar concentration (e.g. 6-10 mmol/L for TCA), i.e. the concentration corresponding to spontaneous formation of micelles[9].

BA TRANSPORT BY EPITHELIAL CELLS

As mentioned above, BAs are efficiently taken up from the lumen of the ileal segment, leaving only approximately 5% (or approximately 0.5 g/d) in the lumen[10]. This fraction is in part passively absorbed in the colon, a process facilitated by bacterial deconjugation, and in part transformed and extruded with faeces. In contrast, ileal uptake is predominantly an active process carried out by the apical sodium-dependent BA transporter (ASBT, gene symbol *SLC10A2*), which imports BAs coupled to Na^+ absorption (1:2 stoichiometry)[11]. ASBT is highly homologous to the hepatocyte Na^+/taurocholate cotransporting polypeptide transporter (NTCP, gene symbol *SLC10A1*), which plays a pivotal role in BA uptake by the liver from the portal bloodstream. BAs species are not equally transported by ASBT. Thus, conjugated (more hydrophilic) BAs are transported more efficiently than unconjugated forms[12,13]. This is physiologically consistent with the fact that deconjugation normally takes place in the colon. The affinity of ASBT is also higher for dihydroxy BAs (CDCA and DCA) than for trihydroxy BAs (CA, taurocholic acid-TCA-, glycocholic acid-GCA-)[12].

BAs are believed to be transferred directly from ASBT to an intracellular 14 kDa protein called ileal BA binding protein (IBABP/FABP6) through the formation of a 2:1 stoichiometric complex[14]. IBABP is supposed to facilitate transport of BAs within the cell to the basolateral membrane. This is suggested by data showing coordinated expression of both ASBT and IBABP in the postnatal development in the intestine and in cholangyocytes, as well as by the fact that IBABP and ASBT form complexes with a defined stoichiometry[15]. It should be noted however that ASBT is expressed in the kidney without being accompanied by IBABP[16], and also that FXR knockout mice, which do not express IBABP, show enhanced rather than inhibited intestinal BA absorption, suggesting that IBABP may function as a negative regulator of intestinal BA reabsorption, at least in the mouse[17]. Interestingly, low IBABP expression has been linked to the risk of necrotizing enterocolitis in an animal model, suggesting that inefficient transfer of BA to the basolateral membrane may ultimately result in epithelial damage and inflammation[18].

Finally, BAs exit the enterocyte *via* the recently characterized OSTα/β transporter[19], an obligate heterodimer which functions in a Na^+-independent manner and also transports prostaglandin E_2, estrone-3-sulfate, dehydroepiandrosterone sulfate[13].

Developmentally ileal transport has been described to be preceded by active colonic absorption in rabbits[20]. This differs markedly from adult animals, which show net colonic secretion. The expression of IBABP and ABST is known to be subjected to distinct changes early in life, which are dependent on the species and protein considered. Thus, in rats and mice, ASBT is highly expressed in the ileum of the fetus before birth but is downregulated or entirely absent in the newborn and later upregulated again. IBABP shows a similar ontogenic profile in mice, while in rats it first appears postnatally[20]. The induction of ABST after birth is stimulated by thyroxine in rats[21].

It is interesting to examine the effects of interference with normal transporter function in the intestine. Genetic disruption of ASBT activity or pharmacological inhibition with SC-435 results in BA malabsorption and diarrhea[22-24]. Conversely, Ostα -/- mice show reduced intestinal capacity to take up BAs but unaffected fecal BA output, which is secondary to a marked shrinkage of the BA pool[19]. On the other hand, there are significant numbers of patients with idiopathic ileal BA malabsoprtion who suffer of unexplained chronic diarrhea[25].

The exact role of other transporters is controversial. They may play a minor role in BA handling by the intestine. These include MRP3 and an alternatively spliced form of ASBT, t-ASBT[13].

REGULATION OF INTESTINAL BA TRANSPORT

The intestinal (ileal) absorption of BAs is tightly regulated to meet physiological demands. In addition, intestinal BA uptake has direct and indirect impact on hepatic BA homeostasis. The main factor involved in both functions is the farnesoid X receptor (FXR/NR1H4), which was originally identified as an orphan nuclear receptor that was activated by farnesol, an intermediate in the mevalonate biosynthetic pathway[26]. FXR is expressed in ileal enterocytes and also in the liver, as well as in other tissues, such as the adrenal gland and the kidney[27]. Interestingly, the intestine seems to have the most intense FXR expression in the body[28]. Agonists of the FXR include BAs, particularly CDCA, followed by DCA, LCA and many other BAs with minor efficacy (conjugation does not affect binding)[29]. The α-position of OH groups in BA molecule is very important for interaction with FXR[30]. Upon activation, FXR modulates gene transcription acting in concert with another nuclear receptor, the retinoid X receptor alpha (RXRα), by recognizing a specific promoter sequence called the FXR responsive element. FXR is pivotal in the BA regulation both in the liver and in the intestine. In hepatocytes FXR increases expression of the bile salt export pump (BSEP) and downregulates the expression of NTCP and CYP7A. Since NTCP and BSEP mediate BA uptake from blood and export to bile in hepatocytes and CYP7A catalyzes the limiting-rate step in the classical BA biosynthetic pathway, this leads to reduced BA uptake, decreased synthesis and enhanced export to bile. Thus BA accumulation in hepatocytes tends to be self-limiting. In enterocytes, FXR is coupled to reduced ASBT and increased IBABP and OSTα/β expression, resulting in inhibition of intestinal absorption of BAs and prevention of intracellular BA accumulation.

The repressing effects of FXR are mediated by the transcription factor SHP (Small Heterodimer Partner), which is induced by FXR but lacks a DNA binding domain[31,32]. Instead, SHP binds to other nuclear receptors such as RXR/RAR (retinoic acid receptor),

LRH1 (liver receptor homolog 1) and LXR (liver X receptor), inhibiting their transcriptional effects[11,33]. IBABP seems to interact with ASBT and FXR to promote FXR transcription[34].

An important feature of FXR role in regulation of BA homeostasis is that it is not limited to local effects. Rather, sensing of the enterocyte BA pool by FXR affects the liver by way of the endocrine factor FGF19 (Fgf15 in mice)[32,33]. FGF19 is released to the portal circulation and activates fibroblast growth factor receptor 4 (FGFR4) in hepatocytes, which results in downregulation of CYP7A1 and therefore inhibition of the classical BA synthetic pathway, both by SHP induction and possibly other pathways[33]. Thus BAs modulate their own synthesis both by local hepatic and remote intestinal negative feedback. Tissue specific FXR gene knockdown experiments suggest that both pathways are similarly important[27]. The importance of the latter pathway is exemplified by the fact that administration of TCA downregulates CYP7A1 in the liver only when administered intraduodenally, but not after intravenous or portal instillation[35]. However, alternative pathways for feedback control have been proposed[33].

Physiologically, lack of BA uptake by ASBT inhibition leads to increased fecal BA (and diarrhea) and reduced FXR stimulation, lower FGF19 synthesis, and consequently enhanced BA synthesis, expanding the BA pool and lowering plasma cholesterol[24]. In contrast, Ostα -/- mice do not exhibit increased fecal BA output and have downregulated Cyp7a1 expression and a reduced BA pool[19]. This is due to increased Fgf15, secondary to FXR activation by "trapped" BAs.

In addition, FXR activation has been claimed to participate in the regulation of bacterial growth within the intestine. This hypothesis is supported by the findings that cholestasis results in bacterial overgrowth in the small intestine and increased translocation, which are counteracted in experimental models by oral BAs[36,37]. The FXR mediated induction by BAs of antibacterial genes such as angiogenin, carbonic anhydrase 12 and inducible nitric oxide synthase may account for this effect[38].

Other nuclear factors are regulated by BAs, including the pregnane X receptor (PXR/NR1I2), the Vitamin D receptor (VDR/NR1I1) and the androstane constitutive receptor (CAR)[33]. Thus LCA binds and activates intestinal and systemic VDR[39]. It has been proposed that the effects brought about by LCA are essentially local, directed to induce CYP3A genes and to aid in detoxification[40], but at any rate it can substitute for Vitamin D systemically. PXR and CAR activation leads to the upregulation of secondary BA transporters such as MRP2, MRP3 and MDR1. For instance, CA induces MRP2 and MRP3 in the intestine[41]. In addition, BAs have been reported to activate a G-protein associate receptor, named after the fact G protein-coupled BA receptor 1 (also known as TGR5), which is expressed in many tissues including the gastrointestinal tract[42].

IBABP is also regulated by PPARα/β in humans but not mice[43], and can also be indirectly up-regulated by cholesterol through the activation of sterol-responsive element-binding protein 1c (SREBP1c) by LXR[44]. ASBT is also regulated by PPARα[45]. These changes are expected to increase BA uptake and possibly reduce cholesterol absorption, a putative mechanism of action of the hypolipemic drugs fibrates[43]. Corticoids are also known to upregulate ASBT expression[46]. Because BA ileal uptake is inhibited in intestinal inflammation and probably contributes to diarrhea, corticoid treatment may be specifically useful in this setting.

Among the pathological conditions affecting BA homeostasis, cholestasis downregulates ASBT expression[47,48]. The mechanism is unclear, but it may be related to PPARα inhibition by BAs (possibly because of high blood levels), given that PPARα has been reported to transactivate ASBT transcription, as mentioned above[45,49]. Intestinal MRP2 but not MRP3 is decreased by cholestasis in rats[50] and in humans[48,51], although the significance of these findings is uncertain. In contrast, increased absorption has been reported in primary biliary cirrhosis, thus contributing to cholestasis in this condition[52]. Hypertriglicerydemia also reduces ASBT expression and inhibits BA absorption[53], an effect which in turn might exacerbate hypertriglyceridemia[54]. Interestingly, gallstones have been associated with lower intestinal ASBT and IBABP expression in normal weight but not overweight women[55]. These changes are accounted for by lower hepatic FXR and thereby increased BA synthesis.

BA AND COLORECTAL CANCER

There is wide epidemiological evidence linking BA exposure (for instance due to high fat diet) and gastrointestinal (specially colorectal) cancer[56-58]. Patients with colorectal adenomas and carcinomas exhibit high blood and fecal levels of secondary BAs[59,60]. Diets rich in fat are powerful stimulants of BA secretion, as mentioned above. Thus many investigators have studied the effects of BAs, particularly secondary BAs (DCA and LCA) on intestinal epithelial cell proliferation, apoptosis and mutagenesis *in vitro*, as well as on cancer promotion *in vivo*. Paradoxically DCA, but not CA or UDCA, exhibit proapoptotic effects on cell lines, which appear to depend on a variety of mechanisms[61,62]. The ability of BAs to induce apoptosis has been linked to their hydrophobicity, so that unconjugated DCA and CDCA are the most powerful inducers[63]. This makes sense, since only hydrophobic BAs can gain access to colonic cells *via* passive diffusion.

Different mechanisms have been involved in the proapoptotic effect of BAs. Direct increase in mitochondrial membrane permeability has been suggested, leading to mitochondrial swelling, release of cytochrome c and apoptosis[64]. Alterations in plasma membrane composition with subsequent up-regulation of caveolin-1 may underlie also the activation of protein kinase C by BAs[65]. DCA and UDCA have opposing effects on PKC translocation, affecting a number of isoenzymes including PKC alpha, epsilon and beta1[66,67]. DCA also acti-

vates NF-κB and AP-1 in colonic epithelial cells, downstream of PKC stimulation[68]. As expected, UDCA has the opposite effect. ERK activation has been involved in DCA proapoptotic effects, inasmuch as genetic or pharmacological inhibition blocks them[69]. In addition, DCA (and CDCA) induce c-Fos and COX2 in intestinal epithelial cells[70].

Despite these observations, it is important to note that epithelial cells may develop resistance to DCA induced apoptosis, which is achieved partly *via* the NO pathway[71] and is correlated with shifted expression of multiple proteins, as assessed by proteomic analysis[72]. Alternatively, additional factors may protect against DCA induced apoptosis, such as glutathione-S-transferase P1-1[73]. The expected result would be the selection of transformed cells, favoring the formation of adenomas and predisposing to subsequent development of cancer.

BAs also exert direct actions that can lead to tumorigenesis. Thus, DCA has genotoxic effects, which are believed to be secondary to induction of oxidative stress in the cell[74], and suppresses the p53 response to DNA damage, an action that is at least partly dependent on ERK signaling[75]. Moreover, inhibition of BRCA-1 by relatively high DCA concentrations contributes to defective DNA repair[76]. Recently DCA and LCA (in conjugated form) were shown to elicit transactivation of the epidermal growth factor receptor *via* interaction with muscarinic receptors and phosphorylation of ERK[77]. Another gene target of DCA *via* ERK is the tumor marker EphA2 receptor protein tyrosine kinase[78]. In general, these actions are not shared by UDCA and may be opposed by it[61,79,80]. Taken together, these data indicate that DCA may behave as a co-carcinogenic and/or cancer promoter agent, which may potentiate the activity of any primary carcinogen or cancer initiator. In addition, DCA may increase tumor invasiveness by activation of beta-catenin signaling[81]. An interesting observation is that FXR expression is diminished in colon cancer[82]. Under these circumstances, FXR-mediated mechanisms involved in the prevention of BA accumulation in these cells could be expected to be completely or partly inactive, thus exacerbating BA-induced effects.

We also count on substantial *in vivo* evidence about the effects of BAs on colorectal cancer. Thus colonic grafts from mice with an APC gene mutation do not develop adenomas if they are removed from the fecal stream[83]. In the standard Min mouse model UDCA produces a dose dependent decrease in the number of intestinal tumors, showing synergism with the cycloxygenase 2 inhibitor sulindac[84]. In the azoxymethane model of cancer associated to chronic colitis, UDCA lowered the multiplicity of colonic adenocarcinoma, while sulfasalazine had no significant effect[85]. Similar results were obtained in the regular (without colitis) azoxymethane model[86]. The chemopreventive effect of UDCA is associated with decreased Ras activation and COX2 expression[87].

This type of observations can be extended to human disease. Thus, a study carried out in patients with primary biliary cirrhosis undergoing surveillance by colonoscopy revealed a non-significant reduction in the prevalence of colorectal adenomas and, more importantly, a lower probability of recurrence (7% *vs* 28% at 3 years, $P = 0.04$)[88]. UDCA lowers cancer mortality (but not incidence) in ulcerative colitis patients with sclerosing cholangitis[89]. In a clinical trial on the secondary prevention of colorectal cancer, UDCA caused a non-significant 12% decrease in recurrence rate but a significant reduction (39%) in the subgroup with high-grade dysplasia[90]. In clinical trials of cancer associated to inflammatory bowel disease, a condition which increases the risk of developing cancer, UDCA has been shown to be beneficial, ranging from a mild chemoprotective effect[91] to a clear decrease in the relative risk of developing colorectal dysplasia or cancer[92]. Mechanistically, UDCA has been reported to reduce mucosal proliferation in cancer naive patients[88] but to have no effect in adenoma patients[93]. From a pharmacokinetic point of view, the main effect of UDCA administration in humans is an increase of luminal (fecal) UDCA/DCA ratio, although DCA absolute levels remain unaltered[94].

An intriguing possibility is that taurine, which is bound to a substantial fraction of the BA pool, contributes to cancer risk. Taurine, which can be released in the intestinal lumen from conjugated BAs due to the metabolic activity of several bacterial strains, is metabolized by the intestinal flora yielding hydrogen sulfite, which increases colonocyte turnover and inhibits butyrate metabolism. Although these cells oxidize efficiently this compound to thiosulfate, taurine derived hydrogen sulfite may be involved in carcinogenesis. In fact, defects in the hydrogen sulfite detoxification pathway may increase the risk of UC, a significant risk factor for colon cancer[95,96]. It is interesting to note that taurine conjugation and sulfite production are increased in meat consumers, thus providing another link to colon cancer[2]. In addition, sulfite promotes DCA generation by bacteria through stimulation of 7α-dehydroxylation.

EFFECT OF BA ON ION TRANSPORT

It is well known that BAs elicit fluid secretion and also increased permeability in the gastrointestinal tract[97-101]. BAs also affect intestinal motility, although this field of study has received relatively little attention[1,102]. The effect of BAs on permeability is primarily due to their detergent action on tight junctions, which is reversible. However, at high BA concentrations epithelial lesions may occur. This effect is to a large extent indirect, induced by intramural reflexes containing nicotinic receptors, but probably it does not involve histamine or nitric oxide pathways[97,103,104]. However, this question is controversial, because in some cases histamine has been suggested to be involved[98].

The secretory effect of BAs has been studied in the small and large intestine, and in both cases the mechanism of secretion appears to be largely indirect[99]. In the small intestine BAs elicit serotonin release by enterochromaffin cells in the mucosa by a Ca^{2+}-dependent mechanism, initiating a neural reflex that stimulates ion secretion, as well as inhibited absorption[105]. In ileal

perfusion experiments permeability and fluid transport were studied in parallel, finding that the effect is dependent on nicotinic receptors in both cases[97,103]. On the other hand, in the colon BA-induced secretion has been claimed to be prostaglandin dependent[106]. More specifically, prostaglandins may account for the early response to BA stimulation observed *in vitro*[98]. Mast cells have also been involved in this process[98]. Indeed, BAs have been shown to induce prostaglandin[107] and histamine secretion[108] *in vitro*. Some of these actions may be secondary to direct mucosal injury[100,109]. Considering the variety of mediators proposed to participate in these events, it is not surprising that multiple intracellular signaling pathways have also been involved.

As with other biological activities of BAs, not all molecular species are equivalent. Dihydroxy BAs, and in particular DCA and CDCA, exhibit prosecretory/antiabsorptive and mucosal damaging effects in the colon, particularly in their unconjugated forms[100,101]. Conversely, other important BAs such as CA and UDCA are generally considered to have no significant bioactivity at this regard. In the small intestine, these differential species-dependent effects are not so well characterized, but they certainly differ from those in the colon. For instance, CDCA is a relatively poor secretagogue in the ileum[110-113]. It should be noted that ileal BA absorption is electrogenic, even in the absence of chloride/bicarbonate secretion, due to the 2:1 stoichiometry of ASBT-mediated Na^+:BA symport[111,114,115].

However, BAs also exert direct actions on intestinal epithelial cells. Using the prototypic T84 cell line TDCA was shown to elicit chloride secretion by a Ca^{2+} dependent mechanism[116]. This pertains to the actions of BAs in the large intestine, since T84 cells have a colonic epithelium phenotype. More recently, BA were described to induce ion secretion *via* transactivation of the cystic fibrosis transmembrane conductance regulator (CFTR)[113]. CFTR is the main chloride/bicarbonate channel in intestinal epithelial cells and is pivotal to ion secretion in the gastrointestinal tract, among other tissues. Transactivation requires apical colocalization of both CFTR and ASBT, which does occur in the distal ileum but also in cholangiocytes. Moreover, CFTR also plays a role in bile flow, as suggested the presence of plugging and dilatation of bile ducts in cystic fibrosis. Interestingly, BA ileal uptake is compromised in this condition, leading to BA waste and diarrhea[113,114,117], suggesting that CFTR has a reciprocal influence on ASBT. Moreover, this provides an additional meaningful link to inhibited BA absorption in intestinal inflammation, where CFTR has been shown to be downregulated, as is ASBT itself[118,119]. Although the relationship between the expression of CFTR and that of ASBT has been previously demonstrated also for other transporters[120], the underlying mechanism has not been elucidated yet.

One interesting question arises as to what is the physiological role of BA-induced ileal and colonic secretion. The simplest explanation is that ileal secretion, which is evoked in normal conditions, may be useful to aid in intestinal propulsion or to prevent the formation of micelles and the consequent epithelial damage during the absorptive process[113]. In contrast, colonic secretion occurs only in pathologic conditions and may be part of a nonspecific mechanism aimed to eliminate invading microorganisms. In addition, BA ileal absorption is compromised in conditions such as irritable bowel syndrome, Crohn's disease, cystic fibrosis and surgical resection (short bowel syndrome)[117], producing diarrhea because of the presence of high (millimolar) concentrations in the colonic lumen. BAs (CDCA, UDCA) can also induce diarrhea in their own right when given to gallstone patients[121,122].

BA AND INTESTINAL INFLAMMATION

Certain BAs have been shown to exert intestinal antiinflammatory actions *in vivo*. Thus UDCA reduces intestinal permeability and oxidative stress in the indomethacin model of ileitis in the rat[123]. Similarly, UDCA counteracts ibuprofen intestinal ulceration in rats[124]. These effects may be related to the actions of BAs on intestinal epithelial cells. Thus DCA induces IL-8 and activates NF-κB in HT29 cells, actions that are opposed by taurine-conjugated UDCA[125,126]. The mechanism for IL-8 induction is probably *via* the classical NF-κB pathway for DCA and *via* RelA phosphorylation in the case of TDCA[126]. The stimulatory effect of DCA is reproduced in other[127,128] but not all cell lines[129]. Thus UDCA might exert antiinflammatory actions in the intestine by inhibiting epithelial stimulation. Conversely, DCA is predicted to aggravate inflammation, but this has not been tested. On the other hand, BAs have also been described to enhance epithelial wound healing, an action dependent on NF-κB activation and the release of transforming growth factor β[130]. This effect is shared by TDCA, DCA and TCA. Unfortunately UDCA was not investigated[130]. It cannot be ruled out that this effect may also form part of the mechanism of action of this beneficial compound.

INTESTINAL APPLICATIONS OF BA

The main clinical application of BAs is in the management of gallstones and cholestasis (primary biliary cirrhosis, cystic fibrosis liver disease, drug induced cholestasis)[88,131,132]. UDCA and to a lesser extent CDCA have been used. Cholylsarcosine is an artificial derivative that has been proposed as an alternative. However, BAs have no current application for intestinal conditions. UDCA has been studied in the clinical setting for cancer chemoprevention as discussed above. Specific artificial BA derivatives have also been studied for chemopreventive application[133]. Interestingly, it may be feasible to increase UDCA intestinal exposure by the use of a specific type of probiotic (living bacteria) that epimerizes CDCA to UDCA within the intestinal lumen[134]. BAs have been used in some cases as a replacement to reduce steatorrhea due to short bowel syndrome or secondary to metabolic genetic diseases, frequently at the price of enhanced diarrhea[135,136]. Conversely, diarrhea without steatorrhea benefits from treatment with ion exchange

resins such as cholestiramine that bind and act as sequestrant of BAs during their intestinal transit[137].

Moreover, BAs are being considered as galenic agents to improve intestinal absorption of drugs compounds such as nucleotides, heparin or insulin[138-140].

CONCLUSION

Owing to their tensioactive properties BAs play an important role in the intestine, facilitating fat digestion and the absorption of lipids and liposoluble vitamins. Efficient intestinal uptake, mainly at the ileum, permits to recover most of the secreted BA molecules, which are sent back to the liver with the portal blood. The existence of the enterohepatic circulation maintains appropriate levels of BAs ready to be used after meals and prevents exposure of other tissues to high levels of these dangerous detergents. In this respect, due to the potential toxicity of BAs and, at the same time, their biological relevance the homeostasis of BAs is tightly regulated, in part by specific plasma membrane receptors and nuclear receptors. The function, transport and regulatory mechanisms regarding BAs and the intestine have been elucidated in great detail, although some questions remain unanswered, such as the exact physiological role of IBABP. Some BA species have peculiar biological or pharmacological effects, which have been characterized to a great extent. Nevertheless, their role in colon cancer and intestinal inflammation requires further study, which is especially interesting considering the potential therapeutical applications.

REFERENCES

1 **Portincasa P**, Peeters TL, van Berge-Henegouwen GP, van Solinge WW, Palasciano G, van Erpecum KJ. Acute intraduodenal bile salt depletion leads to strong gallbladder contraction, altered antroduodenal motility and high plasma motilin levels in humans. *Neurogastroenterol Motil* 2000; **12**: 421-430

2 **Ridlon JM**, Kang DJ, Hylemon PB. Bile salt biotransformations by human intestinal bacteria. *J Lipid Res* 2006; **47**: 241-259

3 **Thomas LA**, Veysey MJ, Bathgate T, King A, French G, Smeeton NC, Murphy GM, Dowling RH. Mechanism for the transit-induced increase in colonic deoxycholic acid formation in cholesterol cholelithiasis. *Gastroenterology* 2000; **119**: 806-815

4 **Thomas LA**, Veysey MJ, Murphy GM, Russell-Jones D, French GL, Wass JA, Dowling RH. Octreotide induced prolongation of colonic transit increases faecal anaerobic bacteria, bile acid metabolising enzymes, and serum deoxycholic acid in patients with acromegaly. *Gut* 2005; **54**: 630-635

5 **Stadler J**, Stern HS, Yeung KS, McGuire V, Furrer R, Marcon N, Bruce WR. Effect of high fat consumption on cell proliferation activity of colorectal mucosa and on soluble faecal bile acids. *Gut* 1988; **29**: 1326-1331

6 **Hardison WG**. Hepatic taurine concentration and dietary taurine as regulators of bile acid conjugation with taurine. *Gastroenterology* 1978; **75**: 71-75

7 **Sadik R**, Abrahamsson H, Ung KA, Stotzer PO. Accelerated regional bowel transit and overweight shown in idiopathic bile acid malabsorption. *Am J Gastroenterol* 2004; **99**: 711-718

8 **Hofmann AF**. Detoxification of lithocholic acid, a toxic bile acid: relevance to drug hepatotoxicity. *Drug Metab Rev* 2004; **36**: 703-722

9 **Roda A**, Hofmann AF, Mysels KJ. The influence of bile salt structure on self-association in aqueous solutions. *J Biol Chem* 1983; **258**: 6362-6370

10 **Dietschy JM**, Turley SD. Control of cholesterol turnover in the mouse. *J Biol Chem* 2002; **277**: 3801-3804

11 **Pellicoro A**, Faber KN. Review article: The function and regulation of proteins involved in bile salt biosynthesis and transport. *Aliment Pharmacol Ther* 2007; **26** Suppl 2: 149-160

12 **Craddock AL**, Love MW, Daniel RW, Kirby LC, Walters HC, Wong MH, Dawson PA. Expression and transport properties of the human ileal and renal sodium-dependent bile acid transporter. *Am J Physiol* 1998; **274**: G157-G169

13 **Alrefai WA**, Gill RK. Bile acid transporters: structure, function, regulation and pathophysiological implications. *Pharm Res* 2007; **24**: 1803-1823

14 **Tochtrop GP**, Bruns JL, Tang C, Covey DF, Cistola DP. Steroid ring hydroxylation patterns govern cooperativity in human bile acid binding protein. *Biochemistry* 2003; **42**: 11561-11567

15 **Kramer W**, Wess G, Bewersdorf U, Corsiero D, Girbig F, Weyland C, Stengelin S, Enhsen A, Bock K, Kleine H, Le Dreau MA, Schafer HL. Topological photoaffinity labeling of the rabbit ileal Na+/bile-salt-cotransport system. *Eur J Biochem* 1997; **249**: 456-464

16 **Gong YZ**, Everett ET, Schwartz DA, Norris JS, Wilson FA. Molecular cloning, tissue distribution, and expression of a 14-kDa bile acid-binding protein from rat ileal cytosol. *Proc Natl Acad Sci USA* 1994; **91**: 4741-4745

17 **Kok T**, Hulzebos CV, Wolters H, Havinga R, Agellon LB, Stellaard F, Shan B, Schwarz M, Kuipers F. Enterohepatic circulation of bile salts in farnesoid X receptor-deficient mice: efficient intestinal bile salt absorption in the absence of ileal bile acid-binding protein. *J Biol Chem* 2003; **278**: 41930-41937

18 **Halpern MD**, Dvorak B. Does abnormal bile acid metabolism contribute to NEC? *Semin Perinatol* 2008; **32**: 114-121

19 **Rao A**, Haywood J, Craddock AL, Belinsky MG, Kruh GD, Dawson PA. The organic solute transporter alpha-beta, Ostalpha-Ostbeta, is essential for intestinal bile acid transport and homeostasis. *Proc Natl Acad Sci USA* 2008; **105**: 3891-3896

20 **Weihrauch D**, Kanchanapoo J, Ao M, Prasad R, Piyachaturawat P, Rao MC. Weanling, but not adult, rabbit colon absorbs bile acids: flux is linked to expression of putative bile acid transporters. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G439-G450

21 **Monteiro I**, David ES, Ferraris RP. Ontogenetic development of rat intestinal bile acid transport requires thyroxine but not corticosterone. *Pediatr Res* 2004; **55**: 611-621

22 **Wong MH**, Oelkers P, Dawson PA. Identification of a mutation in the ileal sodium-dependent bile acid transporter gene that abolishes transport activity. *J Biol Chem* 1995; **270**: 27228-27234

23 **Dawson PA**, Haywood J, Craddock AL, Wilson M, Tietjen M, Kluckman K, Maeda N, Parks JS. Targeted deletion of the ileal bile acid transporter eliminates enterohepatic cycling of bile acids in mice. *J Biol Chem* 2003; **278**: 33920-33927

24 **Li H**, Xu G, Shang Q, Pan L, Shefer S, Batta AK, Bollineni J, Tint GS, Keller BT, Salen G. Inhibition of ileal bile acid transport lowers plasma cholesterol levels by inactivating hepatic farnesoid X receptor and stimulating cholesterol 7 alpha-hydroxylase. *Metabolism* 2004; **53**: 927-932

25 **Balesaria S**, Pell RJ, Abbott LJ, Tasleem A, Chavele KM, Barley NF, Khair U, Simon A, Moriarty KJ, Brydon WG, Walters JR. Exploring possible mechanisms for primary bile acid malabsorption: evidence for different regulation of ileal bile acid transporter transcripts in chronic diarrhoea. *Eur J Gastroenterol Hepatol* 2008; **20**: 413-422

26 **Forman BM**, Goode E, Chen J, Oro AE, Bradley DJ, Perlmann T, Noonan DJ, Burka LT, McMorris T, Lamph WW, Evans RM, Weinberger C. Identification of a nuclear receptor that is activated by farnesol metabolites. *Cell* 1995; **81**: 687-693

27 **Kim I**, Ahn SH, Inagaki T, Choi M, Ito S, Guo GL, Kliewer SA, Gonzalez FJ. Differential regulation of bile acid homeostasis by the farnesoid X receptor in liver and intestine. *J Lipid Res* 2007; **48**: 2664-2672

28 **Houten SM**, Volle DH, Cummins CL, Mangelsdorf DJ, Auwerx J. In vivo imaging of farnesoid X receptor activity reveals the ileum as the primary bile acid signaling tissue. *Mol Endocrinol* 2007; **21**: 1312-1323

29 **Makishima M**, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ, Shan B. Identification of a nuclear receptor for bile acids. *Science* 1999; **284**: 1362-1365

30 **Shea HC**, Head DD, Setchell KD, Russell DW. Analysis of HSD3B7 knockout mice reveals that a 3alpha-hydroxyl stereochemistry is required for bile acid function. *Proc Natl Acad Sci USA* 2007; **104**: 11526-11533

31 **Neimark E**, Chen F, Li X, Shneider BL. Bile acid-induced negative feedback regulation of the human ileal bile acid transporter. *Hepatology* 2004; **40**: 149-156

32 **Inagaki T**, Choi M, Moschetta A, Peng L, Cummins CL, McDonald JG, Luo G, Jones SA, Goodwin B, Richardson JA, Gerard RD, Repa JJ, Mangelsdorf DJ, Kliewer SA. Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. *Cell Metab* 2005; **2**: 217-225

33 **Lee FY**, Lee H, Hubbert ML, Edwards PA, Zhang Y. FXR, a multipurpose nuclear receptor. *Trends Biochem Sci* 2006; **31**: 572-580

34 **Nakahara M**, Furuya N, Takagaki K, Sugaya T, Hirota K, Fukamizu A, Kanda T, Fujii H, Sato R. Ileal bile acid-binding protein, functionally associated with the farnesoid X receptor or the ileal bile acid transporter, regulates bile acid activity in the small intestine. *J Biol Chem* 2005; **280**: 42283-42289

35 **Nagano M**, Kuroki S, Mizuta A, Furukawa M, Noshiro M, Chijiiwa K, Tanaka M. Regulation of bile acid synthesis under reconstructed enterohepatic circulation in rats. *Steroids* 2004; **69**: 701-709

36 **Ding JW**, Andersson R, Soltesz V, Willen R, Bengmark S. The role of bile and bile acids in bacterial translocation in obstructive jaundice in rats. *Eur Surg Res* 1993; **25**: 11-19

37 **Lorenzo-Zuniga V**, Bartoli R, Planas R, Hofmann AF, Vinado B, Hagey LR, Hernandez JM, Mane J, Alvarez MA, Ausina V, Gassull MA. Oral bile acids reduce bacterial overgrowth, bacterial translocation, and endotoxemia in cirrhotic rats. *Hepatology* 2003; **37**: 551-557

38 **Inagaki T**, Moschetta A, Lee YK, Peng L, Zhao G, Downes M, Yu RT, Shelton JM, Richardson JA, Repa JJ, Mangelsdorf DJ, Kliewer SA. Regulation of antibacterial defense in the small intestine by the nuclear bile acid receptor. *Proc Natl Acad Sci USA* 2006; **103**: 3920-3925

39 **Nehring JA**, Zierold C, DeLuca HF. Lithocholic acid can carry out in vivo functions of vitamin D. *Proc Natl Acad Sci USA* 2007; **104**: 10006-10009

40 **Jurutka PW**, Thompson PD, Whitfield GK, Eichhorst KR, Hall N, Dominguez CE, Hsieh JC, Haussler CA, Haussler MR. Molecular and functional comparison of 1,25-dihydroxyvitamin D(3) and the novel vitamin D receptor ligand, lithocholic acid, in activating transcription of cytochrome P450 3A4. *J Cell Biochem* 2005; **94**: 917-943

41 **Kamisako T**, Ogawa H, Yamamoto K. Effect of cholesterol, cholic acid and cholestyramine administration on the intestinal mRNA expressions related to cholesterol and bile acid metabolism in the rat. *J Gastroenterol Hepatol* 2007; **22**: 1832-1837

42 **Kawamata Y**, Fujii R, Hosoya M, Harada M, Yoshida H, Miwa M, Fukusumi S, Habata Y, Itoh T, Shintani Y, Hinuma S, Fujisawa Y, Fujino M. A G protein-coupled receptor responsive to bile acids. *J Biol Chem* 2003; **278**: 9435-9440

43 **Landrier JF**, Thomas C, Grober J, Zaghini I, Petit V, Poirier H, Niot I, Besnard P. The gene encoding the human ileal bile acid-binding protein (I-BABP) is regulated by peroxisome proliferator-activated receptors. *Biochim Biophys Acta* 2005; **1735**: 41-49

44 **Landrier JF**, Grober J, Demydchuk J, Besnard P. FXRE can function as an LXRE in the promoter of human ileal bile acid-binding protein (I-BABP) gene. *FEBS Lett* 2003; **553**: 299-303

45 **Jung D**, Fried M, Kullak-Ublick GA. Human apical sodium-dependent bile salt transporter gene (SLC10A2) is regulated by the peroxisome proliferator-activated receptor alpha. *J Biol Chem* 2002; **277**: 30559-30566

46 **Jung D**, Fantin AC, Scheurer U, Fried M, Kullak-Ublick GA. Human ileal bile acid transporter gene ASBT (SLC10A2) is transactivated by the glucocorticoid receptor. *Gut* 2004; **53**: 78-84

47 **Sauer P**, Stiehl A, Fitscher BA, Riedel HD, Benz C, Kloters-Plachky P, Stengelin S, Stremmel W, Kramer W. Downregulation of ileal bile acid absorption in bile-duct-ligated rats. *J Hepatol* 2000; **33**: 2-8

48 **Hruz P**, Zimmermann C, Gutmann H, Degen L, Beuers U, Terracciano L, Drewe J, Beglinger C. Adaptive regulation of the ileal apical sodium dependent bile acid transporter (ASBT) in patients with obstructive cholestasis. *Gut* 2006; **55**: 395-402

49 **Sinal CJ**, Yoon M, Gonzalez FJ. Antagonism of the actions of peroxisome proliferator-activated receptor-alpha by bile acids. *J Biol Chem* 2001; **276**: 47154-47162

50 **Kamisako T**, Ogawa H. Alteration of the expression of adenosine triphosphate-binding cassette transporters associated with bile acid and cholesterol transport in the rat liver and intestine during cholestasis. *J Gastroenterol Hepatol* 2005; **20**: 1429-1434

51 **Dietrich CG**, Geier A, Salein N, Lammert F, Roeb E, Oude Elferink RP, Matern S, Gartung C. Consequences of bile duct obstruction on intestinal expression and function of multidrug resistance-associated protein 2. *Gastroenterology* 2004; **126**: 1044-1053

52 **Lanzini A**, De Tavonatti MG, Panarotto B, Scalia S, Mora A, Benini F, Baisini O, Lanzarotto F. Intestinal absorption of the bile acid analogue 75Se-homocholic acid-taurine is increased in primary biliary cirrhosis, and reverts to normal during ursodeoxycholic acid administration. *Gut* 2003; **52**: 1371-1375

53 **Duane WC**, Hartich LA, Bartman AE, Ho SB. Diminished gene expression of ileal apical sodium bile acid transporter explains impaired absorption of bile acid in patients with hypertriglyceridemia. *J Lipid Res* 2000; **41**: 1384-1389

54 **Duane WC**. Abnormal bile acid absorption in familial hypertriglyceridemia. *J Lipid Res* 1995; **36**: 96-107

55 **Bergheim I**, Harsch S, Mueller O, Schimmel S, Fritz P, Stange EF. Apical sodium bile acid transporter and ileal lipid binding protein in gallstone carriers. *J Lipid Res* 2006; **47**: 42-50

56 **Armstrong B**, Doll R. Environmental factors and cancer incidence and mortality in different countries, with special reference to dietary practices. *Int J Cancer* 1975; **15**: 617-631

57 **Bernstein H**, Bernstein C, Payne CM, Dvorakova K, Garewal H. Bile acids as carcinogens in human gastrointestinal cancers. *Mutat Res* 2005; **589**: 47-65

58 **Chen YQ**, Edwards IJ, Kridel SJ, Thornburg T, Berquin IM. Dietary fat-gene interactions in cancer. *Cancer Metastasis Rev* 2007; **26**: 535-551

59 **Wynder EL**, Reddy BS, McCoy GD, Weisburger JH, Williams GM. Diet and cancer of the gastrointestinal tract. *Adv Intern Med* 1977; **22**: 397-419

60 **Bayerdorffer E**, Mannes GA, Richter WO, Ochsenkuhn T, Wiebecke B, Kopcke W, Paumgartner G. Increased serum deoxycholic acid levels in men with colorectal adenomas. *Gastroenterology* 1993; **104**: 145-151

61 **Martinez JD**, Stratagoules ED, LaRue JM, Powell AA, Gause PR, Craven MT, Payne CM, Powell MB, Gerner EW, Earnest DL. Different bile acids exhibit distinct biological effects: the tumor promoter deoxycholic acid induces apoptosis and the chemopreventive agent ursodeoxycholic acid inhibits cell proliferation. *Nutr Cancer* 1998; **31**: 111-118

62 **Yui S**, Saeki T, Kanamoto R, Iwami K. Characteristics of apoptosis in HCT116 colon cancer cells induced by deoxycholic acid. *J Biochem* 2005; **138**: 151-157

63 **Powell AA**, LaRue JM, Batta AK, Martinez JD. Bile acid hydrophobicity is correlated with induction of apoptosis and/or growth arrest in HCT116 cells. *Biochem J* 2001; **356**: 481-486

64 **Wachs FP**, Krieg RC, Rodrigues CM, Messmann H, Kullmann F, Knuchel-Clarke R, Scholmerich J, Rogler G, Schlottmann K. Bile salt-induced apoptosis in human colon cancer cell lines involves the mitochondrial transmembrane potential but not the CD95 (Fas/Apo-1) receptor. *Int J Colorectal Dis* 2005; **20**: 103-113

65 **Akare S**, Martinez JD. Bile acid induces hydrophobicity-dependent membrane alterations. *Biochim Biophys Acta* 2005; **1735**: 59-67

66 **Shah SA**, Looby E, Volkov Y, Long A, Kelleher D. Ursodeoxycholic acid inhibits translocation of protein kinase C in human colonic cancer cell lines. *Eur J Cancer* 2005; **41**: 2160-2169

67 **Looby E**, Long A, Kelleher D, Volkov Y. Bile acid deoxycholate induces differential subcellular localisation of the PKC isoenzymes beta 1, epsilon and delta in colonic epithelial cells in a sodium butyrate insensitive manner. *Int J Cancer* 2005; **114**: 887-895

68 **Shah SA**, Volkov Y, Arfin Q, Abdel-Latif MM, Kelleher D. Ursodeoxycholic acid inhibits interleukin 1 beta [corrected] and deoxycholic acid-induced activation of NF-kappaB and AP-1 in human colon cancer cells. *Int J Cancer* 2006; **118**: 532-539

69 **Qiao D**, Stratagouleas ED, Martinez JD. Activation and role of mitogen-activated protein kinases in deoxycholic acid-induced apoptosis. *Carcinogenesis* 2001; **22**: 35-41

70 **Jurek D**, Fleckl E, Marian B. Bile acid induced gene expression in LT97 colonic adenoma cells. *Food Chem Toxicol* 2005; **43**: 87-93

71 **Payne CM**, Waltmire CN, Crowley C, Crowley-Weber CL, Dvorakova K, Bernstein H, Bernstein C, Holubec H, Garewal H. Caspase-6 mediated cleavage of guanylate cyclase alpha 1 during deoxycholate-induced apoptosis: protective role of the nitric oxide signaling module. *Cell Biol Toxicol* 2003; **19**: 373-392

72 **Bernstein H**, Payne CM, Kunke K, Crowley-Weber CL, Waltmire CN, Dvorakova K, Holubec H, Bernstein C, Vaillancourt RR, Raynes DA, Guerriero V, Garewal H. A proteomic study of resistance to deoxycholate-induced apoptosis. *Carcinogenesis* 2004; **25**: 681-692

73 **Nobuoka A**, Takayama T, Miyanishi K, Sato T, Takanashi K, Hayashi T, Kukitsu T, Sato Y, Takahashi M, Okamoto T, Matsunaga T, Kato J, Oda M, Azuma T, Niitsu Y. Glutathione-S-transferase P1-1 protects aberrant crypt foci from apoptosis induced by deoxycholic acid. *Gastroenterology* 2004; **127**: 428-443

74 **Powolny A**, Xu J, Loo G. Deoxycholate induces DNA damage and apoptosis in human colon epithelial cells expressing either mutant or wild-type p53. *Int J Biochem Cell Biol* 2001; **33**: 193-203

75 **Qiao D**, Gaitonde SV, Qi W, Martinez JD. Deoxycholic acid suppresses p53 by stimulating proteasome-mediated p53 protein degradation. *Carcinogenesis* 2001; **22**: 957-964

76 **Romagnolo DF**, Chirnomas RB, Ku J, Jeffy BD, Payne CM, Holubec H, Ramsey L, Bernstein H, Bernstein C, Kunke K, Bhattacharyya A, Warneke J, Garewal H. Deoxycholate, an endogenous tumor promoter and DNA damaging agent, modulates BRCA-1 expression in apoptosis-sensitive epithelial cells: loss of BRCA-1 expression in colonic adenocarcinomas. *Nutr Cancer* 2003; **46**: 82-92

77 **Cheng K**, Raufman JP. Bile acid-induced proliferation of a human colon cancer cell line is mediated by transactivation of epidermal growth factor receptors. *Biochem Pharmacol* 2005; **70**: 1035-1047

78 **Li Z**, Tanaka M, Kataoka H, Nakamura R, Sanjar R, Shinmura K, Sugimura H. EphA2 up-regulation induced by deoxycholic acid in human colon carcinoma cells, an involvement of extracellular signal-regulated kinase and p53-independence. *J Cancer Res Clin Oncol* 2003; **129**: 703-708

79 **Im E**, Akare S, Powell A, Martinez JD. Ursodeoxycholic acid can suppress deoxycholic acid-induced apoptosis by stimulating Akt/PKB-dependent survival signaling. *Nutr Cancer* 2005; **51**: 110-116

80 **Im E**, Martinez JD. Ursodeoxycholic acid (UDCA) can inhibit deoxycholic acid (DCA)-induced apoptosis via modulation of EGFR/Raf-1/ERK signaling in human colon cancer cells. *J Nutr* 2004; **134**: 483-486

81 **Pai R**, Tarnawski AS, Tran T. Deoxycholic acid activates beta-catenin signaling pathway and increases colon cell cancer growth and invasiveness. *Mol Biol Cell* 2004; **15**: 2156-2163

82 **De Gottardi A**, Touri F, Maurer CA, Perez A, Maurhofer O, Ventre G, Bentzen CL, Niesor EJ, Dufour JF. The bile acid nuclear receptor FXR and the bile acid binding protein IBABP are differently expressed in colon cancer. *Dig Dis Sci* 2004; **49**: 982-989

83 **Gould KA**, Dove WF. Action of Min and Mom1 on neoplasia in ectopic intestinal grafts. *Cell Growth Differ* 1996; **7**: 1361-1368

84 **Jacoby RF**, Cole CE, Hawk ET, Lubet RA. Ursodeoxycholate/Sulindac combination treatment effectively prevents intestinal adenomas in a mouse model of polyposis. *Gastroenterology* 2004; **127**: 838-844

85 **Kohno H**, Suzuki R, Yasui Y, Miyamoto S, Wakabayashi K, Tanaka T. Ursodeoxycholic acid versus sulfasalazine in colitis-related colon carcinogenesis in mice. *Clin Cancer Res* 2007; **13**: 2519-2525

86 **Narahara H**, Tatsuta M, Iishi H, Baba M, Uedo N, Sakai N, Yano H, Ishiguro S. K-ras point mutation is associated with enhancement by deoxycholic acid of colon carcinogenesis induced by azoxymethane, but not with its attenuation by all-trans-retinoic acid. *Int J Cancer* 2000; **88**: 157-161

87 **Khare S**, Cerda S, Wali RK, von Lintig FC, Tretiakova M, Joseph L, Stoiber D, Cohen G, Nimmagadda K, Hart J, Sitrin MD, Boss GR, Bissonnette M. Ursodeoxycholic acid inhibits Ras mutations, wild-type Ras activation, and cyclooxygenase-2 expression in colon cancer. *Cancer Res* 2003; **63**: 3517-3523

88 **Serfaty L**, De Leusse A, Rosmorduc O, Desaint B, Flejou JF, Chazouilleres O, Poupon RE, Poupon R. Ursodeoxycholic acid therapy and the risk of colorectal adenoma in patients with primary biliary cirrhosis: an observational study. *Hepatology* 2003; **38**: 203-209

89 **Wolf JM**, Rybicki LA, Lashner BA. The impact of ursodeoxycholic acid on cancer, dysplasia and mortality in ulcerative colitis patients with primary sclerosing cholangitis. *Aliment Pharmacol Ther* 2005; **22**: 783-788

90 **Alberts DS**, Martinez ME, Hess LM, Einspahr JG, Green SB, Bhattacharyya AK, Guillen J, Krutzsch M, Batta AK, Salen G, Fales L, Koonce K, Parish D, Clouser M, Roe D, Lance P. Phase III trial of ursodeoxycholic acid to prevent colorectal adenoma recurrence. *J Natl Cancer Inst* 2005; **97**: 846-853

91 **Sjoqvist U**, Tribukait B, Ost A, Einarsson C, Oxelmark L, Lofberg R. Ursodeoxycholic acid treatment in IBD-patients with colorectal dysplasia and/or DNA-aneuploidy: a prospective, double-blind, randomized controlled pilot study. *Anticancer Res* 2004; **24**: 3121-3127

92 **Pardi DS**, Loftus EV Jr, Kremers WK, Keach J, Lindor KD. Ursodeoxycholic acid as a chemopreventive agent in patients with ulcerative colitis and primary sclerosing cholangitis. *Gastroenterology* 2003; **124**: 889-893

93 **Ochsenkuhn T**, Marsteller I, Hay U, Diebold J, Paumgartner G, Goke B, Sackmann M. Does ursodeoxycholic acid change the proliferation of the colorectal mucosa?. A randomized, placebo-controlled study. *Digestion* 2003; **68**: 209-216

94 **Hess LM**, Krutzsch MF, Guillen J, Chow HH, Einspahr J, Batta AK, Salen G, Reid ME, Earnest DL, Alberts DS. Results of a phase I multiple-dose clinical study of ursodeoxycholic Acid. *Cancer Epidemiol Biomarkers Prev* 2004; **13**: 861-867

95 **Levine J**, Ellis CJ, Furne JK, Springfield J, Levitt MD. Fecal hydrogen sulfide production in ulcerative colitis. *Am J Gastroenterol* 1998; **93**: 83-87

96 **Levitt MD**, Furne J, Springfield J, Suarez F, DeMaster E. Detoxification of hydrogen sulfide and methanethiol in the cecal mucosa. *J Clin Invest* 1999; **104**: 1107-1114

97 **Fihn BM**, Sjoqvist A, Jodal M. Involvement of enteric nerves in permeability changes due to deoxycholic acid in rat jejunum in vivo. *Acta Physiol Scand* 2003; **178**: 241-250

98 **Gelbmann CM**, Schteingart CD, Thompson SM, Hofmann AF, Barrett KE. Mast cells and histamine contribute to bile acid-stimulated secretion in the mouse colon. *J Clin Invest* 1995; **95**: 2831-2839

99 **Karlstrom L**. Evidence of involvement of the enteric nervous system in the effects of sodium deoxycholate on small-intestinal transepithelial fluid transport and motility. *Scand J Gastroenterol* 1986; **21**: 321-330

100 **Chadwick VS**, Gaginella TS, Carlson GL, Debongnie JC, Phillips SF, Hofmann AF. Effect of molecular structure on bile acid-induced alterations in absorptive function, permeability, and morphology in the perfused rabbit colon. *J Lab Clin Med* 1979; **94**: 661-674

101 **Mekjian HS**, Phillips SF, Hofmann AF. Colonic secretion of water and electrolytes induced by bile acids: perfusion studies in man. *J Clin Invest* 1971; **50**: 1569-1577

102 **Fang P**, Dong L, Zhang WJ, Luo JY. Relationship between entero-hepatic bile acid circulation and interdigestive migrating myoelectrical activity in rats. *World J Gastroenterol* 2005; **11**: 5377-5380

103 **Karlstrom L**, Cassuto J, Jodal M, Lundgren O. The importance of the enteric nervous system for the bile-salt-induced secretion in the small intestine of the rat. *Scand J Gastroenterol* 1983; **18**: 117-123

104 **Karlstrom L**, Jodal M, Lundgren O. Blood flow distribution, lymph flow, villus tissue osmolality and fluid and electrolyte transport after exposing the cat small intestine to sodium deoxycholate. *Acta Physiol Scand* 1986; **128**: 83-96

105 **Bossmann B**, Haschen RJ. Release of enzymes from rat jejunal mucosa by bile salts. *J Clin Chem Clin Biochem* 1983; **21**: 1-9

106 **Binder HJ**, Filburn C, Volpe BT. Bile salt alteration of colonic electrolyte transport: role of cyclic adenosine monophosphate. *Gastroenterology* 1975; **68**: 503-508

107 **Zhu Y**, Hua P, Rafiq S, Waffner EJ, Duffey ME, Lance P. Ca2+- and PKC-dependent stimulation of PGE2 synthesis by deoxycholic acid in human colonic fibroblasts. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G503-G510

108 **Quist RG**, Ton-Nu HT, Lillienau J, Hofmann AF, Barrett KE. Activation of mast cells by bile acids. *Gastroenterology* 1991; **101**: 446-456

109 **Camilleri M**, Murphy R, Chadwick VS. Dose-related effects of chenodeoxycholic acid in the rabbit colon. *Dig Dis Sci* 1980; **25**: 433-438

110 **Hardcastle J**, Hardcastle PT, Chapman J, Taylor CJ. Taurocholic acid-induced secretion in normal and cystic fibrosis mouse ileum. *J Pharm Pharmacol* 2001; **53**: 711-719

111 **Hardcastle J**, Hardcastle PT, Chapman J, Taylor CJ. Ursodeoxycholic acid action on the transport function of the small intestine in normal and cystic fibrosis mice. *J Pharm Pharmacol* 2001; **53**: 1457-1467

112 **Volpe BT**, Binder HJ. Bile salt alteration of ion transport across jejunal mucosa. *Biochim Biophys Acta* 1975; **394**: 597-604

113 **Bijvelds MJ**, Jorna H, Verkade HJ, Bot AG, Hofmann F, Agellon LB, Sinaasappel M, de Jonge HR. Activation of CFTR by ASBT-mediated bile salt absorption. *Am J Physiol Gastrointest Liver Physiol* 2005; **289**: G870-G879

114 **Hardcastle J**, Harwood MD, Taylor CJ. Absorption of taurocholic acid by the ileum of normal and transgenic DeltaF508 cystic fibrosis mice. *J Pharm Pharmacol* 2004; **56**: 445-452

115 **Wall MJ**, Baker RD. Effect of taurocholate on electrical potential difference across rat small intestine. *Life Sci* I 1972; **11**: 375-386

116 **Devor DC**, Sekar MC, Frizzell RA, Duffey ME. Taurodeoxycholate activates potassium and chloride conductances via an IP3-mediated release of calcium from intracellular stores in a colonic cell line (T84) *J Clin Invest* 1993; **92**: 2173-2181

117 **Balistreri WF**, Heubi JE, Suchy FJ. Bile acid metabolism: relationship of bile acid malabsorption and diarrhea. *J Pediatr Gastroenterol Nutr* 1983; **2**: 105-121

118 **Sanchez de Medina F**, Perez R, Martinez-Augustin O, Gonzalez R, Lorente MD, Galvez J, Zarzuelo A. Disturbances of colonic ion secretion in inflammation: role of the enteric nervous system and cAMP. *Pflugers Arch* 2002; **444**: 378-388

119 **Chen F**, Ma L, Sartor RB, Li F, Xiong H, Sun AQ, Shneider B. Inflammatory-mediated repression of the rat ileal sodium-dependent bile acid transporter by c-fos nuclear translocation. *Gastroenterology* 2002; **123**: 2005-2016

120 **Zachos NC**, Tse M, Donowitz M. Molecular physiology of intestinal Na+/H+ exchange. *Annu Rev Physiol* 2005; **67**: 411-443

121 **Cotting J**, Lentze MJ, Reichen J. Effects of ursodeoxycholic acid treatment on nutrition and liver function in patients with cystic fibrosis and longstanding cholestasis. *Gut* 1990; **31**: 918-921

122 **Talamini MA**, Gadacz TR. Gallstone dissolution. *Surg Clin North Am* 1990; **70**: 1217-1230

123 **Bernardes-Silva CF**, Damiao AO, Sipahi AM, Laurindo FR, Iriya K, Lopasso FP, Buchpiguel CA, Lordello ML, Agostinho CL, Laudanna AA. Ursodeoxycholic acid ameliorates experimental ileitis counteracting intestinal barrier dysfunction and oxidative stress. *Dig Dis Sci* 2004; **49**: 1569-1574

124 **Lloyd-Still JD**, Beno DW, Uhing MR, Jiyamapa-Serna VA, Kimura RE. Ursodeoxycholic acid ameliorates ibuprofen-induced enteropathy in the rat. *J Pediatr Gastroenterol Nutr* 2001; **32**: 270-273

125 **Lee DK**, Park SY, Baik SK, Kwon SO, Chung JM, Oh ES, Kim HS. [Deoxycholic acid-induced signal transduction in HT-29 cells: role of NF-kappa B and interleukin-8] *Korean J Gastroenterol* 2004; **43**: 176-185

126 **Muhlbauer M**, Allard B, Bosserhoff AK, Kiessling S, Herfarth H, Rogler G, Scholmerich J, Jobin C, Hellerbrand C. Differential effects of deoxycholic acid and taurodeoxycholic acid on NF-kappa B signal transduction and IL-8 gene expression in colonic epithelial cells. *Am J Physiol Gastrointest Liver Physiol* 2004; **286**: G1000-G1008

127 **Payne CM**, Crowley C, Washo-Stultz D, Briehl M, Bernstein H, Bernstein C, Beard S, Holubec H, Warneke J. The stress-response proteins poly(ADP-ribose) polymerase and NF-kappaB protect against bile salt-induced apoptosis. *Cell Death Differ* 1998; **5**: 623-636

128 **Strauch ED**, Bass BL, Rao JN, Vann JA, Wang JY. NF-kappaB regulates intestinal epithelial cell and bile salt-induced migration after injury. *Ann Surg* 2003; **237**: 494-501

129 **Hirano F**, Tanada H, Makino Y, Okamoto K, Hiramoto M, Handa H, Makino I. Induction of the transcription factor AP-1 in cultured human colon adenocarcinoma cells following exposure to bile acids. *Carcinogenesis* 1996; **17**: 427-433

130 **Strauch ED**, Yamaguchi J, Bass BL, Wang JY. Bile salts regulate intestinal epithelial cell migration by nuclear factor-kappa B-induced expression of transforming growth factor-

beta. *J Am Coll Surg* 2003; **197**: 974-984
131 **Lazaridis KN**, Gores GJ, Lindor KD. Ursodeoxycholic acid 'mechanisms of action and clinical use in hepatobiliary disorders'. *J Hepatol* 2001; **35**: 134-146
132 **Paumgartner G**. Medical treatment of cholestatic liver diseases: From pathobiology to pharmacological targets. *World J Gastroenterol* 2006; **12**: 4445-4451
133 **Park SE**, Choi HJ, Yee SB, Chung HY, Suh H, Choi YH, Yoo YH, Kim ND. Synthetic bile acid derivatives inhibit cell proliferation and induce apoptosis in HT-29 human colon cancer cells. *Int J Oncol* 2004; **25**: 231-236
134 **Lepercq P**, Hermier D, David O, Michelin R, Gibard C, Beguet F, Relano P, Cayuela C, Juste C. Increasing ursodeoxycholic acid in the enterohepatic circulation of pigs through the administration of living bacteria. *Br J Nutr* 2005; **93**: 457-469
135 **Kapral C**, Wewalka F, Praxmarer V, Lenz K, Hofmann AF. Conjugated bile acid replacement therapy in short bowel syndrome patients with a residual colon. *Z Gastroenterol* 2004; **42**: 583-589
136 **Akobeng AK**, Clayton PT, Miller V, Super M, Thomas AG. An inborn error of bile acid synthesis (3beta-hydroxy-delta5-C27-steroid dehydrogenase deficiency) presenting as malabsorption leading to rickets. *Arch Dis Child* 1999; **80**: 463-465
137 **Eusufzai S**. Bile acid malabsorption: mechanisms and treatment. *Dig Dis* 1995; **13**: 312-321
138 **Tsutsumi K**, Li SK, Hymas RV, Teng CL, Tillman LG, Hardee GE, Higuchi WI, Ho NF. Systematic studies on the paracellular permeation of model permeants and oligonucleotides in the rat small intestine with chenodeoxycholate as enhancer. *J Pharm Sci* 2008; **97**: 350-367
139 **Lane ME**, O'driscoll CM, Corrigan OI. Quantitative estimation of the effects of bile salt surfactant systems on insulin stability and permeability in the rat intestine using a mass balance model. *J Pharm Pharmacol* 2005; **57**: 169-175
140 **Lee DY**, Lee J, Lee S, Kim SK, Byun Y. Liphophilic complexation of heparin based on bile acid for oral delivery. *J Control Release* 2007; **123**: 39-45

S- Editor Li DL **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5641

World J Gastroenterol 2008 October 7; 14(37): 5641-5649
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Jose JG Marin, Professor, *Series Editor*

Physiology of bile secretion

Alejandro Esteller

Alejandro Esteller, Department of Physiology and Pharmacology, University of Salamanca, Campus Miguel de Unamuno, ED, Salamanca 37007, Spain
Author contributions: Esteller A contributed all to this paper.
Correspondence to: Alejandro Esteller, Department of Physiology and Pharmacology, University of Salamanca, Campus Miguel de Unamuno, ED, Salamanca 37007, Spain. aep@usal.es
Telephone: +34-92-3294529 Fax: +34-92-3294664
Received: July 24, 2008 Revised: September 16, 2008
Accepted: September 23, 2008
Published online: October 7, 2008

Abstract

The formation of bile depends on the structural and functional integrity of the bile-secretory apparatus and its impairment, in different situations, results in the syndrome of cholestasis. The structural bases that permit bile secretion as well as various aspects related with its composition and flow rate in physiological conditions will first be reviewed. Canalicular bile is produced by polarized hepatocytes that hold transporters in their basolateral (sinusoidal) and apical (canalicular) plasma membrane. This review summarizes recent data on the molecular determinants of this primary bile formation. The major function of the biliary tree is modification of canalicular bile by secretory and reabsorptive processes in bile-duct epithelial cells (cholangiocytes) as bile passes through bile ducts. The mechanisms of fluid and solute transport in cholangiocytes will also be discussed. In contrast to hepatocytes where secretion is constant and poorly controlled, cholangiocyte secretion is regulated by hormones and nerves. A short section dedicated to these regulatory mechanisms of bile secretion has been included. The aim of this revision was to set the bases for other reviews in this series that will be devoted to specific issues related with biliary physiology and pathology.

Key words: Hepatocytes; Cholangiocytes; Bile flow; Bile acid; Transport

Peer reviewers: Milan Jirsa, PhD, Laboratory of Experimental Medicine-building Z1, Institute for Clinical and Experimental Medicine, Videnska 1958/9, Praha 414000, Czech; Liqing Yu, MD, PhD, Assistant Professor, Department of Pathology, Lipid Sciences, Director of Transgenic Mouse Core Facility Wake Forest University School of Medicine, Medical Center Blvd Winston-Salem, NC 27157-1040, United States

Esteller A. Physiology of bile secretion. *World J Gastroenterol* 2008; 14(37): 5641-5649 Available from: URL: http://www.wjgnet.com/1007-9327/14/5641.asp DOI: http://dx.doi.org/10.3748/wjg.14.5641

INTRODUCTION

In 1924 Cramer and Ludford[1] published a paper in which they indicated: "liver cells present an unusual problem of secretory cellular activity. The cells of all other secreting glands are functionally unipolar, but hepatocytes are bipolar". Furthermore they wrote that "all the known facts compel a return to the old conception of Claude Bernard that glycogenic function represents an internal secretion of the liver". It is clear that bile secretion is the external one. Finally, regarding this functional bipolarity, those authors reported "it is difficult to form a mental conception of the cellular mechanism which enables one cell to pass two different specific secretions in different directions"; towards sinusoids or towards canaliculi. In that paper they offered morphological evidence that the Golgi apparatus is involved in secretion by hepatocytes in both directions.

Since then our understanding of bile secretion has evolved, and differerent attempts have been made to resolve diverging conceptual theories. In the fifties we learned that bile flow is not the result of a hydrostatic perfusion[2] but the result of osmotic forces[3]. In the sixties we began to distinguish between canalicular and ductular bile[4,5] and showed that bile flow is related to the amount of bile salts secreted to the canaliculi[6]. Shortly after, it was shown that the canalicular bile may be either dependent or independent of bile salts[7,8].

The use of different methods and techniques, such as chronic bile fistula[9], isolated and perfused livers[10], couplets of hepatocytes[11], isolated bile duct units[12], separation of membranes from basolateral and apical domains[13] and several other experimental approaches have helped us to advance our knowledge of the different mechanism involved in the formation of bile flow.

Over the last two decades molecular biology

techniques have allowed the cloning of different proteins expressed in cholangiocytes[14] and/or hepatocytes; such proteins may be involved in the transport of endogenous or exogenous organic anions and cations[15]. The overall role of these transporters depends on whether they are targeted from the Golgi apparatus to the apical or basolateral poles of the epithelial liver cells[16,17]. This may help to resolve the conceptual problem of bipolarity, pointed out by Cramer and Ludford. The discovery of nuclear receptors and plasma membrane receptors for bile acids (BAs)[18,19] has opened a new field of investigation regarding the role of these molecules in the control of secretory and metabolic mechanisms[20,21] that somehow links different aspects of endogenous and exogenous secretions to metabolic functions in parenchymal liver cells. This issue will be the matter of a separate review in this series.

In this introductory review, a brief revision is made of the structural bases that permit bile secretion as well as several aspects related to its composition and flow rate under certain physiological conditions. Knowledge of the role in bile flow generation of cholangiocytes has increased exponentially over the last few years[22,23] and is therefore reviewed here. Finally, a short section dedicated to the regulatory mechanisms of bile secretion is included. This should set the bases for other reviews in this series that will be devoted to specific issues related to biliary physiology and pathology.

STRUCTURAL BASES

The biliary apparatus is a convergent system of canals that begins in the canaliculi, followed by the bile ducts, and ending with the common bile duct (coledochus). Bile secretion depends on the function of membrane transport systems in hepatocytes and cholangiocytes and on the structural and functional integrity of the biliary tree. The hepatocytes, constituting the most abundant liver cell population (65%), generate the so-called primary bile in their canaliculi[4]. Biliary canaliculi are blind tubular structures, with a very high surface/volume ratio that favors-by means of osmotic gradients-the formation of bile flow[5]. Cholangiocytes, which constitute 3%-5% of the liver cells[24], modify the canalicular bile by secretory and reabsorptive processes as bile passes through the bile ducts[22], and they are responsible for approximately 30% of bile volume[25]. In contrast to hepatocytes, where secretion is constant and poorly controlled[26], cholangiocytes secretion is broadly regulated[22,27].

Hepatocytes exhibit structural and functional polarity, three different zones being distinguished in their membranes. The sinusoidal membrane, which faces Disse's space, covers 37% of the total surface of the hepatocyte. There is no lamina basal between hepatocytes and endothelial cells, which are fenestrated and show abundant vacuole of endocytosis and exocytosis, accounting for the intense exchange of substances between blood and hepatocytes[28]. In the lateral membrane (50% of the total surface) there are specialized structures that allow adhesion (desmosomes and tight-junctions) and comunication (gap-junctions) between adjacent hepatocytes. The tight-junctions determine the exchange of fluids and electrolytes between Disse's space and the canalicular space through the paracelular pathway[28]. Together, the sinusoidal and lateral membranes cover the basolateral surface (basolateral membrane). The canaliculi are tiny ducts delimited by the canalicular or apical membrane membrane of two adjacent hepatocytes[28] and they represent a small fraction of the total hepatocyte surface area.

Table 1 Membrane transporters in hepatocytes

Abbreviation	Function
Basolateral membrane (sinusoidal)	
NTCP/SLC10A1[1]	Takes up BAs
OATP/SLC21A[1]	Takes up BAs and OA^- and exports BAs, GSH, HCO_3^-
MRP3/ABCC3	Export OA^- conjugates, GSH
MRP4/ABCC4	Export BAs, GSH
OSTa /OSTb	Organic solute transporter: Exports BAs
NBC4c/SLC4A5[1]	Na^+-HCO_3^- symporter, acid extruder
NHE1/SLC9A1[1]	Na^+/H^+ exchanger, acid extruder
SK2	Potassium channel, potassium efflux
SLC12A2	Na^+-K^+-Cl^- symporter: Sodium, potassium, chloride uptake
Apical membrane (canalicular)	
BSEP/ABCB11[1], formerly SPGE	Bile salt export pump
MRP2/ABCC2[1], formerly cMOAT	Export non BAs OA^-, GSH
MDR1	Efflux of lipofilic cations
MDR3/ABCB4	Phospholipid flipase
ABCG5/ABCG8	Export sterols
AE2/SLC4A2[1]	Cl^-/HCO_3^- anion exchanger: Acid loader
Cl- channel[1]	Export chloride
AQP8[1]	Water channel
NHE3/SLC9A3[1]	Na^+/H^+ exchanger: Acid extruder

[1]Transporters relevant to bile flow under physiological basal conditions.

Currently, the molecular biology and genetic characteristics of many transporters of the basolateral and apical membranes are known (Table 1). Such transporters take part in the transfer of substances between blood and hepatocytes and between hepatocytes and bile, respectively. Likewise, many transporters expressed in cholangiocyte membranes are also known (Table 2). In this review only those transporters with a clear role in the genesis of physiological bile flow are addressed. More comprehensive reviews are available elsewhere[29-31].

The Golgi complex and the network of microtubules and microfilaments are important structures for the exocrine function of hepatocytes and also for the mechanisms of bile formation. The pericanalicular space, free of cellular organelles, contains actin microfilaments that reach to the microvilli of the canalicular membrane. Microtubules are distributed throughout the cytoplasm. The vesicles from the Golgi complex are vehicles for substances to be excreted in bile as well as plasma proteins, including transporters, to be placed at apical or basolateral membranes[17,32]. Newly synthesized apical

Table 2 Membrane transporters in cholangiocytes: Abbreviations and function

Abbreviation	Function
Basolateral membrane	
NDCBE/SLC4A8[1]	Na^+-dependent Cl^-/HCO_3^- exchanger: Import HCO_3^- and Na^+, Export H^+ and Cl^-
NHE/SLC9[1]	Na^+/H^+ exchanger: Acid extruder
AQP4[1]	Water channel
tASBT/SLC10A2	Export BAs and Na^+
SK2	Potassium channel, potassium efflux
SLC12A2	Import Na^+-K^+-$2Cl^-$
MRP3/ABCC3	Export OA^- conjugates, GSH
MRP4/ABCC4	Export BAs, GSH
Apical membrane	
AE2/SLC4A2[1]	Cl^-/HCO_3^- anion exchanger: Acid loader
CFTR[1]	Cl^- channel: Export chloride
AQP1[1]	Water channel
NBCe/NBC4/SLC4A5	Na^+-HCO_3^- symporter: Acid extruder
ASBT/SLC10A2	BAs-Na^+, symport: Uptake of BAs and Na^+

[1]Transporters relevant to bile flow under physiological basal conditions.

ABC (ATP-binding cassette) transporters are transferred from the Golgi apparatus to the canalicular membrane[16]. This traffic is dependent on intact microtubule and microfilament systems[17,32].

The hepatocytes and the biliary system are closely related to the blood vascular elements, both forming a functional unit: the hepatic acinus[33]. The blood flow generates concentrative gradients of oxygen and nutrients along the sinusoids. These gradients allow a division of the hepatic acinus into three different zones according to its distance from the portal space: the periportal or zone Ⅰ, zone Ⅱ and the centrilobular or zone Ⅲ. Moreover, these gradients cause functional heterogeneity between the hepatocytes of the different zones[34,35].

BILE COMPOSITION

Bile mainly consists of water, in which there are organic and inorganic substances in suspension, dissolved, or in equilibrium between both states. In bile samples, collected from the human common bile duct, the concentrations of the inorganic electrolytes sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and bicarbonate (HCO_3^-) are slightly higher than their plasmatic concentrations, whereas biliary chloride (Cl^-) concentrations are slightly lower than these found in plasma. BAs concentrations range between 2 and 45 mmol/L. The concentrations of biliary pigments range from 50 to 200 mg/100 mL. Proteins and peptides, such as glutathione, are also found in bile[36]. It is also possible to detect glucose and small amounts of endogenous substances such as thyroid and steroid hormones[37]. Human bile is rich in lipids. Thus, phospholipids concentrations seem to range between 25 and 810 mg/100 mL, whereas these of cholesterol vary between 60 and 320 mg/100 mL, with average ratios of phospholipids to BA of 0.3 and cholesterol to BA of 0.07 (Figure 1). Humans differ from other animals in the fact that our species eliminates cholesterol from the body to a greater extent as cholesterol itself rather than by converting it into BAs[38].

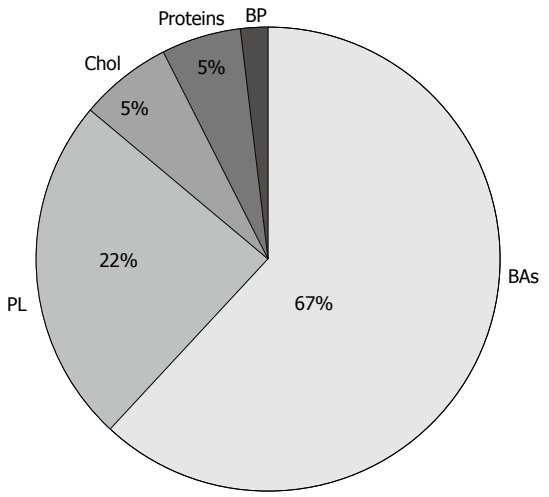


Figure 1 Organic composition of bile. Perceptual distribution of BAs, Phospholipids (PL), Cholesterol (Chol), Proteins and biliary pigments (BP).

BILE FLOW

The mean basal flow of bile in humans is approximately 620 mL/d. One portion of this flow (220 mL/d, 35%) is determined by the secretion of BAs and is called the BAs-dependent canalicular fraction -BADFc-[8]. In addition, there is a linear correlation between the amount of BAs secreted into bile and the amount of water that follows them (7-25 mL/mmol). This choleretic activity of individual BAs is species-dependent and varies according to its chemical structure, conjugational condition and relative concentrations. Choleretic activity is lower for BA species that have a higher tendency to form micellar aggregates in bile[39]. These findings explain the different contributions of BADFc to the bile flow among species (30%-60%)[3,40]. Certain BAs (ursodesoxycholic and the nor-derivatives of ursodesoxycholic and quenodeoxycholic acids) generate a volume of bile higher than that expected from their osmotic force. In order to explain such hypercholeresis, it has been proposed that these BAs would be reabsorbed, in their protonated form, by cholangiocytes. From there, they would be effluxed to blood to reach the sinusoids, where they would be taken up again by hepatocytes and re-secreted to bile, increasing the magnitude of the BADFc[41,42]. This phenomenon, so-called "the cholehepatic shunt pathway" is discussed below in the section devoted to ductular processes.

The amount of canalicular bile independent of the osmotic force of BAs (235 mL/d, 38%) has been designated the BA-independent canalicular fraction (BAIFc)[7,8]. Quantitatively, in humans the BAIFc represents up to a 40% of total primarily formed bile[43]. In other species it varies between 30% and 60%[5,40,44]. The ductular fraction of bile flow has a high value (30%) in humans[25], although it also varies among different species[8,24].

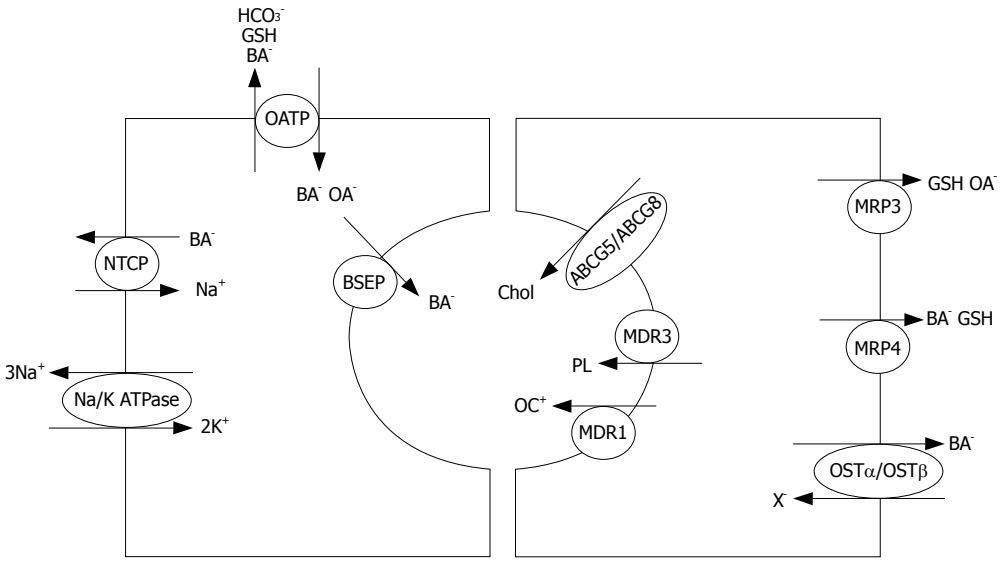


Figure 2 Sinusoidal transport and canalicular secretion. Left: BA dependent canalicular fraction of bile (BADFc). Right: canalicular and sinusoidal secretion of anionic and cationic organic molecules.

THE BA-DEPENDENT CANALICULAR FRACTION (BADFc)

BAs are supplied either by synthesis in liver cells or from the sinusoidal blood as part of *de* enterohepatic circulation (EHC).

Sinusoidal uptake

The BAs in sinusoidal blood are efficiently taken up by hepatocytes from Disse's space despite being highly albumin bound, due to the existence in the basolateral membrane of transporters[45,46]. This uptake is carried out against an electrochemical gradient, is saturable[37] and depends on the structure of the BA. Thus, it is more efficient for trihydroxyl- than for dihydroxyl-BA and for conjugated more than for unconjugated BAs[47]. The sodium taurocholate-cotransporting polypeptide (NTCP), the main Na^+-dependent BAs transporter[30,48] is only expressed in the basolateral membrane[49]. The uptake of BAs by Na^+-independent mechanisms seems to be mediated by less specific transporters, known as organic anion-transporting polypeptides (OATPs), which exchange these molecules for other anions, such as HCO_3^-, glutathione (GSH) or even other BAs[30,47]. These transporters may take up BAs (mainly non conjugated forms), endogenous OA^- (thyroid hormones, monoconjugated bilirubin) and xenobiotic compounds (toxins, drugs, food components, *etc*)[50]. The quantitative relevance of the different isoforms of these transporters in sodium-independent BA uptake by hepatocytes is still not completely understood (Figure 2). The heterodimeric protein OSTalpha/OSTbeta is expressed at the basal membrane of hepatocytes and cholangiocytes[51]. This is a sodium-independent BA transporter that may play a role in BA efflux from hepatocytes toward blood when these compounds get accumulated in cholestatic conditions. Moreover, in cholangiocytes, in addition to play a similar role, this transporter may also be involved in the cholehepatic shunting of BAs.

Transcellular transport

In order to explain the transit of BAs from the sinusoidal membrane to the pericanalicular region, two different, not mutually excluding, mechanisms have been proposed: (1) simple diffusion of BAs bound to intracellular proteins[52]; (2) and/or vesicular transport of BAs driven by cytoskeleton contractile activity[53,54]. Two arguments have been raised against the role of the second mechanism. One is that hepatic transit of labeled BAs is too fast[54]. The second one is that the baseline secretion of BAs is not modified by microtubules disruption[53]. However, the overload of BAs intensify the vesicular trafficking from the Golgi complex to the pericanalicular zone[55], and under these circumstances the alteration in the functional integrity of the cytoskeleton results in impaired BA secretion[56] and subsequently cholestasis[57].

The quantity of ABC transporters in the apical membrane is regulated by the amount of biliary components available for secretion[58,59]. The regulated intracellular vesicular traffic of canalicular ABC transporters[59,60] is crucial for normal bile secretion. The bile salt export pump (BSEP, formerly SPGE, a sister of P-glycoprotein) is the main, if not the only, canalicular BA transporter[61], and it is also located in subcanalicular vesicles that may act as an intracellular pool. It is therefore probable that the impaired secretion of BAs observed in overloaded conditions would be an indirect result of the distortion of the increased vesicular traffic of transporters to the canalicular membrane[56]. These and other studies[17,62] have established not only the actual role of vesicular trafficking in hepatocytes, but also that a specific vesicle trafficking machinery is required for membrane polarity. The overall functions based on hepatocyte polarity are not attributable to the mere presence of transporters in both poles of these cells[63] but also to their intracellular trafficking and temporary anchorage to the different hepatocyte membranes (Figure 2).

Canalicular secretion

At the end of the eighties it was believed that BAs were extruded to the canalicular lumen by an electric gradient, being negative inside hepatocytes (around -37 mV)[64]. However, this gradient is not strong enough

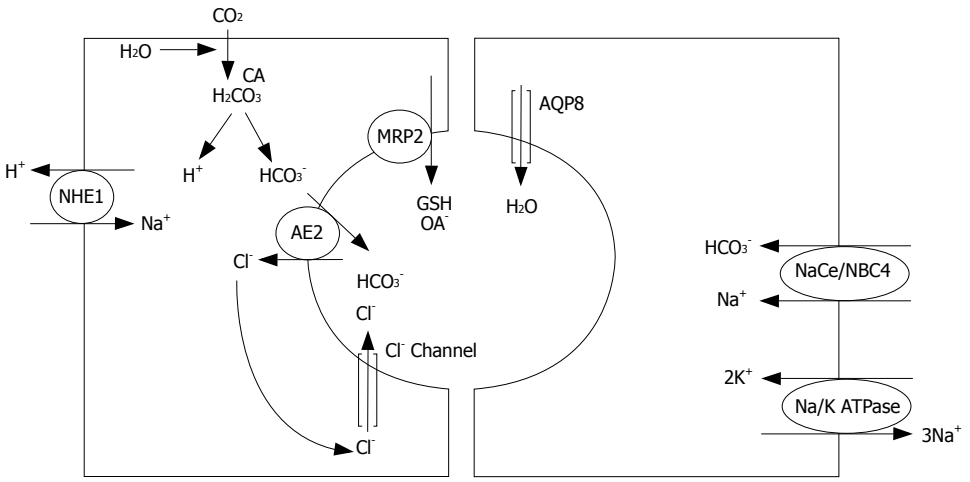


Figure 3 Sinusoidal transport and canalicular secretion. Left: BA independent canalicular fraction of bile (BAIFc). Right: Water an electrolyte movement. CA: carbonic anhydrase.

to impose higher concentrative differences, such as BAs up to 200 times more concentrated in canaliculus than in hepatocyte[26]. It is now known that the secretion of BAs to the canalicular lumen is a saturable phenomenon mediated by a transporter[65]. The energy-dependent bile secretion of BAs is mediated by BSEP[30,58,63]. This export pump was cloned in1998[54] and since them it has been studied in detail[66,67]. It is currently known that BSEP transports both conjugated and unconjugated BAs, sulfated lithocholyl conjugates[68], and a variety of drugs[69]. Other apical ATP binding cassette (ABC) transporters are required: multidrug resistance-P glycoprotein 3 (MDR3) for phospholipids, MDR1 for lipofilic cationic drugs, multidrug resistance-associated protein 2 (MRP2) for non-BA organic anions[70,71] and the heterodimeric protein ABCG5/G8 involved in the secretion of cholesterol and other sterols, such as fitosterols[72] (Figure 2).

BAIFc

The osmotic activity of BAs is not the only determinant of bile flow. Certain substances with osmotic activity, both endogenous and exogenous, may also play a role in canalicular bile generation, without modifying the BA secretion rate[73]. In certain situations, such as diabetes mellitus, there appears to be a reduction in bile flow, without impairment in BA secretion[74]. Glutathione and bicarbonate seem to play a similar quantitative role in BAIFc formation[13].

Multidrug resistance-associated protein 2 (MRP2, formerly cMOAT, canalicular multispecific organic-anion transporter) transports a broad range of amphipathic anionic substrates, including glutathione conjugates, glucuronosyl bilirubin and sulfated BA derivatives[63]. The ATP-dependent canalicular excretion of GSH is one of the main forces responsible for the generation of BAIFc[75]. Under basal conditions, the biliary levels of this tripeptide reache up to 5 mmol/L. This is a sufficient amount to account for the formation of bile by osmotic force[36]. Rats heterozygous and homozygous for inactivating mutations in *Mrp2* secrete less GSH to bile, 37% and 99% below control levels, respectively[76].

The secretion of HCO_3^- is carried out by the canalicular antiporter named AE2[13,77]. This system functions in connection with the canalicular water channel aquaporin (AQP8) and the apical chloride channel, the cystic fibrosis transmembrane regulator (CF^-TR)[13,77]. However, the force that maintains favorable Cl^- gradients remains to be defined. The AE2 antiporter requires the existence of suitable internal levels of HCO_3^- through cotransport of the anion with sodium in the basolateral membrane by the NBCe symporter[78,79] and/or by its formation activated by the carbonic anhydrase (CA) pathway[80]. This latter mechanism is linked to H^+ extrusion *via* Na^+/H^+ antiporters (NHE) working in both the basolateral (NHE1)[81] and canalicular (NHE3)[82] membranes. In turn, sodium cations are extruded *via* a sodium pump. This is why bicarbonate secretion is said to be is a concentrative mechanism that indirectly requires metabolic energy. Canalicular bicarbonate excretion is upregulated by glucagon[83], which also enhances AQP8-mediated water permeability at the canaliculi[84]. These choleretic effects are microtubular-dependent and involve mobilization of intracellular vesicles[83,84]. The other osmotically active inorganic components of bile are not as important as HCO_3^- in generating BAIFc[37,85] (Figure 3).

DUCTULAR PROCESSES

Cholangiocytes exert a series of reabsorptive and secretory processes that dilute and alkalinize the primary (canalicular) bile during its passage along the biliary tract[24,86,87]. Cholangiocytes secrete fluid, HCO_3^-, Cl^- and carry out the reabsorption of glucose, BAs (cholehepatic shunt), glutamate, conjugated bilirubin, BSP, and small OAs^-[87]. As a result, osmotic gradients generate an extra flow of bile known as the ductular BA-independent fraction (BAIFd). These processes are regulated by bile constituents, nerves and hormones[86,88]. The biliary transport of bicarbonate is a relevant function of the cholangiocytes. An electroneutral sodium-independent Cl^-/HCO_3^- exchange activity (AE2) has been observed in the apical membrane of cholangiocytes[77]. There is also a cAMP-responsive Cl^- channel (CFTR) that is coordinated with AE2 to play a role in biliary excretion of HCO_3^-[27,89]. These apical fluxes of anions, in the

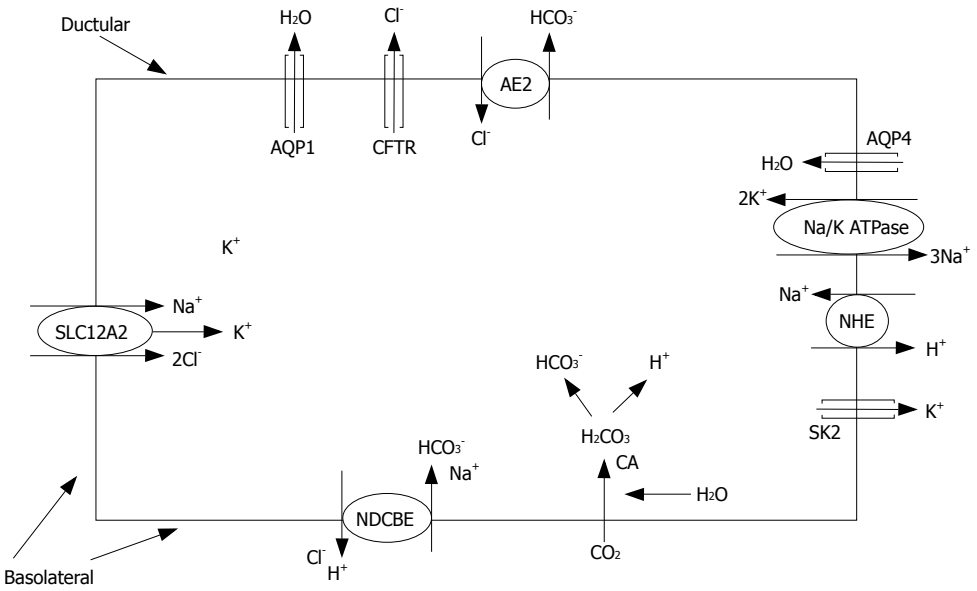


Figure 4 Ductular secretion and reabsorption. BA independent ductular fraction of bile (BAIFd).

presence of aquaporins (AQP1) contribute to the BAIFd[90]. This coordinated function became more evident after discovering that a pool of AE2, CFTR and AQP1 is stored in cholangiocyte intracellular vesicles, which are co-redistributed to the apical membrane under secretin stimulation[90]. The CA pathway and an Cl^-/HCO_3^- exchanger provide the required level of HCO_3^- and the H^+ is subsequently extruded by a coupled carrier-mediated basolateral H^+/Na^+ exchanger (NHE)[91,92]. In humans, the import of HCO_3^- into cholangiocytes occurs mainly through electroneutral Na^+-dependent Cl^-/HCO_3^- anion exchange (NDCBE)[93] (Figure 4).

Lipophilic, unconjugated BAs, such as ursodeoxycholic acids, are passively reabsorbed in cholangiocytes, which constitutes the first essential step in the cholehepatic shunt model[94]. An active transport for conjugated BAs has been described in rat cholangiocytes[95], which expands the idea of cholehepatic shunting of BAS. With the identification of apical (ASBT) and basolateral (tASBT) BAs carriers in cholangiocytes[96], the hypothesis of the cholehepatic BA shunt received additional support[97].

Regulatory factors

Secretin receptors (SCTRs) are exclusively expressed at the basolateral membrane of cholangiocytes[98] and when they are stimulated intracellular levels of cAMP are increased[99]. The pool of AE2, CFTR and AQP1 stored in intracellular vesicles is redistributed to the apical membrane under cAMP or secretin stimulation[90] and secretin stimulation activates CFTR through cAMP. Both effects together explain the increase in HCO_3^- efflux[37]. Most experiments with rats and rabbits have used animals with induced bile duct proliferation[100], since normal rats[23,90] and rabbits[101] respond very poorly to secretin. ASBT activity increases acutely upon secretin stimulation[102], which may accentuate the cholehepatic BAs shunting in the postprandial period.

On cholangiocytes, acetylcholine increases both secretin-stimulated cAMP synthesis and Cl^-/HCO_3^- exchanger activity[103,104]. Vagotomy in BDL rats inhibits secretin-stimulated ductal secretion and decrease cholangiocyte cAMP levels[88]. Bombesin can act either by increasing the secretin release in dogs[105], or inducing ductal secretion with activated Cl^-/HCO_3^- exchange *via* secretin-independent mechanisms in isolated rat cholangiocytes[86]. VIP increases secretin-stimulated bile flow and HCO_3^- excretion in humans[106]. Dopamine, somatostatin and, gastrin to some extent, inhibit basal and secretin-stimulated bicarbonate-rich choleresis[86,107,108].

REFERENCES

1 **Cramer W**, Ludford RJ. On the cellular mechanism of bile secretion and its relation to the Golgi apparatus of the liver cell. *J Physiol* 1926; **62**: 74-80
2 **Brauer RW**, Leong GF, Holloway RJ. Mechanics of bile secretion; effect of perfusion pressure and temperature on bile flow and bile secretion pressure. *Am J Physiol* 1954; **177**: 103-112
3 **Sperber I**. Secretion of organic anions in the formation of urine and bile. *Pharmacol Rev* 1959; **11**: 109-134
4 **Forker EL**. Two sites of bile formation as determined by mannitol and erythritol clearance in the guinea pig. *J Clin Invest* 1967; **46**: 1189-1195
5 **Wheeler HO**, Ross ED, Bradley SE. Canalicular bile production in dogs. *Am J Physiol* 1968; **214**: 866-874
6 **Preisig R**, Cooper H, Wheeler HO. The relationship between taurocholate secretion rate and bile production in the unanesthetized dog during cholinergic blockade and during secretin administration. *J Clin Invest* 1962; **41**: 1152-1162
7 **Boyer JL**, Klatskin G. Canalicular bile flow and bile secretory pressure. Evidence for a non-bile salt dependent fraction in the isolated perfused rat liver. *Gastroenterology* 1970; **59**: 853-859
8 **Erlinger S**, Dhumeaux D, Berthelot P, Dumont M. Effect of inhibitors of sodium transport on bile formation in the rabbit. *Am J Physiol* 1970; **219**: 416-422
9 **Jimenez R**, Esteller A, Lopez MA. Biliary secretion in conscious rabbits: surgical technique. *Lab Anim* 1982; **16**: 182-185
10 **Reichen J**, Paumgartner G. Kinetics of taurocholate uptake by the perfused rat liver. *Gastroenterology* 1975; **68**: 132-136
11 **Graf J**, Gautam A, Boyer JL. Isolated rat hepatocyte couplets: a primary secretory unit for electrophysiologic

studies of bile secretory function. *Proc Natl Acad Sci USA* 1984; **81**: 6516-6520
12 **Boyer JL**. Isolated hepatocyte couplets and bile duct units--novel preparations for the in vitro study of bile secretory function. *Cell Biol Toxicol* 1997; **13**: 289-300
13 **Meier PJ**, Knickelbein R, Moseley RH, Dobbins JW, Boyer JL. Evidence for carrier-mediated chloride/bicarbonate exchange in canalicular rat liver plasma membrane vesicles. *J Clin Invest* 1985; **75**: 1256-1263
14 **Tietz P**, de Groen PC, Anderson NL, Sims C, Esquer-Blasco R, Meheus L, Raymackers J, Dauwe M, LaRusso NF. Cholangiocyte-specific rat liver proteins identified by establishment of a two-dimensional gel protein database. *Electrophoresis* 1998; **19**: 3207-3212
15 **Suzuki H**, Sugiyama Y. Transporters for bile acids and organic anions. *Pharm Biotechnol* 1999; **12**: 387-439
16 **Sai Y**, Nies AT, Arias IM. Bile acid secretion and direct targeting of mdr1-green fluorescent protein from Golgi to the canalicular membrane in polarized WIF-B cells. *J Cell Sci* 1999; **112** (Pt 24): 4535-4545
17 **Wakabayashi Y**, Lippincott-Schwartz J, Arias IM. Intracellular trafficking of bile salt export pump (ABCB11) in polarized hepatic cells: constitutive cycling between the canalicular membrane and rab11-positive endosomes. *Mol Biol Cell* 2004; **15**: 3485-3496
18 **Makishima M**, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ, Shan B. Identification of a nuclear receptor for bile acids. *Science* 1999; **284**: 1362-1365
19 **Parks DJ**, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, Stimmel JB, Willson TM, Zavacki AM, Moore DD, Lehmann JM. Bile acids: natural ligands for an orphan nuclear receptor. *Science* 1999; **284**: 1365-1368
20 **Chiang JY**. Bile acid regulation of gene expression: roles of nuclear hormone receptors. *Endocr Rev* 2002; **23**: 443-463
21 **Chiang JY**. Nuclear receptor regulation of lipid metabolism: potential therapeutics for dyslipidemia, diabetes, and chronic heart and liver diseases. *Curr Opin Investig Drugs* 2005; **6**: 994-1001
22 **Alpini G**, Phillips JO, LaRusso NF. The biology of the biliary epithelia. In: Arias IM, Boyer JL, Fausto N, Jakoby WB, Schachter DA, Shafritz DA, editors. The Liver: Biology and Pathobiology, 3rd edn. New York: Raven Press, 1994; 623-653
23 **Banales JM**, Prieto J, Medina JF. Cholangiocyte anion exchange and biliary bicarbonate excretion. *World J Gastroenterol* 2006; **12**: 3496-3511
24 **Tavoloni N**. The intrahepatic biliary epithelium: an area of growing interest in hepatology. *Semin Liver Dis* 1987; **7**: 280-292
25 **Nathanson MH**, Boyer JL. Mechanisms and regulation of bile secretion. *Hepatology* 1991; **14**: 551-566
26 **Arrese M**, Accatino L. From blood to bile: recent advances in hepatobiliary transport. *Ann Hepatol* 2002; **1**: 64-71
27 **Alvaro D**, Cho WK, Mennone A, Boyer JL. Effect of secretion on intracellular pH regulation in isolated rat bile duct epithelial cells. *J Clin Invest* 1993; **92**: 1314-1325
28 **Jones AL**, Schmucker DL, Renston RH, Murakami T. The architecture of bile secretion. A morphological perspective of physiology. *Dig Dis Sci* 1980; **25**: 609-629
29 **Hagenbuch B**, Meier PJ. The superfamily of organic anion transporting polypeptides. *Biochim Biophys Acta* 2003; **1609**: 1-18
30 **Meier PJ**, Stieger B. Bile salt transporters. *Annu Rev Physiol* 2002; **64**: 635-661
31 **Trauner M**, Boyer JL. Bile salt transporters: molecular characterization, function, and regulation. *Physiol Rev* 2003; **83**: 633-671
32 **Kipp H**, Arias IM. Trafficking of canalicular ABC transporters in hepatocytes. *Annu Rev Physiol* 2002; **64**: 595-608
33 **Rappaport AM**. The microcirculatory acinar concept of normal and pathological hepatic structure. *Beitr Pathol* 1976; **157**: 215-243
34 **Gonzalez J**, Esteller A. Heterogeneity of rabbit hepatocytes for bile secretion after acinar zone 3 damage induced by bromobenzene. Effect of bilirubin and bile salt infusions. *Biochem Pharmacol* 1985; **34**: 507-514
35 **Katz NR**. Metabolic heterogeneity of hepatocytes across the liver acinus. *J Nutr* 1992; **122**: 843-849
36 **Inoue M**, Kinne R, Tran T, Arias IM. The mechanism of biliary secretion of reduced glutathione. Analysis of transport process in isolated rat-liver canalicular membrane vesicles. *Eur J Biochem* 1983; **134**: 467-471
37 **Erlinger S**. Bile flow. In: Arias IM, Popper H, Jacoby WB, Schachter D, Shafritz DA, eds. The Liver: Biology and Pathobiology. New York: Raven Press, 1988: 643-664
38 **Quintao E**, Grundy SM, Ahrens EH Jr. Effects of dietary cholesterol on the regulation of total body cholesterol in man. *J Lipid Res* 1971; **12**: 233-247
39 **Zsembery A**, Thalhammer T, Graf J. Bile Formation: a Concerted Action of Membrane Transporters in Hepatocytes and Cholangiocytes. *News Physiol Sci* 2000; **15**: 6-11
40 **Erlinger S**, Dhumeaux D. Mechanisms and control of secretion of bile water and electrolytes. *Gastroenterology* 1974; **66**: 281-304
41 **Dumont M**, Erlinger S, Uchman S. Hypercholeresis induced by ursodeoxycholic acid and 7-ketolithocholic acid in the rat: possible role of bicarbonate transport. *Gastroenterology* 1980; **79**: 82-89
42 **Garcia-Marin JJ**, Corbic M, Dumont M, de Couet G, Erlinger S. Role of H+ transport in ursodeoxycholate-induced biliary HCO-3 secretion in the rat. *Am J Physiol* 1985; **249**: G335-G341
43 **Erlinger S**. Does Na+-K+-atpase have any role in bile secretion? *Am J Physiol* 1982; **243**: G243-G247
44 **Boyer JL**. Canalicular bile formation in the isolated perfused rat liver. *Am J Physiol* 1971; **221**: 1156-1163
45 **Frimmer M**, Ziegler K. The transport of bile acids in liver cells. *Biochim Biophys Acta* 1988; **947**: 75-99
46 **Hagenbuch B**, Meier PJ. Sinusoidal (basolateral) bile salt uptake systems of hepatocytes. *Semin Liver Dis* 1996; **16**: 129-136
47 **Meier PJ**, Eckhardt U, Schroeder A, Hagenbuch B, Stieger B. Substrate specificity of sinusoidal bile acid and organic anion uptake systems in rat and human liver. *Hepatology* 1997; **26**: 1667-1677
48 **Arrese M**, Ananthanarayanan M, Suchy FJ. Hepatobiliary transport: molecular mechanisms of development and cholestasis. *Pediatr Res* 1998; **44**: 141-147
49 **Hagenbuch B**, Meier PJ. Molecular cloning, chromosomal localization, and functional characterization of a human liver Na+/bile acid cotransporter. *J Clin Invest* 1994; **93**: 1326-1331
50 **Kullak-Ublick GA**, Hagenbuch B, Stieger B, Schteingart CD, Hofmann AF, Wolkoff AW, Meier PJ. Molecular and functional characterization of an organic anion transporting polypeptide cloned from human liver. *Gastroenterology* 1995; **109**: 1274-1282
51 **Ballatori N**. Biology of a novel organic solute and steroid transporter, OSTalpha-OSTbeta. *Exp Biol Med* (Maywood) 2005; **230**: 689-698
52 **Kaplowitz N**. Physiological significance of glutathione S-transferases. *Am J Physiol* 1980; **239**: G439-G444
53 **Crawford JM**, Berken CA, Gollan JL. Role of the hepatocyte microtubular system in the excretion of bile salts and biliary lipid: implications for intracellular vesicular transport. *J Lipid Res* 1988; **29**: 144-156
54 **Lamri Y**, Roda A, Dumont M, Feldmann G, Erlinger S. Immunoperoxidase localization of bile salts in rat liver cells. Evidence for a role of the Golgi apparatus in bile salt transport. *J Clin Invest* 1988; **82**: 1173-1182
55 **Crawford JM**, Vinter DW, Gollan JL. Taurocholate induces pericanalicular localization of C6-NBD-ceramide in isolated

hepatocyte couplets. *Am J Physiol* 1991; **260**: G119-G132
56 **Dubin M**, Maurice M, Feldmann G, Erlinger S. Influence of colchicine and phalloidin on bile secretion and hepatic ultrastructure in the rat. Possible interaction between microtubules and microfilaments. *Gastroenterology* 1980; **79**: 646-654
57 **Trauner M**, Meier PJ, Boyer JL. Molecular pathogenesis of cholestasis. *N Engl J Med* 1998; **339**: 1217-1227
58 **Gerloff T**, Stieger B, Hagenbuch B, Madon J, Landmann L, Roth J, Hofmann AF, Meier PJ. The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. *J Biol Chem* 1998; **273**: 10046-10050
59 **Kipp H**, Pichetshote N, Arias IM. Transporters on demand: intrahepatic pools of canalicular ATP binding cassette transporters in rat liver. *J Biol Chem* 2001; **276**: 7218-7224
60 **Gatmaitan ZC**, Nies AT, Arias IM. Regulation and translocation of ATP-dependent apical membrane proteins in rat liver. *Am J Physiol* 1997; **272**: G1041-G1049
61 **Strautnieks SS**, Bull LN, Knisely AS, Kocoshis SA, Dahl N, Arnell H, Sokal E, Dahan K, Childs S, Ling V, Tanner MS, Kagalwalla AF, Nemeth A, Pawlowska J, Baker A, Mieli-Vergani G, Freimer NB, Gardiner RM, Thompson RJ. A gene encoding a liver-specific ABC transporter is mutated in progressive familial intrahepatic cholestasis. *Nat Genet* 1998; **20**: 233-238
62 **Lapierre LA**, Kumar R, Hales CM, Navarre J, Bhartur SG, Burnette JO, Provance DW Jr, Mercer JA, Bahler M, Goldenring JR. Myosin vb is associated with plasma membrane recycling systems. *Mol Biol Cell* 2001; **12**: 1843-1857
63 **Paulusma CC**, Bosma PJ, Zaman GJ, Bakker CT, Otter M, Scheffer GL, Scheper RJ, Borst P, Oude Elferink RP. Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. *Science* 1996; **271**: 1126-1128
64 **Weinman SA**, Graf J, Boyer JL. Voltage-driven, taurocholate-dependent secretion in isolated hepatocyte couplets. *Am J Physiol* 1989; **256**: G826-G832
65 **Sippel CJ**, Ananthanarayanan M, Suchy FJ. Isolation and characterization of the canalicular membrane bile acid transport protein of rat liver. *Am J Physiol* 1990; **258**: G728-G737
66 **Stieger B**, Meier Y, Meier PJ. The bile salt export pump. *Pflugers Arch* 2007; **453**: 611-620
67 **Suchy FJ**, Ananthanarayanan M. Bile salt excretory pump: biology and pathobiology. *J Pediatr Gastroenterol Nutr* 2006; **43** Suppl 1: S10-S16
68 **Hayashi H**, Takada T, Suzuki H, Onuki R, Hofmann AF, Sugiyama Y. Transport by vesicles of glycine- and taurine-conjugated bile salts and taurolithocholate 3-sulfate: a comparison of human BSEP with rat Bsep. *Biochim Biophys Acta* 2005; **1738**: 54-62
69 **Hirano M**, Maeda K, Hayashi H, Kusuhara H, Sugiyama Y. Bile salt export pump (BSEP/ABCB11) can transport a nonbile acid substrate, pravastatin. *J Pharmacol Exp Ther* 2005; **314**: 876-882
70 **Nies AT**, Gatmaitan Z, Arias IM. ATP-dependent phosphatidylcholine translocation in rat liver canalicular plasma membrane vesicles. *J Lipid Res* 1996; **37**: 1125-1136
71 **Thiebaut F**, Tsuruo T, Hamada H, Gottesman MM, Pastan I, Willingham MC. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc Natl Acad Sci USA* 1987; **84**: 7735-7738
72 **Kidambi S**, Patel SB. Cholesterol and non-cholesterol sterol transporters: ABCG5, ABCG8 and NPC1L1: a review. *Xenobiotica* 2008; **38**: 1119-1139
73 **Aza MJ**, Gonzalez J, Esteller A. Effect of diethyl maleate pretreatment on biliary excretion and choleretic action of sulfobromophthalein in rats. *Arch Int Pharmacodyn Ther* 1986; **281**: 321-330
74 **Garcia-Marin JJ**, Villanueva GR, Esteller A. Diabetes-induced cholestasis in the rat: possible role of hyperglycemia and hypoinsulinemia. *Hepatology* 1988; **8**: 332-340
75 **Ballatori N**, Truong AT. Glutathione as a primary osmotic driving force in hepatic bile formation. *Am J Physiol* 1992; **263**: G617-G624
76 **Paulusma CC**, van Geer MA, Evers R, Heijn M, Ottenhoff R, Borst P, Oude Elferink RP. Canalicular multispecific organic anion transporter/multidrug resistance protein 2 mediates low-affinity transport of reduced glutathione. *Biochem J* 1999; **338** (Pt 2): 393-401
77 **Martinez-Anso E**, Castillo JE, Diez J, Medina JF, Prieto J. Immunohistochemical detection of chloride/bicarbonate anion exchangers in human liver. *Hepatology* 1994; **19**: 1400-1406
78 **Fitz JG**, Persico M, Scharschmidt BF. Electrophysiological evidence for Na+-coupled bicarbonate transport in cultured rat hepatocytes. *Am J Physiol* 1989; **256**: G491-G500
79 **Renner EL**, Lake JR, Scharschmidt BF, Zimmerli B, Meier PJ. Rat hepatocytes exhibit basolateral Na+/HCO3-cotransport. *J Clin Invest* 1989; **83**: 1225-1235
80 **Buanes T**, Grotmol T, Veel T, Landsverk T, Ridderstrale Y, Raeder MG. Importance of carbonic anhydrase for canalicular and ductular choleresis in the pig. *Acta Physiol Scand* 1988; **133**: 535-544
81 **Moseley RH**, Meier PJ, Aronson PS, Boyer JL. Na-H exchange in rat liver basolateral but not canalicular plasma membrane vesicles. *Am J Physiol* 1986; **250**: G35-G43
82 **Mennone A**, Biemesderfer D, Negoianu D, Yang CL, Abbiati T, Schultheis PJ, Shull GE, Aronson PS, Boyer JL. Role of sodium/hydrogen exchanger isoform NHE3 in fluid secretion and absorption in mouse and rat cholangiocytes. *Am J Physiol Gastrointest Liver Physiol* 2001; **280**: G247-G254
83 **Benedetti A**, Strazzabosco M, Ng OC, Boyer JL. Regulation of activity and apical targeting of the Cl-/HCO3- exchanger in rat hepatocytes. *Proc Natl Acad Sci USA* 1994; **91**: 792-796
84 **Gradilone SA**, Garcia F, Huebert RC, Tietz PS, Larocca MC, Kierbel A, Carreras FI, Larusso NF, Marinelli RA. Glucagon induces the plasma membrane insertion of functional aquaporin-8 water channels in isolated rat hepatocytes. *Hepatology* 2003; **37**: 1435-1441
85 **Scharschmidt BF**, Van Dyke RW. Mechanisms of hepatic electrolyte transport. *Gastroenterology* 1983; **85**: 1199-1214
86 **Kanno N**, LeSage G, Glaser S, Alpini G. Regulation of cholangiocyte bicarbonate secretion. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G612-G625
87 **Strazzabosco M**. New insights into cholangiocyte physiology. *J Hepatol* 1997; **27**: 945-952
88 **LeSagE G**, Alvaro D, Benedetti A, Glaser S, Marucci L, Baiocchi L, Eisel W, Caligiuri A, Phinizy JL, Rodgers R, Francis H, Alpini G. Cholinergic system modulates growth, apoptosis, and secretion of cholangiocytes from bile duct-ligated rats. *Gastroenterology* 1999; **117**: 191-199
89 **Cohn JA**, Strong TV, Picciotto MR, Nairn AC, Collins FS, Fitz JG. Localization of the cystic fibrosis transmembrane conductance regulator in human bile duct epithelial cells. *Gastroenterology* 1993; **105**: 1857-1864
90 **Tietz PS**, Marinelli RA, Chen XM, Huang B, Cohn J, Kole J, McNiven MA, Alper S, LaRusso NF. Agonist-induced coordinated trafficking of functionally related transport proteins for water and ions in cholangiocytes. *J Biol Chem* 2003; **278**: 20413-20419
91 **Henry RP**. Multiple roles of carbonic anhydrase in cellular transport and metabolism. *Annu Rev Physiol* 1996; **58**: 523-538
92 **Spirli C**, Granato A, Zsembery K, Anglani F, Okolicsanyi L, LaRusso NF, Crepaldi G, Strazzabosco M. Functional polarity of Na+/H+ and Cl-/HCO3- exchangers in a rat cholangiocyte cell line. *Am J Physiol* 1998; **275**: G1236-G1245
93 **Grubman SA**, Perrone RD, Lee DW, Murray SL, Rogers LC, Wolkoff LI, Mulberg AE, Cherington V, Jefferson DM. Regulation of intracellular pH by immortalized human intrahepatic biliary epithelial cell lines. *Am J Physiol* 1994; **266**: G1060-G1070
94 **Hofmann AF**. Biliary secretion and excretion in health and

disease: current concepts. *Ann Hepatol* 2007; **6**: 15-27
95 **Lazaridis KN**, Pham L, Tietz P, Marinelli RA, deGroen PC, Levine S, Dawson PA, LaRusso NF. Rat cholangiocytes absorb bile acids at their apical domain via the ileal sodium-dependent bile acid transporter. *J Clin Invest* 1997; **100**: 2714-2721
96 **Lazaridis KN**, Tietz P, Wu T, Kip S, Dawson PA, LaRusso NF. Alternative splicing of the rat sodium/bile acid transporter changes its cellular localization and transport properties. *Proc Natl Acad Sci USA* 2000; **97**: 11092-11097
97 **Alpini G**, Glaser S, Francis H, Marzioni M, Venter J, LeSage G. Bile acid interaction with cholangiocytes. In: Alpini G, Alvaro D, Marzioni M, LeSage G, LaRusso N, eds. The Pathophysiology of the Biliary Epithelia, Georgetown, TX: Landes Bioscience, 2004: 112-126
98 **Farouk M**, Vigna SR, McVey DC, Meyers WC. Localization and characterization of secretin binding sites expressed by rat bile duct epithelium. *Gastroenterology* 1992; **102**: 963-968
99 **Levine RA**, Hall RC. Cyclic AMP in secretin choleresis. Evidence for a regulatory role in man and baboons but not in dogs. *Gastroenterology* 1976; **70**: 537-544
100 **Jimenez R**, Torres D, Gomez-Bautista M, Esteller A. Basal and secretin induced hipercholeresis in experimental biliary cirrhosis: The role of ductular-duct proliferation. *J Hepatol* 1991; **13** Sup 2, S38
101 **Esteller A**, Lopez MA. The effect of secretin and cholecystokinin-pancreozymin on the secretion of bile in the anaesthetized rabbit. *Q J Exp Physiol Cogn Med Sci* 1977; **62**: 353-359
102 **Alpini G**, Glaser S, Baiocchi L, Francis H, Xia X, Lesage G. Secretin activation of the apical Na+-dependent bile acid transporter is associated with cholehepatic shunting in rats. *Hepatology* 2005; **41**: 1037-1045
103 **Hirata K**, Nathanson MH. Bile duct epithelia regulate biliary bicarbonate excretion in normal rat liver. *Gastroenterology* 2001; **121**: 396-406
104 **Nathanson MH**, Burgstahler AD, Mennone A, Boyer JL. Characterization of cytosolic Ca2+ signaling in rat bile duct epithelia. *Am J Physiol* 1996; **271**: G86-G96
105 **Kaminski DL**, Deshpande YG. Effect of somatostatin and bombesin on secretin-stimulated ductular bile flow in dogs. *Gastroenterology* 1983; **85**: 1239-1247
106 **Nyberg B**, Einarsson K, Sonnenfeld T. Evidence that vasoactive intestinal peptide induces ductular secretion of bile in humans. *Gastroenterology* 1989; **96**: 920-924
107 **Glaser S**, Alvaro D, Roskams T, Phinizy JL, Stoica G, Francis H, Ueno Y, Barbaro B, Marzioni M, Mauldin J, Rashid S, Mancino MG, LeSage G, Alpini G. Dopaminergic inhibition of secretin-stimulated choleresis by increased PKC-gamma expression and decrease of PKA activity. *Am J Physiol Gastrointest Liver Physiol* 2003; **284**: G683-G694
108 **Tietz PS**, Alpini G, Pham LD, Larusso NF. Somatostatin inhibits secretin-induced ductal hypercholeresis and exocytosis by cholangiocytes. *Am J Physiol* 1995; **269**: G110-G118

S- Editor Li DL **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5650

World J Gastroenterol 2008 October 7; 14(37): 5650-5656
World Journal of Gastroenterology ISSN 1007-9327

GASTRIC CANCER

Overexpression of the receptor tyrosine kinase EphA4 in human gastric cancers

Mariko Oki, Hiroyuki Yamamoto, Hiroaki Taniguchi, Yasushi Adachi, Kohzoh Imai, Yasuhisa Shinomura

Mariko Oki, Hiroyuki Yamamoto, Hiroaki Taniguchi, Yasushi Adachi, Yasuhisa Shinomura, First Department of Internal Medicine, Sapporo Medical University School of Medicine, Sapporo 060-8543, Japan
Kohzoh Imai, Sapporo Medical University, Sapporo 060-8556, Japan
Author contributions: Oki M and Yamamoto H contributed equally to this paper and designed research; Oki M, Yamamoto H, Taniguchi H, and Adachi Y performed research; Oki M, Taniguchi H, Yamamoto H, Imai K, and Shinomura Y analyzed data; and Oki M, Yamamoto H, and Shinomura Y wrote the paper.
Supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (H.Y. and K.I.) and Grants-in-Aid for Cancer Research and for the Third Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare of Japan (H.Y. and K.I.)
Correspondence to: Hiroyuki Yamamoto, MD, FJSIM, PhD, First Department of Internal Medicine, Sapporo Medical University, S.-1, W.-16, Chuo-ku, Sapporo 060-8543, Japan. h-yama@sapmed.ac.jp
Telephone: +81-11-6112111 Fax: +81-11-6112282
Received: March 28, 2008 Revised: August 26, 2008
Accepted: September 2, 2008
Published online: October 7, 2008

Abstract

AIM: To clarify the expression and role of Ephrin receptor A4 (EphA4) in gastric cancer in relation to clinicopathological characteristics and the expression of fibroblast growth factor receptor 1 (FGFR1) and ephrin ligands.

METHODS: Eleven gastric carcinoma cell lines, 24 paired surgical fresh specimens of gastric adenocarcinoma and adjacent nontumor tissue, 74 conventional formalin-fixed, paraffin-embedded tumor specimens, and 55 specimens spotted on tissue microarray (TMA) were analyzed. Reverse transcription-PCR (RT-PCR), real-time RT-PCR, immunohistochemistry, and cell growth assays were performed.

RESULTS: Overexpression of EphA4 mRNA expression was observed in 8 (73%) of 11 gastric cancer cell lines and 10 (42%) of 24 gastric cancer tissues. Overexpression of EphA4, analyzed by immunohistochemistry, was observed in 62 (48%) of 129 gastric cancer tissues. EphA4 overexpression, at the protein level, was significantly associated with depth of invasion and recurrence. EphA4 overexpression was also correlated with FGFR1 overexpression. Patients with EphA4-positive cancer had significantly shorter overall survival periods than did those with EphA4-negative cancer (P = 0.0008). The mRNAs for ephrin ligands were coexpressed in various combinations in gastric cancer cell lines and cancer tissues. Downregulation of EphA4 expression by siRNA in EphA4-overexpressing gastric cancer cell lines resulted in a significant decrease in cell growth.

CONCLUSION: Our results suggest that overexpression of EphA4 plays a role in gastric cancer.

Key words: Ephrin receptor A4; Gastric cancer; Ephrin; Fibroblast growth factor receptor 1; Prognosis

Peer reviewer: Roberto Mazzanti, Professor, Department of Internal Medicine, University of Florence, viale Morgagni 85, Firenze 50134, Italy

Oki M, Yamamoto H, Taniguchi H, Adachi Y, Imai K, Shinomura Y. Overexpression of the receptor tyrosine kinase EphA4 in human gastric cancers. *World J Gastroenterol* 2008; 14(37): 5650-5656 Available from: URL: http://www.wjgnet.com/1007-9327/14/5650.asp DOI: http://dx.doi.org/10.3748/wjg.14.5650

INTRODUCTION

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death in the world[1]. Genetic and epigenetic alterations play important roles in the development and progression of gastric cancer[1,2]. Considerable attention has been given to the potential role of tyrosine kinases in gastric cancer[3-6].

The Ephrin (Eph) receptors constitute the largest family of tyrosine kinase receptors[7-10]. The Eph receptors interact with a second family of cell surface-anchored ligands, ephrins. The Eph receptors and ephrin ligands are divided into the two subclasses, A and B. Type A ephrins and type B ephrins are attached to the plasma membrane *via* glycosyl phosphatidyl inositol linkages and transmembrane sequences, respectively.

Eight EphA receptors (EphA1-A8), five EphB receptors (EphB1-B4, B6), five type A ephrins (EphfrinA1-A5), and three type B ephrins (ephrinB1-3) are known in the human genome. EphA receptors usually bind to type A ephrins and EphB receptors binds to type B ephrins. The combinations for the Eph receptors and ephrin ligands are considered to occur in a tissue-type and/or cancer-type specific manner[7-10].

The potential role of Eph receptor and ephrin ligand family in human cancer is receiving increasing attention. Altered expression patterns of Eph/ephrin have been correlated with tumor behavior, such as invasiveness, vascularization, metastatic potential, and patients' prognosis[7-10]. Generally, the upregulation of Eph/ephrin has been reported in various types of cancer[7-10]. Overexpression of EphB2, ephrinB1, EphA2, and ephrinA1 has been reported in gastric cancer[11-13]. On the other hand, the concept that Eph receptors are oncogenes needs a new look on the basis of recent findings of downregulation of Eph receptors in certain types of cancer[14-17]. However, because functions of Eph receptors can overlap, loss of one receptor can be partially compensated for by other Eph receptors that have similar ligand-binding specificities and expression patterns[7].

Thus, it seems important to characterize the role of Eph/ephrin with specific characteristics. In this regard, EphA4 is an engaging target for research. Compared with other Eph receptors, EphA4 is distinguished by its ability to bind to both type A ephrins and most type B ephrins[7-10]. Indeed, overexpression of EphA4 has been recently reported in human prostate and pancreatic cancers[18,19]. Moreover, it has been reported that EphA4 forms a hetero receptor complex with fibroblast growth factor receptor (FGFR) 1 and that EphA4/FGFR1 complex potentiates FGFR-mediated downstream signal transduction. It is well known that FGFR signal pathway plays important roles in gastric cancer[20,21].

Thus, it seems important to clarify the relevance of EphA4 in gastric cancer. Using reverse transcription-PCR (RT-PCR), real-time RT-PCR, immunohistochemistry, and cell growth assays, we analyzed the expression and role of EphA4 in gastric cancer, in relation to clinicopathological characteristics and the expression of FGFR1 and ephrin ligands.

MATERIALS AND METHODS

Cell culture

Gastric carcinoma cell lines, NUGC3, NUGC4, SNU1, SNU638, MKN28, MKN45, MKN74, KATOⅢ, HGC27, GC1Y, and AZ521 were purchased from the Japanese Cancer Research Resources Bank (Tokyo, Japan), Riken Cell Bank (Tokyo), or the American Type Culture Collection (Rockville, MD), and were grown in Dulbecco's modified Eagle's medium or RPMI1640 supplemented with 10% fetal bovine serum (Cansera, Ontario, Canada). Cells were maintained at 37℃ in an atmosphere of humidified air with 5% CO_2.

Tissue samples

Twenty-four paired surgical fresh specimens of Japanese gastric adenocarcinoma and adjacent nontumor tissue and 74 formalin-fixed, paraffin-embedded tumor specimens were obtained from Japanese patients who had undergone surgical treatment. pTNM stages were as follows: 14 stage I cancers; 24 stage Ⅱ cancers, 33 stage Ⅲ cancers, and 3 stage Ⅳ cancers. No patients received chemotherapy or radiation therapy before surgery. No patients received adjuvant treatment until diagnosis of the recurrence of cancer. Recurrent patients received chemotherapy (fluorouracil, S-1, or S-1/cisplatin). An analysis of the effect of chemotherapy for recurrent patients showed no significant effect on survival in this study (data not shown). Tissue microarray (TMA) of Korean gastric cancer tissues was purchased from SuperBioChips Laboratories (Seoul, Korea). pTNM stages were as follows: 23 stage I cancers, 13 stage Ⅱ cancers, 9 stage Ⅲ cancers, and 10 stage Ⅳ cancers. Each fresh tissue specimen was divided into two pieces after resection. For total RNA extraction, one sample was immediately frozen in liquid nitrogen at the time of surgery and stored at -80℃ until extraction. The other sample was processed for pathological examination using hematoxylin and eosin staining for the evaluation of the tumor cell content. Only specimens containing more than 80% tumor cells were used for analysis. The tumor-node-metastasis (TNM) system of the American Joint Committee on Cancer and the International Union against Cancer was used for the pathologic diagnosis and classification of variables. Informed consent was obtained from each patient.

Semiquantitative RT-PCR and real-time RT-PCR

Semiquantitative RT-PCR was done as described previously[22]. Briefly, total RNA was extracted from cancer cell lines and tissue specimens using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' instructions. Extracted RNA was treated with DNase I (Roche, Mannheim, Germany) and reverse-transcribed to cDNA using random hexamers with Superscript Ⅱ reverse transcriptase (Invitrogen). PCR amplification was performed with published PCR primers. The primer sequences used were 5'-CGTTATGTGGGAAGTGATGTCATA-3' and 5'-TTCCTCAATGGCTTTAATCACA TCT-3' for EphA4[9] and 5'-TCCTAGTGCCACAATCCCAGTCCT-3' and 5'-AGGGCATTAGAGGCCCAGAGA-3' for FGFR1[23]. The other primer sequences were provided by Dr. Nakagawa[19]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control of the reaction. Standard curves for semiquantitative RT-PCR were drawn as described previously[22]. PCR products were resolved on 2.5% agarose gels and signal intensities were quantified using a computer imaging system. All reactions were controlled without reverse transcriptase. The levels of gene transcripts were quantified as the ratio of the intensity of the target gene to the intensity of GAPDH. Overexpression was judged when the target

gene expression in the tumor sample was at least 3 times higher than that in the corresponding normal sample. In cases without target gene expression in the corresponding normal sample, overexpression was judged when the target gene expression in the tumor sample was at least 3 times higher than the highest ratio among the 24 normal samples analyzed. Downexpression was judged in a reversed way. Real-time RT-PCR for EphA4 was done by using TaqMan real-time PCR system as described previously[9]. The amount of EphA4 mRNA was displayed as the x-fold gene expression compared with the corresponding normal tissue.

Immunohistochemistry

Five mm-thick sections of formalin-fixed and paraffin-embedded tissue and TMA were dewaxed in xylene and rehydrated in alcohol. The sections were then heated to 105℃ in target retrieval solution (DakoCytomation, Carpinteria, CA) in an autoclave for 10 min for antigen retrieval. Endogenous peroxidase activity was suppressed using a solution of 3% hydrogen peroxide in methanol for 5 min. After being rinsed twice in phosphate-buffered saline (PBS), the sections were incubated for 18 h at 4℃ with a rabbit anti-EphA4 polyclonal antibody (Santa Cruz, CA, USA) or a mouse anti-FGFR1 monoclonal antibody (Abcam, Cambridge, UK). The antibodies were diluted in antibody diluent with background reducing components (0.05 mol/L Tris-HCl buffer containing 0.1% Tween and 0.015 mol/L sodium azide, DakoCytomation). Normal rabbit or mouse immunoglobulins were substituted for each primary antibody as negative controls. After washing 3 times in PBS, the sections were treated with biotinylated goat anti-rabbit or anti-mouse immunoglobulin (DakoCytomation) for 10 min and then with horseradish peroxidase-avidin complex, diluted as recommended by the manufacturer, for 10 min. The slides were then washed in PBS and developed in 0.05 mol/L Tris-HCl (pH 7.5) containing 0.6 mg/mL 3-3' diaminobenzidine at room temperature. The sections were counterstained in Mayer's hematoxylin and mounted.

siRNA transfection and cell growth analysis

siRNAs for EphA4 mRNA (Silencer® Pre-designed siRNA) were purchased from Ambion (Austiin, TX). Unrelated nonspecific siRNAs (Silencer® Negative Control siRNA) were used as control. siRNA transfection was performed following manufacturers' instructions. After incubation for 72 h, siRNA-transfected SNU638 and AZ521 cells were analyzed by RT-PCR to validate knockdown effect on EphA4. These cells were used for cell growth assay. Tumor cell growth assay was done using the tetrazolium compound WST-8 (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan).

Statistical analysis

The results were assessed for associations with clinicopathological parameters using the following statistical tests: Student's *t* test for age, the Mann-Whitney test for depth of invasion, lymph node metastasis, and pTNM stage, and the Chi-square test or Fisher's exact test for the remaining parameters. Survival analysis was carried out for 71 patients (pTNM stages were as follows: 14 stage Ⅰ cancers; 24 stage Ⅱ cancers and 33 stage Ⅲ cancers). Since information on preoperative and postoperative treatment was not available for 55 specimens spotted on TMA, these patients were not involved in survival analysis. Cumulative survival rates were calculated by the Kaplan-Meier method. The difference between survival curves was analyzed by the logrank test. Multivariate analysis was performed using Cox's proportional hazard model. A *P* value of less than 0.05 was considered significant.

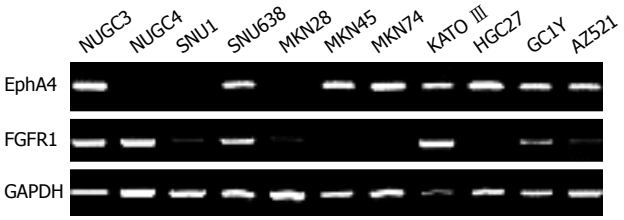


Figure 1 RT-PCR analysis of the EphA4 and FGFR1 in gastric cancer cell lines.

RESULTS

Expression of EphA4 and FGFR1 mRNA in gastric cancer cell lines

Expression of EphA4 mRNA was analyzed in 11 gastric cancer cell lines by RT-PCR. EphA4 expression was detected in 8 (73%) of 11 gastric cancer cell lines (Figure 1). There was no correlation between EphA4 positivity and histopathology. Expression of FGFR1 mRNA was detected in 8 (73%) of 11 gastric cancer cell lines (Figure 1). Expression levels of FGFR1 were low in SNU1, MKN28, GC1Y, and AZ521. There was no correlation between FGFR1 positivity and histopathology. Five of 11 gastric cancer cell lines expressed both EphA4 and FGFR1.

Overexpression of EphA4 and FGFR1 mRNA in gastric cancer tissues

Expression of EphA4 mRNA was analyzed in 4 paired gastric cancer and corresponding normal tissues by both real-time RT-PCR and semiquantitative RT-PCR. The mRNA expression of EphA4, analyzed by real-time RT-PCR, was upregulated in cancer tissues compared with normal tissues (Figure 2A). Concordant results were obtained by semiquantitative RT-PCR (data not shown). Therefore, expression of EphA4 mRNA was further analyzed in 20 paired gastric cancer and corresponding normal tissues by semiquantitative RT-PCR. In 10 (42%) of total 24 cases, the mRNA expression of EphA4 was upregulated in cancer tissues compared with normal tissues (Figure 2B). Similarly, in 12 (50%) of 24 cases, the mRNA expression of FGFR1 was upregulated in cancer tissues compared with normal tissues (Figure 2B). Overexpression of EphA4 was significantly correlated

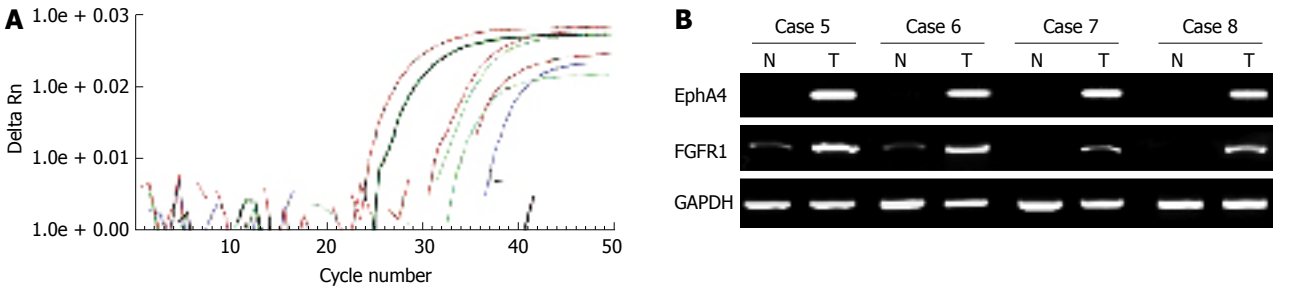


Figure 2 Real time RT-PCR (**A**) and semiquantitative RT-PCR (**B**) analysis of the EphA4 in gastric cancer tissues. **A**: From left to right; case 1 tumor, case 2 tumor, case 3 tumor, case 4 tumor, case 3 normal, case 1 normal, and case 2 normal; **B**: N and T = matched samples from non-tumor and tumor tissue, respectively.

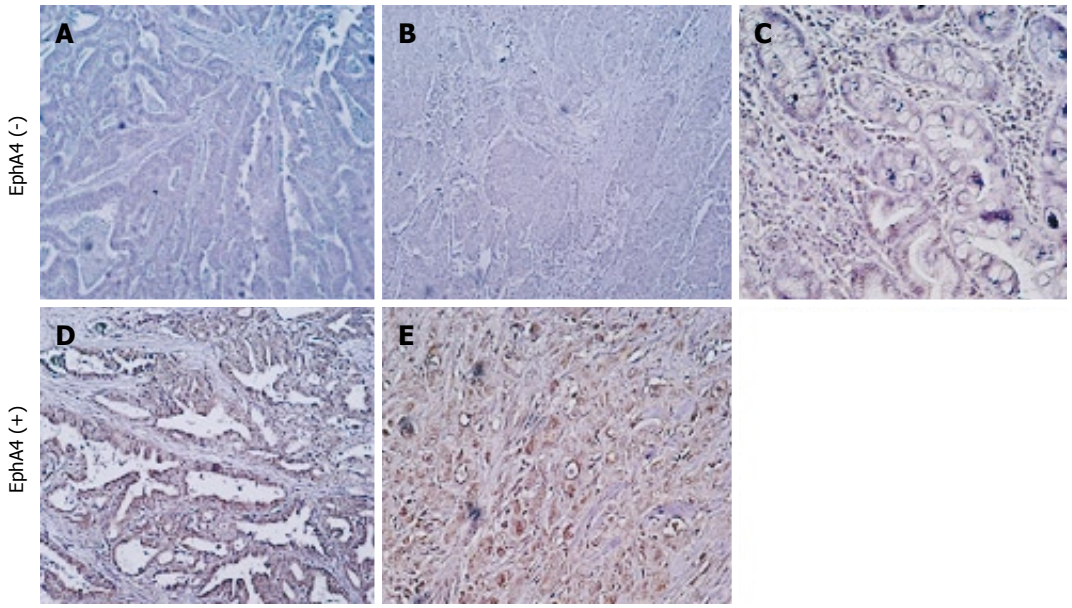


Figure 3 Immunohistochemistry for EphA4 in gastric cancer tissues (x 200). **A**: Well differentiated gastric cancer negative for EphA4; **B**: Poorly differentiated gastric cancer negative for EphA4; **C**: Normal gastric epithelia negative for EphA4; **D**: Moderately differentiated gastric cancer positively stained for EphA4; **E**: Poorly differentiated gastric cancer positively stained for EphA4.

with FGFR1 overexpression ($P < 0.01$; data not shown).

Immunohistochemical expression of EphA4 and FGFR1 in gastric cancer tissues

Figure 3 shows representative results of immunohistochemistry for EphA4 in gastric cancer tissues. The EphA4 protein was strongly expressed in the cytoplasm and/or membrane of cancer cells, whereas normal gastric epithelium from the same patient showed no, or very weak, expression of EphA4 protein. There was no detectable immunoreactivity with the control normal rabbit immunoglobulins (data not shown). EphA4 expression was positive in 62 (48%) of the 129 cases. EphA4 overexpression was confirmed in those cases in which EphA4 mRNA overexpression was shown by using RT-PCR. The relationship between EphA4 positivity and clinicopathological characteristics was then analyzed. EphA4 positivity was significantly associated with depth of invasion and recurrence. Moreover, patients with EphA4-positive cancer had significantly shorter overall survival periods than did those with EphA4-negative cancer ($P = 0.0008$, Figure 4). Multivariate analysis, including EphA4 expression, depth of invasion, lymph node metastasis, and vascular invasion, showed that EphA4 expression was an independent marker of poor survival for gastric cancer (relative risk 2.1, 95% confidence interval 1.0-4.2, $P = 0.038$).

Immunohistochemistry for FGFR1 was done in 55 gastric cancer samples spotted on TMA and six conventional sections of gastric cancer. FGFR1 protein was strongly expressed in the cytoplasm and/or membrane of cancer cells, whereas normal gastric epithelium from the same patient showed no, or very weak, expression of FGFR1 protein. FGFR1 protein was overexpressed in 37 (61%) of 61 gastric cancer specimens. There was no significant correlation between FGFR1 expression and clinicopathological characteristics. FGFR1 expression was significantly correlated with EphA4 expression ($P = 0.009$).

Expression of ephrin ligands in gastric cancer cell lines and tissues

Expression of ephrin A1-A5 and ephrin B1-B3 mRNA was analyzed in 11 gastric cancer cell lines by RT-PCR. The extent of expression of each gene was considerably differed among the cell lines (Figure 5). Expression of ephrin A2 was relatively concordant with that of EphA4. The relative amounts of the transcripts of ephrin ligands also varied considerably among tumor tissue samples. None of the expression of ephrin ligands was clearly concordant with that of EphA4 (data not shown).

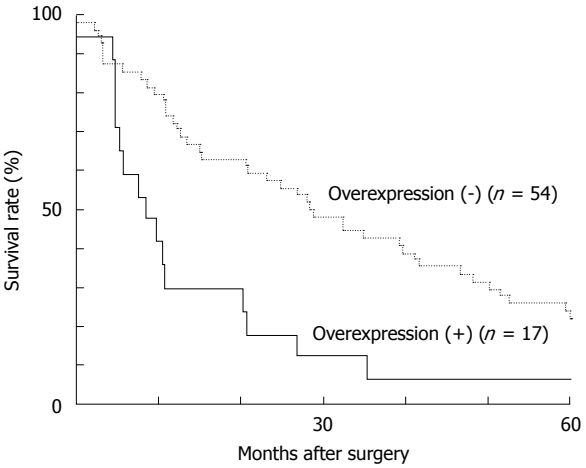


Figure 4 Kaplan-Meier survival curves of patients with gastric cancer according to the expression of EphA4 ($P = 0.0008$).

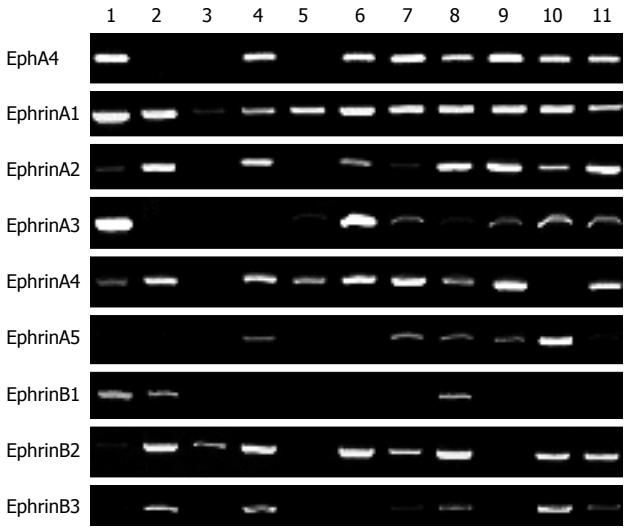


Figure 5 RT-PCR analysis of the type A ephrins and type B ephrins in gastric cancer cell lines. 1: NUGC3; 2: NUGC4; 3: SNU1; 4: SNU638; 5: MKN28; 6: MKN45; 7: MKN74; 8: KATOIII; 9: HGC27; 10: GC1Y; 11: AZ521.

Suppression of cell growth by EphA4 siRNA treatment

Cell growth assay after treatment with specific siRNA for EphA4 gene was done to assess the role of EphA4 expression in gastric cancer cells. Transfection with siRNA resulted in over 80% inhibition of mRNA expression (data not shown). Transfection with EphA4-specific siRNA significantly suppressed cell growth of SNU638 and AZ521cells compared with control siRNA-transfected counterparts ($P = 0.008$; data not shown).

DISCUSSION

In this study, we found overexpression of EphA4 mRNA in gastric cancer cell lines and cancer tissues. Previous microarray and Northern blot analyses have shown that EphA4 expression in normal adult tissues is very restricted[19]. Moreover, overexpression of EphA4 in gastric cancer was observed at the protein level as analyzed by immunohistochemistry and this correlated with depth of invasion. These results suggest that EphA4 plays a role in gastric cancer.

The implication of EphA4 expression in gastric cancer was further substantiated by its correlation with recurrence and short overall survival time. Moreover, EphA4 expression provided significant predictive value for overall survival in the multivariate analysis, suggesting that EphA4 expression could be a useful predictor of recurrence and poor prognosis. Prognostic significance of EphA4 expression has not previously reported in human cancer. Moreover, EphA4 is the first Eph/ephrin family member of which prognostic significance was shown in gastric cancer.

It has been reported that EphA4 forms a hetero receptor complex with FGFR1 and that the EphA4/FGFR1 complex potentiates FGFR-mediated downstream signal transduction. Since FGF/FGFR signal pathway has been reported to play an important role in gastric cancer[20,21], we analyzed the relationship of EphA4 expression with FGFR1 expression. The frequency of FGFR1 overexpression in this study was consistent with the frequencies reported previously[20]. FGFR1 expression was correlated with EphA4 expression. Therefore, EphA4 may play a role in gastric cancer, at least in part, through the interaction with FGFR signaling. EphA4 has also been shown to phosphorylate FGFR2 and 3. Overexpression of mRNA for FGFR-2 and FGFR-4 has been reported in gastric cancer[20]. Further analysis is necessary to clarify the relationship between EphA4 and FGF/FGFR signaling pathway in gastric cancer.

To search for potential candidate ligands for the EphA4 receptor in gastric cancer cells, we examined the expression patterns of members in the type A and type B ephrin ligand families by semiquantitative RT-PCR. The mRNAs of ephrin ligands were coexpressed in various combinations in gastric cancer cell lines and cancer tissues. Expression of ephrinA2 was relatively concordant with that of EphA4 in gastric cancer cell lines. However, expression of ephrin ligands was not clearly concordant with that of EphA4 in cancer tissue samples. On the other hand, coexpression of ephrinA3 and EphA4 has been reported to play an important role in pancreatic cancer[19]. These results further support the notion that combinations for the Eph receptors and ephrin ligands depend on tissue-type and/or cancer-type[7-10]. Moreover, the interaction of EphA4 expressed on gastric cancer cells and ephrin ligands expressed on nontumour cells, such as mesenchymal cells and vascular cells, could play a role in gastric cancer through the interaction between these cells[19]. Further studies are necessary to clarify these issues.

By using the small interfering RNA strategy, we investigated whether EphA4 expression was associated with growth of gastric cancer cells. Downregulation of EphA4 expression in gastric cancer cells resulted in a reduction in cell viability, suggesting that the overexpression of EphA4 plays a role in gastric cancer cell growth.

Taken together, these results suggest that EphA4

overexpression plays a role in gastric cancer. As a target gene for molecular therapy, its expression in normal adult tissues is important. Although EphA4 was found to be highly expressed in the adult brain, it has been thought that therapeutic antibody targeting membrane molecules could not pass through the blood brain barrier and were unlikely to affect the central nervous system[18]. Considering the expression pattern of EphA4 together with its oncogenic function, EphA4 could be a target for molecular therapy by a therapeutic antibody and/or by small molecule approach targeting its kinase activities[24].

COMMENTS

Background

The Ephrin (Eph) receptors constitute the largest family of tyrosine kinase receptors. EphA4 is distinguished by its ability to bind to both type A ephrins and most type B ephrins. It has been reported that EphA4 forms a hetero receptor complex with fibroblast growth factor receptor (FGFR) 1 and that the EphA4/FGFR1 complex potentiates FGFR-mediated downstream signal transduction. The potential role of Eph receptor and ephrin ligand family in human cancer is receiving increasing attention.

Research frontiers

Altered expression patterns of Eph/ephrin have been correlated with tumor behavior, such as invasiveness, vascularization, metastatic potential, and patients' prognosis. Generally, the upregulation of Eph/ephrin has been reported in various types of cancer.

Innovations and breakthroughs

The expression profiles of EphA4, FGFR1, and ephrins were systematically analyzed in gastric cancer cell lines and/or tissues by using RT-PCR, immunohistochemistry, and tissue microarray analysis.

Applications

This study demonstrated that overexpression of EphA4 and FGFR1 may be involved in gastric tumorigenesis. EphA4 overexpression could be a poor prognostic marker for gastric cancer.

Terminology

Ephrin receptor: The Ephrin receptors constitute the largest family of tyrosine kinase receptors. The Eph receptors interact with a second family of cell surface-anchored ligands, ephrins. Eight EphA receptors (EphA1-A8) and five EphB receptors (EphB1-B4, B6) are known in the human genome. Ephrin: The ephrins are divided into the two subclasses, A and B. Type A ephrins and type B ephrins are attached to plasma membrane *via* glycosyl phosphatidyl inositol linkages and transmembrane sequences, respectively. Five type A ephrins (Ephrin A1-A5) and three type B ephrins (Ephrin B1-B3) are known in the human genome.

Peer review

This paper studied expression of EphA4 and FGFR1 in gastric cancer. The authors showed that overexpression of EphA4 is a frequent feature in cell lines (73%) and less common in gastric cancer samples (around 40%-50%). They show a correlation between the expression of such molecular aspect and shorter survival. This study has the potential to become interesting to the scientific community.

REFERENCES

1 **Hamilton JP**, Meltzer SJ. A review of the genomics of gastric cancer. *Clin Gastroenterol Hepatol* 2006; **4**: 416-425

2 **Tamura G**. Alterations of tumor suppressor and tumor-related genes in the development and progression of gastric cancer. *World J Gastroenterol* 2006; **12**: 192-198

3 **Lin W**, Kao HW, Robinson D, Kung HJ, Wu CW, Chen HC. Tyrosine kinases and gastric cancer. *Oncogene* 2000; **19**: 5680-5689

4 **Wu CW**, Li AF, Chi CW, Huang CL, Shen KH, Liu WY, Lin W. Human gastric cancer kinase profile and prognostic significance of MKK4 kinase. *Am J Pathol* 2000; **156**: 2007-2015

5 **Kao HW**, Chen HC, Wu CW, Lin WC. Tyrosine-kinase expression profiles in human gastric cancer cell lines and their modulations with retinoic acids. *Br J Cancer* 2003; **88**: 1058-1064

6 **Becker JC**, Muller-Tidow C, Serve H, Domschke W, Pohle T. Role of receptor tyrosine kinases in gastric cancer: new targets for a selective therapy. *World J Gastroenterol* 2006; **12**: 3297-3305

7 **Kullander K**, Klein R. Mechanisms and functions of Eph and ephrin signalling. *Nat Rev Mol Cell Biol* 2002; **3**: 475-486

8 **Murai KK**, Pasquale EB. 'Eph'ective signaling: forward, reverse and crosstalk. *J Cell Sci* 2003; **116**: 2823-2832

9 **Hafner C**, Schmitz G, Meyer S, Bataille F, Hau P, Langmann T, Dietmaier W, Landthaler M, Vogt T. Differential gene expression of Eph receptors and ephrins in benign human tissues and cancers. *Clin Chem* 2004; **50**: 490-499

10 **Heroult M**, Schaffner F, Augustin HG. Eph receptor and ephrin ligand-mediated interactions during angiogenesis and tumor progression. *Exp Cell Res* 2006; **312**: 642-650

11 **Kiyokawa E**, Takai S, Tanaka M, Iwase T, Suzuki M, Xiang YY, Naito Y, Yamada K, Sugimura H, Kino I. Overexpression of ERK, an EPH family receptor protein tyrosine kinase, in various human tumors. *Cancer Res* 1994; **54**: 3645-3650

12 **Kataoka H**, Tanaka M, Kanamori M, Yoshii S, Ihara M, Wang YJ, Song JP, Li ZY, Arai H, Otsuki Y, Kobayashi T, Konno H, Hanai H, Sugimura H. Expression profile of EFNB1, EFNB2, two ligands of EPHB2 in human gastric cancer. *J Cancer Res Clin Oncol* 2002; **128**: 343-348

13 **Nakamura R**, Kataoka H, Sato N, Kanamori M, Ihara M, Igarashi H, Ravshanov S, Wang YJ, Li ZY, Shimamura T, Kobayashi T, Konno H, Shinmura K, Tanaka M, Sugimura H. EPHA2/EFNA1 expression in human gastric cancer. *Cancer Sci* 2005; **96**: 42-47

14 **Batlle E**, Bacani J, Begthel H, Jonkheer S, Gregorieff A, van de Born M, Malats N, Sancho E, Boon E, Pawson T, Gallinger S, Pals S, Clevers H. EphB receptor activity suppresses colorectal cancer progression. *Nature* 2005; **435**: 1126-1130

15 **Alazzouzi H**, Davalos V, Kokko A, Domingo E, Woerner SM, Wilson AJ, Konrad L, Laiho P, Espin E, Armengol M, Imai K, Yamamoto H, Mariadason JM, Gebert JF, Aaltonen LA, Schwartz S Jr, Arango D. Mechanisms of inactivation of the receptor tyrosine kinase EPHB2 in colorectal tumors. *Cancer Res* 2005; **65**: 10170-10173

16 **Wang J**, Kataoka H, Suzuki M, Sato N, Nakamura R, Tao H, Maruyama K, Isogaki J, Kanaoka S, Ihara M, Tanaka M, Kanamori M, Nakamura T, Shinmura K, Sugimura H. Downregulation of EphA7 by hypermethylation in colorectal cancer. *Oncogene* 2005; **24**: 5637-5647

17 **Davalos V**, Dopeso H, Velho S, Ferreira AM, Cirnes L, Diaz-Chico N, Bilbao C, Ramirez R, Rodriguez G, Falcon O, Leon L, Niessen RC, Keller G, Dallenbach-Hellweg G, Espin E, Armengol M, Plaja A, Perucho M, Imai K, Yamamoto H, Gebert JF, Diaz-Chico JC, Hofstra RM, Woerner SM, Seruca R, Schwartz S Jr, Arango D. High EPHB2 mutation rate in gastric but not endometrial tumors with microsatellite instability. *Oncogene* 2007; **26**: 308-311

18 **Ashida S**, Nakagawa H, Katagiri T, Furihata M, Iiizumi M, Anazawa Y, Tsunoda T, Takata R, Kasahara K, Miki T, Fujioka T, Shuin T, Nakamura Y. Molecular features of the transition from prostatic intraepithelial neoplasia (PIN) to prostate cancer: genome-wide gene-expression profiles of prostate cancers and PINs. *Cancer Res* 2004; **64**: 5963-5972

19 **Iiizumi M**, Hosokawa M, Takehara A, Chung S, Nakamura T, Katagiri T, Eguchi H, Ohigashi H, Ishikawa O, Nakamura Y, Nakagawa H. EphA4 receptor, overexpressed in pancreatic ductal adenocarcinoma, promotes cancer cell growth. *Cancer Sci* 2006; **97**: 1211-1216

20 **Shin EY**, Lee BH, Yang JH, Shin KS, Lee GK, Yun HY, Song YJ, Park SC, Kim EG. Up-regulation and co-expression of fibroblast growth factor receptors in human gastric cancer. *J Cancer Res Clin Oncol* 2000; **126**: 519-528
21 **Katoh M**, Katoh M. FGF signaling network in the gastrointestinal tract (review). *Int J Oncol* 2006; **29**: 163-168
22 **Taniguchi H**, Yamamoto H, Hirata T, Miyamoto N, Oki M, Nosho K, Adachi Y, Endo T, Imai K, Shinomura Y. Frequent epigenetic inactivation of Wnt inhibitory factor-1 in human gastrointestinal cancers. *Oncogene* 2005; **24**: 7946-7952
23 **Jayson GC**, Vives C, Paraskeva C, Schofield K, Coutts J, Fleetwood A, Gallagher JT. Coordinated modulation of the fibroblast growth factor dual receptor mechanism during transformation from human colon adenoma to carcinoma. *Int J Cancer* 1999; **82**: 298-304
24 **Imai K**, Takaoka A. Comparing antibody and small-molecule therapies for cancer. *Nat Rev Cancer* 2006; **6**: 714-727

S- Editor Zhong XY **L- Editor** Rippe RA **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5657

World J Gastroenterol 2008 October 7; 14(37): 5657-5664
World Journal of Gastroenterology ISSN 1007-9327

Comparative proteomics analysis of human gastric cancer

Wei Li, Jian-Fang Li, Ying Qu, Xue-Hua Chen, Jian-Min Qin, Qin-Long Gu, Min Yan, Zheng-Gang Zhu, Bing-Ya Liu

Wei Li, Jian-Fang Li, Ying Qu, Xue-Hua Chen, Qin-Long Gu, Min Yan, Zheng-Gang Zhu, Bing-Ya Liu, Shanghai Key Laboratory of Diagnosis and Treatment for Gastrointestinal Cancer, Department of Surgery, Shanghai Institute of Digestive Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China
Wei Li, Jian-Min Qin, Putuo District Cancer Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200025, China
Author contributions: Liu BY designed the research; Li W, Li JF, Qu Y, Chen XH performed research; Yan M, Gu QL, Zhu ZG, and Qin JM supported the clinical samples and clinical data; Li W and Liu BY wrote the paper.
Supported by National High Technology Research and Development Program of China (863 Program) No. 2006AA 02A301 and No. 2007AA02Z179; Science and Technology Commission of Shanghai Municipality, No. 07jc14041; National Key Fundamental Research 973 Project, No. 2002CB13700
Correspondence to: Bing-Ya Liu, Professor, Shanghai Key Laboratory of Diagnosis and Treatment for Gastrointestinal Cancer, Department of Surgery, Shanghai Institute of Digestive Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China. liubyrj@yahoo.com.cn
Telephone: +86-21-64674654 Fax: +86-21-64373909
Received: May 26, 2008 Revised: August 26, 2008
Accepted: September 2, 2008
Published online: October 7, 2008

Abstract

AIM: To isolate and identify differentially expressed proteins between cancer and normal tissues of gastric cancer by two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

METHODS: Soluble fraction proteins of gastric cancer tissues and paired normal tissues were separated by 2-DE. The differentially expressed proteins were selected and identified by MALDI-TOF-MS and database search.

RESULTS: 2-DE profiles with high resolution and reproducibility were obtained. Twenty-three protein spots were excised from sliver staining gel and digested in gel by trypsin, in which fifteen protein spots were identified successfully. Among the identified proteins, there were ten over-expressed and five under-expressed proteins in stomach cancer tissues compared with normal tissues.

CONCLUSION: In this study, the well-resolved, reproducible 2-DE patterns of human gastric cancer tissue and paired normal tissue were established and optimized and certain differentially-expressed proteins were identified. The combined use of 2-DE and MS provides an effective approach to screen for potential tumor markers.

Key words: Proteome; Two-dimensional polyacrylamide gel electrophoresis; Mass spectrometry; Gastric cancer; Tumor markers

Peer reviewer: Yaron Niv, Professor, Department of Gastroenterology, Rabin Medical Center, Beilinson Campus, Tel Aviv University, 2 Hadekel St., Pardesia 42815, Israel

Li W, Li JF, Qu Y, Chen XH, Qin JM, Gu QL, Yan M, Zhu ZG, Liu BY. Comparative proteomics analysis of human gastric cancer. *World J Gastroenterol* 2008; 14(37): 5657-5664 Available from: URL: http://www.wjgnet.com/1007-9327/14/5657.asp DOI: http://dx.doi.org/10.3748/wjg.14.5657

INTRODUCTION

Gastric carcinoma is one of the most common malignancies worldwide and is the first most common cause of cancer-related death in China[1]. The main barrier for improving the survival rate is the lack of useful markers for early diagnosis. The cells' fate depends on which gene expresses and how much it expresses. When a cell malignantly transforms, its gene expression profile and their respective protein products may have changed. So it is necessary to isolate and identify genes and proteins that are differentially expressed in gastric cancer.

With the completion of human genome project, the practice of medicine was altered fundamentally, which includes the identification of genes that are involved in the appearance, progression, and treatment of cancer, the answers to what those specific genes do and how they interact in communication networks, and the roles played by their protein products in molecular pathways. However, in most cases, although studies of differential mRNA expression are informative, they do not always correlate with protein concentrations because proteins are often subject to proteolytic cleavage or posttranslational modifications (such as phosphorylation or glycosylation). Cancer biomarkers discovery strategies

that target expressed proteins are becoming increasingly popular because proteomic approaches can characterize the proteins, modified or unmodified, involved in cancer progression[2-6].

Recent years, proteomics analysis was has been applied in many kinds of tumors, such as breast cancer[7-9], lung cancer[10,11], prostate cancer[12], liver cancer[13-15] and ovarian cancer[16], *etc*. In this study, we tried to find biomarkers in human gastric cancer tissue *via* proteomics as high throughput method. The results presented here will no doubt provide clues to further study of the carcinogenic mechanisms, diagnosis, and therapy of gastric cancer.

MATERIALS AND METHODS

Materials

Stomach cancer tissues were prepared from ten patients with gastric cancer (the clinical-pathological characteristics are described in Table 1) in Ruijin Hospital. The paired normal gastric tissues were prepared from non cancerous regions at least 5 cm apart from primary tumor. Protean IEF Cell, Protean Plus Multi-Casting Chamber, PROTEAN Plus Dodeca Cell, Fluor-S^{TM} Multimager, Immobilized pH gradient (IPG) strips, iodoacetamide, 40% w/v Bio-Lyte pH 3-10 Ampholyte, CHAPS, DTT, urea, acrylamide, N,N'-methylene-bis-acrylamide and RC DC Protein Assay Kit were purchased from Bio-Rad (Richmond, CA, USA). Tris-base, TEMED and SDS were from Promega. Thiourea, DNase, ammonium persulfate and phenylmethyl-sulfonylfluoride (PMSF) were from Sigma-Aldrich (Missouri, USA). 4700 Proteomics Analyzer was from ABI (USA).

Sample preparation

Proteins were extracted from human gastric tissue as instructed by the Ha[17] method. Gastric tissue (100 mg) was homogenized in 2 mL buffer (50 mmol/L Tris-HCl, pH 7.2) containing 1 mmol/L PMSF using an homogenizer at 26 000 r/min. The mixture was centrifuged at 1000 *g* for 5 min to remove debris. Then, the homogenate was centrifuged at 20 000 r/min for 30 min at 4℃. The supernatant was taken as the soluble fraction and TCA (50% w/v) was added to a final concentration of 10% w/v (containing 20 mmol/L DTT) and the solution was allowed to stand on ice for 30 min. The protein precipitate was collected and centrifuged in a microcentrifuge at 14 000 rpm for 10 min at 4℃, and washed three times in 10% TCA (containing 20 mmol/L DTT). The precipitate was washed twice in acetone (containing 20 mmol/L DTT) and dried under an air stream. The dry pellet was dissolved with vortex in the lysis solution (7 mol/L urea, 2 mol/L thiourea, 2% w/v CHAPS, 2% w/v SB3-10, 40 mmol/L Tris, 2 mmol/L TBP, 0.2% w/v Bio-Lyte pH 3-10, 1050 U/mL DNase, 25 μg/mL RNase) and allowed to stand for 1 h at room temperature. After centrifugation at 14 000 r/min for 10 min at 15℃, the supernatant was used as the two-dimensional polyacrylamide gel electrophoresis (2-DE) sample for the soluble fraction. The protein samples were stored in aliquots at -80℃ until use. Protein concentration of 2-DE samples was determined using RC DC Protein Assay kit.

Table 1 Clinical-pathological characters of 10 gastric cancer patients

Age	Sex	Cell type	Stage	Bommann type
51	M	Moderate	$T_3N_1M_0$	Ⅱ
68	M	Moderate	$T_3N_1M_1$	Ⅲ
53	M	Poor with signet ring	$T_3N_3M_0$	Ⅱ
49	F	Poor	$T_3N_1M_0$	Ⅱ
75	F	Moderate	$T_3N_1M_0$	Ⅰ
40	M	Poor	$T_3N_2M_0$	Ⅲ
62	M	Poor with signet ring	$T_3N_1M_0$	Ⅱ
74	M	Poor with signet ring	$T_3N_1M_0$	Ⅱ
78	M	Poor with signet ring	$T_3N_1M_0$	Ⅲ
67	F	Poor	$T_3N_2M_0$	Ⅲ

2-DE

The first-dimensional gel separation was carried out with 17 cm IPG strips (pH 3-10, pH 4-7, pH 5-8) following the manufacturers' protocol with minor modifications. Samples containing up to 100 μg protein were diluted to 320 μL with rehydration solution (7 mol/L urea, 2 mol/L thiourea, 2% CHAPS, 100 mmol/L DTT, 0.2% w/v Bio-Lyte pH 3-10, trace bromophenol blue), and applied to strips by overnight rehydration at 50 V. Proteins were focused succeedingly for 1 h at 150 V, 1 h at 250 V, 1 h at 1000 V, then a gradient was applied from 1000 to 10 000 V in 5 h, and focusing was continued at 10 000 V for 6 h to give a total of 88 kVh on a Protean IEF Cell. After IEF, strips were equilibrated for 15 min in 6 mol/L urea, 2% SDS, 0.05 mol/L Tris-HCl, pH 8.8, and 30% glycerol containing 2% DTT, and then equilibrated again for 15 min in the same buffer containing 2.5% iodoacetamide. Equilibrated IPG strips were transferred onto 12% uniform polyacrylamide gels and then were run in Protean Plus Dodeca Cell (Bio-Rad, USA) at 24 mA per gel for 5 h and 30 min. The gels were visualized using silver staining method. After staining, 2D gels were scanned using Fluor-S^{TM} Multimager (Bio-Rad, USA) and images were analyzed using PDQuest 7.1.1 (Bio-Rad, USA).

In-gel digestion of proteins

Protein spots of interest were cut manually from the gel and placed into siliconized microcentrifuge tubes. The gel fragments were destained in solution that consisted of 100 mmol/L $Na_2S_2O_3$ and 30 mmol/L $K_3Fe(CN)_6$ (V/V, 1:1) and dehydrated by washing two times for 10-15 min with 25 mmol/L NH_4HCO_3 in 50% acetonitrile until shrunken and white. The protein-containing gel-spots were reduced in the reduction buffer (25 mmol/L NH_4HCO_3 and 10 mmol/L DTT) for 1 h at 56℃ and then alkylated in the alkylation buffer (25 mmol/L NH_4HCO_3 and 55 mmol/L iodoacetamide) in the dark for 30 min at room temperature. The gel pieces were washed twice with 100 mmol/L NH_4HCO_3 and dehydrated

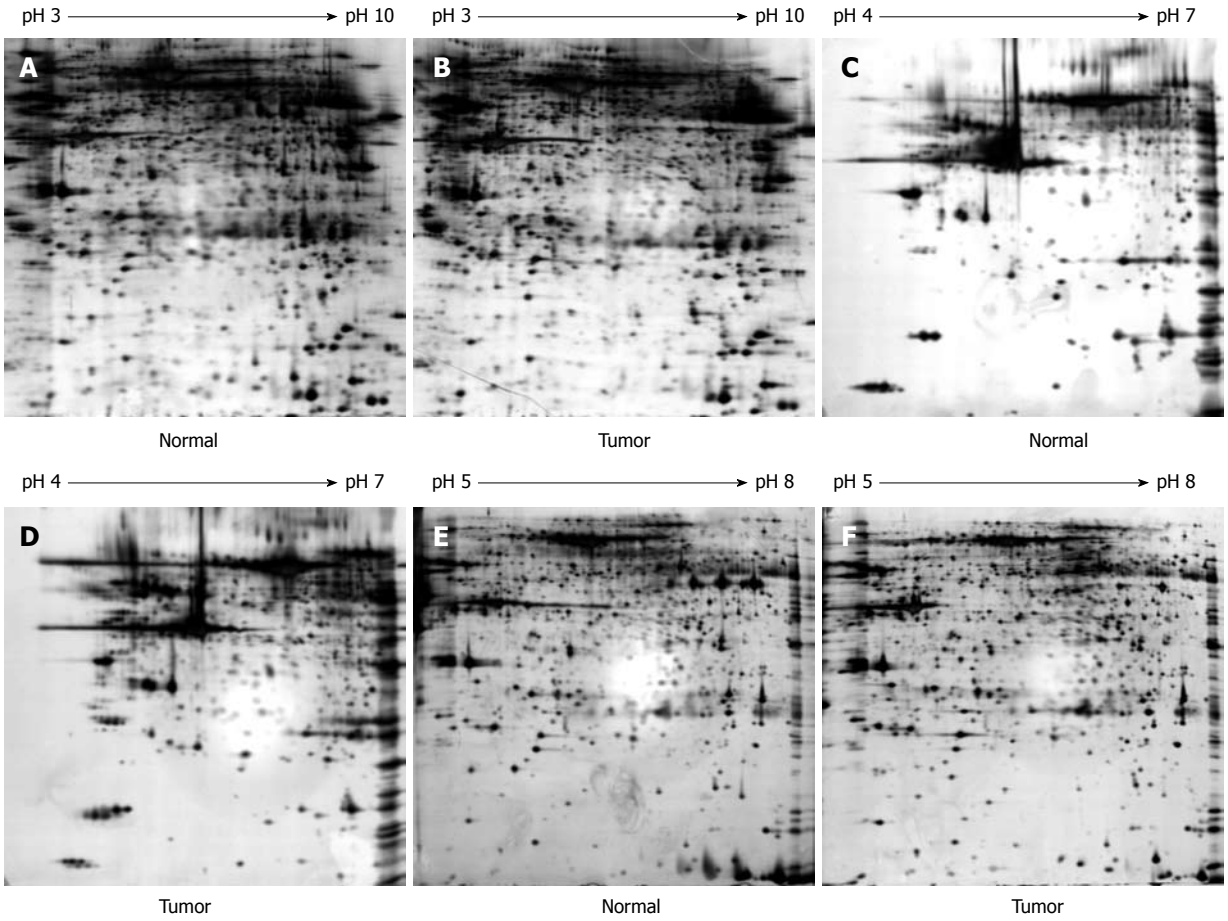


Figure 1 2-DE maps of human gastric tissue separated by IPG with different pH intervals (silver staining). **A** and **B**: Normal and cancer tissues of stomach separated by pH 3-10 IPG strips; **C** and **D**: Normal and cancer tissues of stomach separated by pH 5-8 IPG strips; **E** and **F**: Normal and cancer tissues of stomach separated by pH 4-7 IPG strips.

by 100% acetonitrile. Ten microliters modified trypsin (8 μg/mL) was added to each tube. Tubes were sealed with parafilm and digestion was performed for 16 h at 30℃. When digestion was completed, 100 μL of 100 mmol/L NH_4HCO_3 was added and tubes were sonicated for 10 min. The supernatant was removed from each tube and transferred into a fresh tube. Further recovery of the peptides was accomplished by two extractions with 50% acetonitrile/5% TFA. All supernatants were pooled and concentrated and desalted by ZIPTIPS (Millipore, USA) prior to applying on to the sample plate.

MALDI-TOF MS and database search

A small fraction (0.5 μL) of the sample was mixed with a-cyano-4-hydroxycinnamic acid matrix (1:1, v/v) and the mixture solution was then spotted on the plate and dried, then analyzed on a 4700 MALDI-TOF-TOF mass spectrometer (Applied Biosystems, USA). The laser wavelength was 355 nm, and the laser repetition rate was 200 Hz. External calibration was employed. Samples that did not get positive identification by MALDI were subjected to MALDI-TOF-TOF-MS using a 4700 Proteomics Analyzer equipped with a special analysis software GPS and local databases. The following parameters were used in the searches: SWISS-PROT was used as the protein sequence database; trypsin digest (one missed cleavage allowed); mass accuracy 0.2 Da; species: *Homo sapiens*; acetylation of the N-terminus, alkylation of cysteine by carbamidomethylation and oxidation of methionine were considered as possible modifications.

RESULTS

Overview 2-DE maps of human gastric tissue

Resolution and reproducibility of 2-DE have been improved significantly since the introduction of IPG strips[18,19]. However, it is still a time-consuming and laborious job. In this study, we firstly separate the soluble proteins of human gastric tissue in IPG strips with different pH intervals. All maps (Figure 1) were constructed on silver-stained gels with protein loadings of 100 μg. Proteins from normal and tumor tissues of stomach were separated in pH 3-10 IPG strips. After SDS-PAGE, about 850 protein spots could be detected (Figure 1A and B). While, about 670 protein spots could be detected which separated by pH 4-7 IPG strips (Figure 1C and D). The well-resolved, reproducible 2-DE patterns of human gastric cancer tissues and paired normal tissues were obtained with pH 5-8 IPG strips (Figure 1E and F). About 1400 spots were detected, respectively, and about a total of 1100 spots

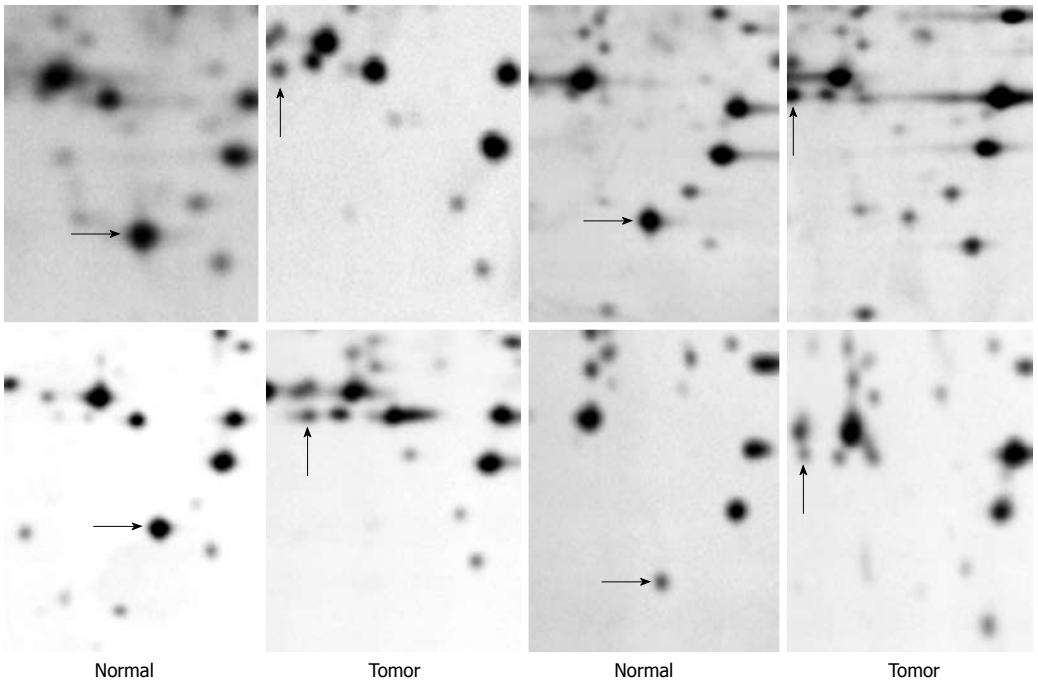


Figure 2 Up-regulation of T8 (Zinc finger protein 134, indicated by vertical arrows) and down-regulation of N4 (Bullous pemphigoid antigen 1 isoforms 6/9/10, indicated by horizontal arrows) in gastric cancer tissue of four patients.

Table 2 Over-expressed proteins in human gastric cancer

Spot ID	Protein name	Accession number[1]	Top score	Theoretic pI	Theoretic Mr (Da)
T1	Bullous pemphigoid antigen1 isoforms 1/2/3/4/5/8	Q03001	85	6.38	371977
T2	Ras-related protein Rab-30	Q15771	64	4.91	23058
T3	Zinc finger protein 268	Q14587	57	9.14	108373
T4	Calcium-binding protein CaBP5	Q9NP86	61	4.46	19812
T5	RGS3	P49796	62	4.79	56566
T6[1]	MAGUK p55 subfamily member 3	Q13368	45	6.27	66168
T7	Zinc finger protein 134	P52741	57	9.18	40297
T8[1]	Actin, alpha cardiac	P04270	154	5.23	42019
T9	Nebulin	P20929	59	9.10	772727
T10	Vacuolar protein sorting 33B	Q9H267	54	6.29	70570

[1]Accession number from SWISS-PROT database, identified by MALDI-TOF-TOF-MS.

were matched each other. We got better resolution and more protein spots with pH 5-8 IPG strips; therefore, in the succeeding studies, we chose pH 5-8 IPG strips to analyze proteins of human gastric tissues.

Comparing proteomic patterns of cancer tissue and normal tissue by 2-DE

After 2-DE, the differential proteins between human gastric cancer tissues and paired normal tissues were found by PDQuest 7.1.1 2-D gel analysis software (BIO-RAD, USA). To compare 2-DE maps, it is important to have a representative sample. Hence, an average electrophoresis map of human gastric cancer tissues was constructed by the combination of the 2-DE maps from 10 tumor tissues with the PDQuest 7.1.1 2-DE gel analysis software. Similarly, an average electrophoresis map of 10 normal gastric tissues was also established. These average electrophoresis maps were used to perform the differential expression analysis. The differential protein spots with at least five-fold discrepancy, which were present in at least four paired tumor and normal tissues, were detected by comparing the 2-DE protein patterns of the average gels of tumor and control tissues. Figure 2 shows differentially expressed proteins of N4, T8 between tumor and control tissues in four patients. Twenty three protein spots were selected to be identified by mass spectrometry (MS). Among these, 16 spots were over expressed and other 7 spots were under expressed in gastric cancer tissue.

Protein identification by MALDI-TOF MS or tandem MS

Fifteen proteins out of the selected 23 proteins were identified successfully by MALDI-TOF MS or tandem MS (Figures 3 and 4) and there were ten over expressed and five under expressed proteins in stomach cancer tissues compared with normal tissues. The identified proteins are shown in Tables 2 and 3.

Proteins which were up-regulated in gastric cancer tissue includes: (1) transcriptional regulation proteins, such as zinc finger protein; (2) signal transduction regulator, such as regulator of G-protein signaling 3 (RGS3); (3) cancer-related proteins, such as ras-related protein; (4) cytoskeleton proteins, such as bullous pemphigoid antigen 1 isoforms 1/2/3/4/5/8; (5) tissue-

Table 3 Under-expressed proteins in human gastric cancer

Spot ID	Protein name	Accession number[1]	Top score	Theoretic pI	Theoretic Mr (Da)
N1[1]	Peroxiredoxin 2	P32119	72	5.66	21892
N2[1]	Zinc finger protein ZFP-36	P16415	44	8.99	66683
N3	Zinc finger protein 64, isoforms 3 and 4	Q9NTW7	58	8.80	72216
N4	Bullous pemphigoid antigen 1, isoforms 6/9/10	O94833	62	5.50	590528
N5	Transcriptional repressor CTCFL	Q8NI51	67	8.58	75668

[1]Accession number from SWISS-PROT database, identified by MALDI-TOF-TOF-MS.

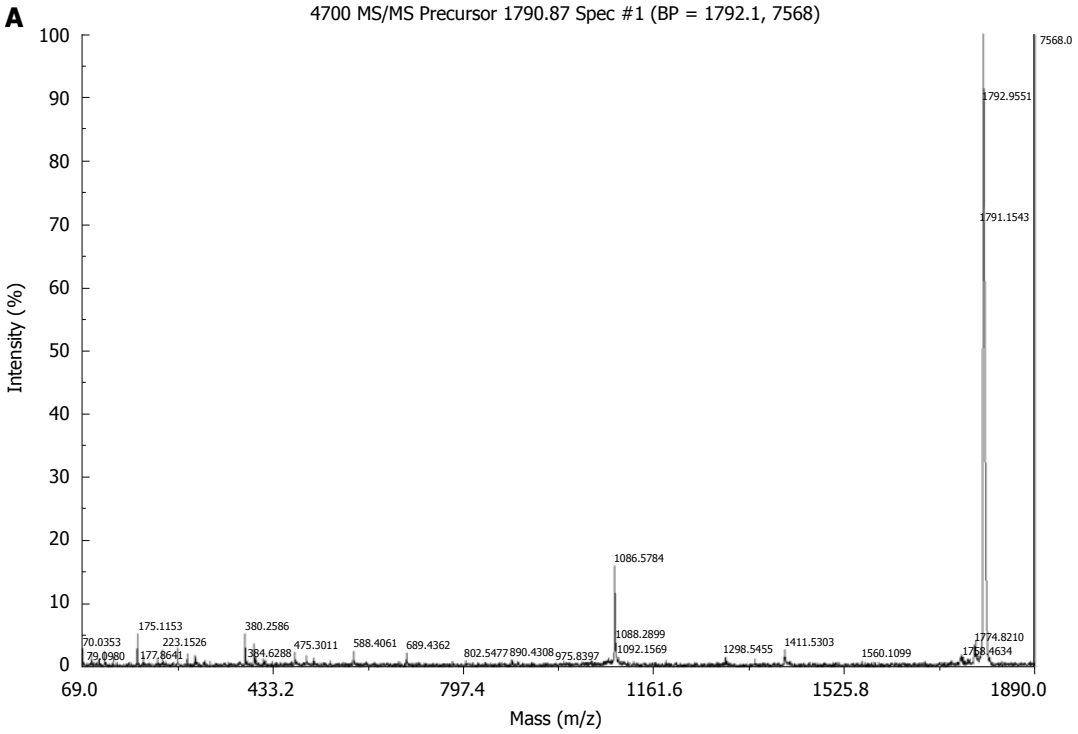


Figure 3 **A**: MALDI-TOF-TOF mass spectrum of the mixture of tryptic peptides derived from T8 (Actin, alpha cardiac); **B**: Matching of protein spot T8 (Actin, alpha cardiac) Peptide Mass Fingerprint data with protein P04270 in database.

specific proteins, such as Calcium-binding protein CaBP5. Proteins which were down-regulated in gastric cancer tissue includes: (1) transcriptional regulation proteins, such as zinc finger protein and transcriptional repressor CTCFL; (2) cytoskeleton proteins, such as bullous pemphigoid antigen 1 isoforms 6/9/10; (3) metabolic enzymes, such as peroxiredoxin 2.

DISCUSSION

Genomics-based techniques, such as expressed sequence tag (EST) sequencing, serial analysis of gene expression (SAGE)[20], cDNA expression array hybridization[21], were widely used to find out potential cancer biomarkers. However, a major limitation to these approaches is detection of high levels of mRNA transcripts of a specific gene may not necessarily mean that high levels of the corresponding protein product will be present. Now, proteomics-based techniques can help us find cancer-related biomarkers at the protein level.

In this study, we obtained ten over expressed proteins (Bullous pemphigoid antigen 1 isoforms 1/2/3/4/5/8,

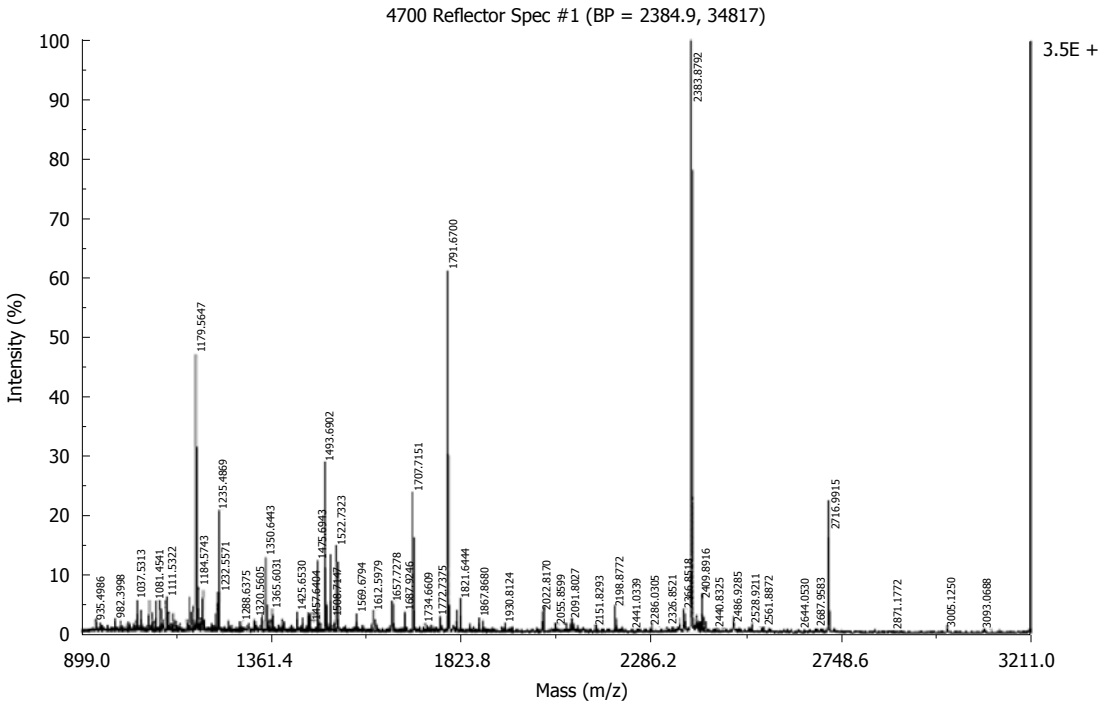


Figure 4 MALDI-TOF mass spectrum of the mixture of tryptic peptides derived from N4 (Bullous pemphigoid antigen 1 isoforms 6/9/10).

Ras-related protein Rab-30, Zinc finger protein 268, Calcium-binding protein CaBP5, RGS3, membrane-associated guanylate kinase (MAGUK) p55 subfamily member 3, Zinc finger protein 134, Actin alpha cardiac, Nebulin, Vacuolar protein sorting 33B) in gastric cancer tissue.

RGS3 inhibits signal transduction by increasing the GTPase activity of G protein alpha subunits thereby driving them into their inactive GDP. A recent study revealed that *RGS3* gene was up-regulated in highly migratory clones selected from U373MG glioma cells compared with that of the original cells using oligonucleotide microarrays comprising 12 625 genes. U373MG glioma cell clones with over-expression of RGS3 or RGS4 showed an increase of both adhesion and migration. These findings expand the spectrum of possible molecular pathways underlying the invasion of neoplastic astrocytes[22].

MAGUK p55 subfamily member 3 (MPP3) belongs to the MAGUK protein family. Shins' study showed MPP3 was identified by cell surface biotinylation and MS in the cell surface proteome of A549 lung adenocarcinoma cells and LoVo colon adenocarcinoma cells[23].

Nebulin may be involved in maintaining the structural integrity of sarcomeres and the membrane system associated with the myofibrils. The nebulin gene was found up-regulated in non small cell lung carcinoma-derived cell lines D51 and lung cancer cell lines (H2170 and H526) compared with human bronchial epithelial cell lines by suppression subtractive hybridization (SSH). But the relations between nebulin and gastric cancer is not clear[24].

We also obtained five under-expressed proteins (Peroxiredoxin 2, Zinc finger protein ZFP-36, Zinc finger protein 64 isoforms 3 and 4, Bullous pemphigoid antigen 1 isoforms 6/9/10, Transcriptional repressor CTCFL).

Peroxiredoxins (Prxs) are a novel group of peroxidases containing high antioxidant efficiency and some of them having also effects on cell differentiation and apoptosis. The mammalian Prx family has six distinct members located in various subcellular locations. Peroxiredoxin 2 (Prx 2) is known not only to protect cells from oxidative damage caused by hydrogen peroxide (H_2O_2), but also to endow cancer cells with resistance to both H_2O_2 and cisplatin and to grant them radioresistance[25]. Somiari *et al*[26] analyzed human breast infiltrating ductal carcinoma (IDCA) using two-dimensional difference gel electrophoresis and MS and found peroxiredoxin 2 was less abundant in four breast IDCAs (Stage Ⅰ, ⅡA, ⅡB and ⅢA) compared with non-neoplastic tissue. But recent studies showed Prx family were over expressed in some kind of carcinomas suggesting that Prx has a proliferative effect and may be related to cancer development or progression[27-30].

The transcriptional repressor, CTCFL, acts as a transcriptional regulator binding to some gene promoters and may be associated with epigenetic reprogramming events in the male germ line. But the role that transcriptional repressor CTCFL plays in gastric cancer is still not demonstrated.

Because of different protein processing and posttranslational modifications, protein isoforms of individual protein can be identified on 2D gels. Interestingly, our study revealed Bullous pemphigoid antigen 1 isoforms 1/2/3/4/5/8 were over expressed in gastric cancer tissue while Bullous pemphigoid antigen 1 isoforms 6/9/10 were under expressed in cancer tissue.

This finding suggests that the different protein isoforms may be correlated with specific features of gastric cancer and play different roles in the progression of cancer.

In conclusion, the identified proteins, both up-regulated and down-regulated, may participate in the progression of malignant growth or in the maintenance of normal conditions of gastric tissue. The functions of these proteins in the carcinogenesis of gastric cancer remain to be studied. We hope they may provide useful information for early detection and therapy of gastric cancer.

ACKNOWLEDGMENTS

We thank Dr. Qun-Ye Zhang and Qiu-Hua Huang (Shanghai Institute of Hematology, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine) for their assistance in the identification of proteins.

COMMENTS

Background

Gastric carcinoma is one of the most common malignancies worldwide and is the most common cause of cancer-related death in China. The main barrier for improving survival rate is the lack of useful markers for early diagnosis.

Research frontiers

Identification differential proteins between tumor tissue and normal tissue by proteomics to be candidate tumor markers for gastric cancer.

Innovations and breakthroughs

Twenty three differential proteins were found between tumor and normal tissues of gastric cancer, among these fifteen proteins were identified.

Applications

The differential proteins may be tumor markers for gastric cancer.

Peer review

The authors utilized two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) to isolate and identify differentially expressed proteins between gastric cancer tissues and paired normal mucosa. This is an interesting paper using new technology to identify gastric cancer characteristics.

REFERENCES

1 **Sun XD**, Mu R, Zhou YS, Dai XD, Zhang SW, Huangfu XM, Sun J, Li LD, Lu FZ, Qiao YL. [Analysis of mortality rate of stomach cancer and its trend in twenty years in China] *Zhonghua Zhongliu Zazhi* 2004; **26**: 4-9
2 **Hanash S**. Disease proteomics. *Nature* 2003; **422**: 226-232
3 **Simpson RJ**, Bernhard OK, Greening DW, Moritz RL. Proteomics-driven cancer biomarker discovery: looking to the future. *Curr Opin Chem Biol* 2008; **12**: 72-77
4 **Wulfkuhle JD**, Liotta LA, Petricoin EF. Proteomic applications for the early detection of cancer. *Nat Rev Cancer* 2003; **3**: 267-275
5 **Bitarte N**, Bandres E, Zarate R, Ramirez N, Garcia-Foncillas J. Moving forward in colorectal cancer research, what proteomics has to tell. *World J Gastroenterol* 2007; **13**: 5813-5821
6 **Cowan ML**, Vera J. Proteomics: advances in biomarker discovery. *Expert Rev Proteomics* 2008; **5**: 21-23
7 **Li J**, Zhang Z, Rosenzweig J, Wang YY, Chan DW. Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. *Clin Chem* 2002; **48**: 1296-1304
8 **Clarke CH**, Buckley JA, Fung ET. SELDI-TOF-MS proteomics of breast cancer. *Clin Chem Lab Med* 2005; **43**: 1314-1320
9 **Rui Z**, Jian-Guo J, Yuan-Peng T, Hai P, Bing-Gen R. Use of serological proteomic methods to find biomarkers associated with breast cancer. *Proteomics* 2003; **3**: 433-439
10 **Li C**, Chen Z, Xiao Z, Wu X, Zhan X, Zhang X, Li M, Li J, Feng X, Liang S, Chen P, Xie JY. Comparative proteomics analysis of human lung squamous carcinoma. *Biochem Biophys Res Commun* 2003; **309**: 253-260
11 **Au JS**, Cho WC, Yip TT, Law SC. Proteomic approach to biomarker discovery in cancer tissue from lung adenocarcinoma among nonsmoking Chinese women in Hong Kong. *Cancer Invest* 2008; **26**: 128-135
12 **Lin JF**, Xu J, Tian HY, Gao X, Chen QX, Gu Q, Xu GJ, Song JD, Zhao FK. Identification of candidate prostate cancer biomarkers in prostate needle biopsy specimens using proteomic analysis. *Int J Cancer* 2007; **121**: 2596-2605
13 **Kim J**, Kim SH, Lee SU, Ha GH, Kang DG, Ha NY, Ahn JS, Cho HY, Kang SJ, Lee YJ, Hong SC, Ha WS, Bae JM, Lee CW, Kim JW. Proteome analysis of human liver tumor tissue by two-dimensional gel electrophoresis and matrix assisted laser desorption/ionization-mass spectrometry for identification of disease-related proteins. *Electrophoresis* 2002; **23**: 4142-4156
14 **Zinkin NT**, Grall F, Bhaskar K, Otu HH, Spentzos D, Kalmowitz B, Wells M, Guerrero M, Asara JM, Libermann TA, Afdhal NH. Serum proteomics and biomarkers in hepatocellular carcinoma and chronic liver disease. *Clin Cancer Res* 2008; **14**: 470-477
15 **Feng Y**, Tian ZM, Wan MX, Zheng ZB. Protein profile of human hepatocarcinoma cell line SMMC-7721: identification and functional analysis. *World J Gastroenterol* 2007; **13**: 2608-2614
16 **Gagnon A**, Kim JH, Schorge JO, Ye B, Liu B, Hasselblatt K, Welch WR, Bandera CA, Mok SC. Use of a combination of approaches to identify and validate relevant tumor-associated antigens and their corresponding autoantibodies in ovarian cancer patients. *Clin Cancer Res* 2008; **14**: 764-771
17 **Ha GH**, Lee SU, Kang DG, Ha NY, Kim SH, Kim J, Bae JM, Kim JW, Lee CW. Proteome analysis of human stomach tissue: separation of soluble proteins by two-dimensional polyacrylamide gel electrophoresis and identification by mass spectrometry. *Electrophoresis* 2002; **23**: 2513-2524
18 **Bjellqvist B**, Ek K, Righetti PG, Gianazza E, Gorg A, Westermeier R, Postel W. Isoelectric focusing in immobilized pH gradients: principle, methodology and some applications. *J Biochem Biophys Methods* 1982; **6**: 317-339
19 **Gorg A**, Postel W, Gunther S. The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* 1988; **9**: 531-546
20 **Yasui W**, Oue N, Ito R, Kuraoka K, Nakayama H. Search for new biomarkers of gastric cancer through serial analysis of gene expression and its clinical implications. *Cancer Sci* 2004; **95**: 385-392
21 **Hibbs K**, Skubitz KM, Pambuccian SE, Casey RC, Burleson KM, Oegema TR Jr, Thiele JJ, Grindle SM, Bliss RL, Skubitz AP. Differential gene expression in ovarian carcinoma: identification of potential biomarkers. *Am J Pathol* 2004; **165**: 397-414
22 **Tatenhorst L**, Senner V, Puttmann S, Paulus W. Regulators of G-protein signaling 3 and 4 (RGS3, RGS4) are associated with glioma cell motility. *J Neuropathol Exp Neurol* 2004; **63**: 210-222
23 **Shin BK**, Wang H, Yim AM, Le Naour F, Brichory F, Jang JH, Zhao R, Puravs E, Tra J, Michael CW, Misek DE, Hanash SM. Global profiling of the cell surface proteome of cancer cells uncovers an abundance of proteins with chaperone function. *J Biol Chem* 2003; **278**: 7607-7616
24 **Difilippantonio S**, Chen Y, Pietas A, Schluns K, Pacyna-Gengelbach M, Deutschmann N, Padilla-Nash HM, Ried T, Petersen I. Gene expression profiles in human non-small

and small-cell lung cancers. *Eur J Cancer* 2003; **39**: 1936-1947
25 **Yo YD**, Chung YM, Park JK, Ahn CM, Kim SK, Kim HJ. Synergistic effect of peroxiredoxin II antisense on cisplatin-induced cell death. *Exp Mol Med* 2002; **34**: 273-277
26 **Somiari RI**, Sullivan A, Russell S, Somiari S, Hu H, Jordan R, George A, Katenhusen R, Buchowiecka A, Arciero C, Brzeski H, Hooke J, Shriver C. High-throughput proteomic analysis of human infiltrating ductal carcinoma of the breast. *Proteomics* 2003; **3**: 1863-1873
27 **Kinnula VL**, Paakko P, Soini Y. Antioxidant enzymes and redox regulating thiol proteins in malignancies of human lung. *FEBS Lett* 2004; **569**: 1-6
28 **Karihtala P**, Mantyniemi A, Kang SW, Kinnula VL, Soini Y. Peroxiredoxins in breast carcinoma. *Clin Cancer Res* 2003; **9**: 3418-3424
29 **Noh DY**, Ahn SJ, Lee RA, Kim SW, Park IA, Chae HZ. Overexpression of peroxiredoxin in human breast cancer. *Anticancer Res* 2001; **21**: 2085-2090
30 **Yanagawa T**, Iwasa S, Ishii T, Tabuchi K, Yusa H, Onizawa K, Omura K, Harada H, Suzuki H, Yoshida H. Peroxiredoxin I expression in oral cancer: a potential new tumor marker. *Cancer Lett* 2000; **156**: 27-35

S- Editor Li DL **L- Editor** Rippe RA **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5665

World J Gastroenterol 2008 October 7; 14(37): 5665-5673
World Journal of Gastroenterology ISSN 1007-9327

Reduced expression of E-cadherin/catenin complex in hepatocellular carcinomas

Bo Zhai, He-Xin Yan, Shu-Qin Liu, Lei Chen, Meng-Chao Wu, Hong-Yang Wang

Bo Zhai, Department of Ultrasonic Intervention, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200438, China
He-Xin Yan, Shu-Qin Liu, Lei Chen, Meng-Chao Wu, Hong-Yang Wang, International Cooperation Laboratory on Signal Transduction, Eastern Hepatobilliary Surgery Institute, Second Military Medical University, Shanghai 200438, China
Author contributions: Zhai B wrote the paper and organized the figures and patients' data; Yan HX and Liu SQ did immunohistochemical staining assays; Chen L analyzed the statistical data; Wu MC and Wang HY helped write, organize, and correct the paper; Wang HY supervised the writing and organization process.
Correspondence to: Hong-Yang Wang, International Cooperation Laboratory on Signal Transduction, Eastern Hepatobilliary Surgery Institute, Second Military Medical University, Shanghai 200438, China. hywangk@online.sh.cn
Telephone: +86-21-25074927 Fax: +86-21-65566851
Received: May 14, 2008 Revised: July 19, 2008
Accepted: July 26, 2008
Published online: October 7, 2008

Abstract

AIM: To examine the immunoreactivity of E-cadherin and four subtypes of catenin family in human hepatocellular carcinomas (HCCs) and to investigate the correlation between expression of E-cadherin/catenin complex and clinicopathologic parameters of HCC patients.

METHODS: An immunohistochemical study for E-cadherin and catenins was performed on 97 formalin-fixed, paraffin-embedded specimens of HCC.

RESULTS: Reduced expression of E-cadherin, α-, β-, γ-catenin and p120 was observed in 69%, 76%, 63%, 71% and 73%, respectively. Both expressions of E-cadherin and catenin components were significantly correlated with tumor grade (P = 0.000). It showed significant difference between expression of catenin members and tumor stage (P = 0.003, P = 0.017, P = 0.007 and P = 0.000, respectively). The reduced expression of E-cadherin in HCCs was significantly correlated with intrahepatic metastasis (IM) and capsular invasion (P = 0.008, P = 0.03, respectively). A close correlation was also observed between the expression of catenins and the tumor size (P = 0.002, P = 0.034, P = 0.016 and P = 0.000, respectively). In addition, the expression of each catenin was found correlated with IM (P = 0.012, P = 0.049, P = 0.026 and P = 0.014, respectively). No statistically significant difference was observed between the expression level of E-cadherin/catenin complex and lymph node permission, vascular invasion and satellite nodules. Interestingly, only expression of p120 showed correlation with AFP value (P = 0.035). The expression of E-cadherin was consistent with α-, β-, γ-catenin and p120 expression (P = 0.000). Finally, the abnormal expression of E-cadherin/catenin complex was significantly associated with patients' survival (P = 0.0253, P = 0.0052, P = 0.003, P = 0.0105 and P = 0.0016, respectively). Nevertheless, no component of E-cadherin/catenin complex was the independent prognostic factor of HCC patients.

CONCLUSION: Down-regulated expressions of E-cadherin, catenins and p120 occur frequently in HCCs and contribute to the progression and development of tumor. It may be more exact and valuable to detect the co-expression of E-cadherin/catenin complex than to explore one of them in predicting tumor invasion, metastasis and patient's survival.

Key words: E-cadherin; Hepatocellular carcinomas; Histologic feature; Survival

Peer reviewer: Yogesh K Chawla, PhD, Professor, Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

Zhai B, Yan HX, Liu SQ, Chen L, Wu MC, Wang HY. Reduced expression of E-cadherin/catenins complex in hepatocellular carcinomas. *World J Gastroenterol* 2008; 14(37): 5665-5673 Available from: URL: http://www.wjgnet.com/1007-9327/14/5665.asp DOI: http://dx.doi.org/10.3748/wjg.14.5665

INTRODUCTION

E-cadherin is a transmembrane glycoprotein and plays an important role in the contact between epithelial cells depending on calcium. Homophilic interaction of the extracellular part of E-cadherin mediates cell-cell adhesion[1]. The adhesion mediated by E-cadherin is significantly implicated in the establishment of cell polarity, the transformation of epithelial/interstitial cells

and the classification of cell types in the development[2,3]. The cytoplasmic part of E-cadherin is linked to the actin cytoskeleton *via* the catenins, including α-catenin, β-catenin, γ-catenin and p120[4]. E-cadherin/catenin complex is widely acknowledged as both tumor and metastasis suppressor, and the search for strategies to repress metastasis has led to intense studies of the mechanisms and molecules regulating E-cadherin function[2,3]. In addition, several recent trials have displayed the bright future of E-cadherin/catenin complex as a targeted therapy for human cancers[5-7].

The expression of E-cadherin/catenin complex has been shown to associate with tumor histological features (tumor size, grade, stage, invasion, metastasis, prognosis, *etc*.) in several cancers[8-12]. For liver cancer, some studies have reported that E-cadherin gene methylation or β-catenin exon 3 mutation occurred frequently and there was significant correlations between these abnormal biologic behavior and tumor development and progression[13-15]. In addition, some studies have explored the relationship between the clinicopathologic parameters of hepatocellular carcinoma (HCC) and the expression of E-cadherin/catenins complex, but the conclusions are still controversial. Endo *et al*[16] revealed over-expression of α-, β-, and γ-catenins in most HCCs, which was inversely correlated with histological grade of HCC, whereas E-cadherin expression was down-regulated and displayed a significant positive correlation with HCC grade. However, Ihara *et al*[17] showed over-expression of E-cadherin in their 66 HCCs, which was inversely correlated with tumor histological grade.

At present, there are little reports to demonstrate the relationship between the co-expression of above-mentioned five cell adhesion molecules and the clinicopathologic parameters (including patients' prognosis) in HCC. Especially, there was no report about expression of p120 catenin in HCC patients. In the present study, we used immunohistochemical staining of E-cadherin/catenin complex in the primary lesion of surgically resected HCC specimens to clarify the correlations between the expression of E-cadherin/cadherin complex and the development and progression of HCC.

MATERIALS AND METHODS

Patient selection and definition of clinicopathologic parameters

In this study, we selected 97 cases of HCC that had been collected and diagnosed at the Eastern Hepatobilliary Surgery Hospital, the Second Military Medical University between October 1998 and March 2004. The patients consisted of 67 men and 30 women and ranged in age from 34 to 72 years, with an average age of 54 years. In each case, HCC tissues and nontumorous liver tissues were obtained for pathological examination. The detailed pathologic results were gained from the department of pathology of our hospital. Background liver showed cirrhosis in 72 cases (74.2%) and chronic hepatitis in 69 cases (71.1%) which included hepatitis B virus in 60 cases and hepatitis C virus (HCV) in 5 cases, and cryptogenic in 4 cases.

Clinicopathologic parameters include histological grades, stages, size, capsular and vascular invasion (portal vein cancer thrombus), satellite nodules, IM, lymph node permission, AFP value and patients' survival. HCC was classified into grade Ⅰ, Ⅱ, Ⅲ and Ⅳ according to Edomndson and Steiner[18]. Histological types of HCC were adopted according to the system of World Health Organization. HCC staging was performed according to TNM staging system of the International Union against Cancer UICC[19]. IM was defined as recurrent tumors consisting of moderately or poorly differentiated HCC with the same or lower degree of differentiation compared with the differentiation of the primary tumors[20]. The satellite nodules of this HCC represent either intrahepatic spread of the tumor or multicentric origin of the tumor. The patients were followed up for 8-68 mo.

Immunohistochemistry

All samples were collected from 5-μm-thick histological sections. They were cut from the formalin-fixed paraffin-embedded material. Dako EnVision™ Kit (Dakocytomation Company, Denmark) was used for the immunohistochemical staining of E-cadherin, α-, β- and γ-catenins and p120. Sections were dewaxed, and incubated with mathenol containing 30% H_2O_2 for 20 min to block endogenous peroxidase activity. To enhance antigen retrieval, sections were treated at this stage in a microwave oven. Briefly, sections were immersed in 0.01 mol/L citrate buffer (pH 6.0) and heated in a microwave oven at 100℃ for 20 min. Subsequently, they were washed three times with distilled water, and then blocked with 1% BSA for 30 min. Sections were incubated overnight at 4℃ with rabbit polyclonal IgG of E-cadherin, α-catenin and p120 (Santa Clauze Corporation, USA, dilution 1:200) and mouse monoclonal IgG of β-catenin and γ-catenin (Santa Clauze Corporation, USA, dilution 1:200). A subsequent reaction was carried out using second antibodies ((Dakocytomation Company, Denmark, dilution 1:200) for 30 min at 37℃. Sections were washed three times with PBS buffer and subsequently displayed color with DAB for about 5 min. Nuclei were lightly counterstained with hematoxylin. No staining was obtained when nonimmune serum or PBS was used instead of the primary antibodies, thus confirming the specificity of each primary antibody.

Evaluation of immunostaining

We used a scoring system[21] to evaluate semiquantitatively the immunoexpression. The expression of non-tumorous tissue served as an internal control. Briefly, immunoractivities were assessed by the extent (broadness) and intensity (color strength). Depending on the percentage of positive cells, the extent score was classified as follows: 0, no positive cell or less than 5%; +1, 5%-25%; +2, 26%-50%; +3, 51%-75% and +4, 76%-100% positive cells. The intensity score was also

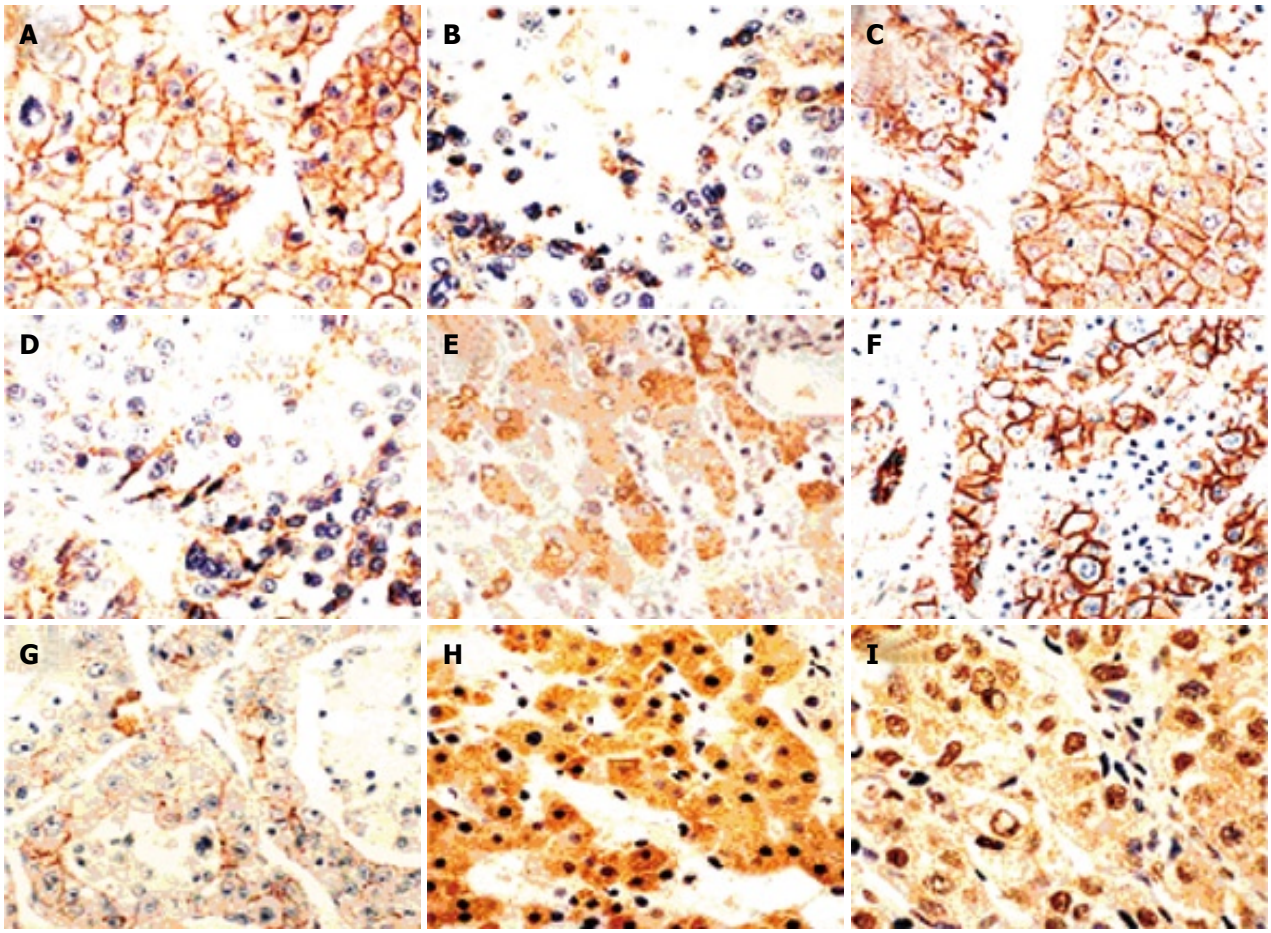


Figure 1 Immunoreactivity of E-cadherin, α- and β-catenins in HCCs. **A** and **B**: Stained for E-cadherin: preserved type (+), reduced type (-); **C-E**: Stained for α-catenin: preserved type (+), reduced type (-) and staining in the cytoplasm; **F-I**: Stained for β-catenin: preserved type (+), reduced type (-) and staining in the cytoplasm and the nucleus (× 200).

Table 1 Expression of five cell adhesion molecules in HCCs *n* (%)

	E-cadherin	α-catenin	β-catenin	γ-catenin	p120
Normal	40 (41.2)	32 (35.0)	42 (35.0)	37 (37.5)	35 (42.5)
Reduced	41 (42.3)	46 (65.0)	42 (65.0)	50 (62.5)	47 (57.5)
Absent	16 (16.5)	19	13	10	15

categorized into four groups: 0, no immunoreaction; +1, mild immunoreaction; +2, moderate immunoreaction; and +3, marked immunoreaction. Preserved E-cadherin or catenin expression was defined when the composite score was 6 or 7. In contrast, the expression of E-cadherin or catenin was also defined as "absent or loss" when the total score was 0.

Statistical analysis

Results from immunohistochemistry were analyzed by Yates' correction or Fisher's exact tests and statistical significance was accepted when $P < 0.05$. For survival analysis, log-rank test was used with a significant level of $P < 0.05$. Survival curves were computed according to the method of Kaplan-Meier. The prognosis value of these five molecules was evaluated with univariate and multivariate analysis. The SPSS 10.1 software package for Windows (SPSS, Inc., Chicago, IL) was used.

RESULTS

Microscopic observations

In nontumorous liver tissues, both E-cadherin and catenins were expressed strongly at cell membrane, but the staining strength gradually weakened from nontumoral tissue to the tumor region. In addition, bile ducts, proliferated ductiles and intrahepatic vessels strongly expressed these two molecules at the cell membrane. There was no expression in other cell types in the liver.

In 97 specimens of HCCs, the abnormal expression of E-cadherin, α-, β-, γ-catenin and p120 was found in 57, 65, 55, 60 and 62 cases (completely absent in 16, 19, 13, 10 and 15 cases), respectively (Table 1). The immunostaining distribution of E-cadherin was only presented at cell membrane, whereas catenins were found at the membrane in the cytoplasm and/or nucleus (Table 2, Figures 1 and 2).

Relationship between expression of E-cadherin/catenin complex and histological features in HCCs

As shown in Table 3, expression of both E-cadherin and catenin components was significantly related with tumor grade ($P = 0.000$). There was a greater tendency for the expression of E-cadherin/catenin complex to reduce in

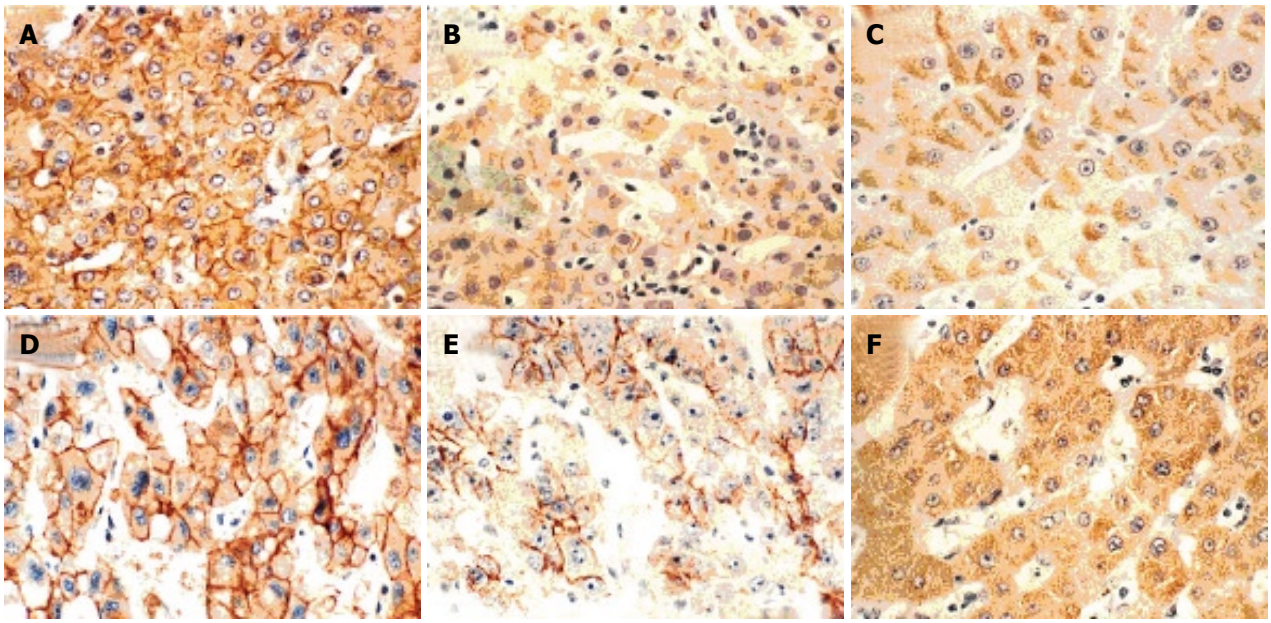


Figure 2 Immunoreactivity of γ-catenin and p120 in HCCs. **A-C**: Stained for γ-catenin: preserved type (+), reduced type (-) and staining in the cytoplasm; **D-F**: Stained for p120: preserved type (+), reduced type (-) and staining in the cytoplasm (× 200).

Table 2 Distribution of complex expression in HCCs

	Membrane	Cytoplasm	Nucleus
E-cadherin	97	0	0
α-catenin	70	27	0
β-catenin	55	36	6
γ-catenin	63	34	0
p120	65	32	0

poorly differentiated tumors than in well and moderately differentiated tumors. Except for E-cadherin, significant difference was found between expression of catenin members and tumor stage ($P = 0.003$, $P = 0.017$, $P = 0.007$ and $P = 0.000$, respectively).

Relationship between expression of E-cadherin/catenin complex and clinical parameters in HCCs

In Table 3, the reduced expression of E-cadherin in HCCs was significantly correlated with IM and capsular invasion ($P = 0.008$ and $P = 0.03$, respectively). A close correlation was also observed between expression of α-, β-, γ-catenin and p120 and the tumor size ($P = 0.002$, $P = 0.034$, $P = 0.016$ and $P = 0.000$, respectively). In addition, the expression of each catenin was found correlated with IM ($P = 0.012$, $P = 0.049$, $P = 0.026$ and $P = 0.014$, respectively). No statistically significant difference was observed between the expression level of E-cadherin/catenin complex and lymph node permission, vascular invasion and satellite nodules. Interestingly, only p120 expression was correlated with AFP value ($P = 0.035$).

Relationship between expression of E-cadherin and catenins in HCCs

As shown in Table 4, the expression of E-cadherin was significantly correlated to the expression of all kinds of catenins ($P = 0.000$). There was a significant concordance between the expression of E-cadherin and catenins.

Relationship between expression of E-cadherin/catenin complex and patients' survival

The overall patient survival according to the expression of E-cadherin and catenins in tumor is shown in Figure 3. Analysis of the survival for all patients showed that abnormal expression of E-cadherin and α-, β-, γ- and p120 catenins were correlated with poor survival ($P = 0.0253$, $P = 0.0052$, $P = 0.003$, $P = 0.0105$ and $P = 0.0016$, respectively). However, when E-cadherin/catenins complex status and other clinicopathological parameters were analyzed by the Cox regression model, abnormal expression of the E-cadherin/catenin complex was not found to be an independent prognostic factor (data not shown).

DISCUSSION

In accordance with previous reports[22,23], the expressions of all the members of E-cadherin/catenin complex were down-regulated in HCC, among of which the reduced expression of α-catenin was found most frequently. The expression of these five molecules was inversely correlated with tumor differentiation degree, thus all of them may be considered as good differentiation markers of HCC. Our result, however, is different from the reports of Endo and Ihara *et al*[16,17], which respectively showed over-expression of E-cadherin or α-, β- and γ-catenins in most HCCs. Therefore, together with CpG methylation of E-cadherin in HCC[13], it can be concluded that E-cadherin may act as a modulator of the maintenance of HCC histological architecture.

In addition, the reduced expression of all catenins,

Table 3 Relationship between expression of E-cadherin/catenin complex and histological features and clinical parameters in HCCs

	n	E-cadherin +/-	α-catenin +/-	β-catenin +/-	γ-catenin +/-	p120ctn +/-
Histological grade						
Ⅰ-Ⅱ	23	20/3	19/4	22/1	20/3	19/4
Ⅲ	58	17/41	11/47	20/38	11/47	15/43
Ⅳ	16	3/13	2/14	2/14	3/13	1/15
P		0.000	0.000	0.000	0.000	0.000
Histological stage						
Ⅰ-Ⅱ	47	24/23	22/25	27/20	25/22	27/20
Ⅲ	37	12/25	5/32	10/27	10/27	6/31
Ⅳ	14	4/10	3/11	5/9	2/12	2/12
P		0.136	0.003	0.017	0.007	0.000
Tumor size						
< 3 cm	29	15/14	17/12	18/11	17/12	19/10
3-10 cm	50	18/32	10/40	16/34	13/37	12/38
> 10 cm	18	7/11	5/13	8/10	7/11	4/14
P		*0.382*	*0.002*	*0.034*	*0.016*	*0.000*
Capsular invasion						
Present	9	7/2	6/3	6/3	5/4	5/4
Absent	88	33/55	26/62	36/52	32/56	30/58
P		0.003	0.055	0.169	0.295	0.277
Satellite nodules						
Present	24	10/14	8/16	12/12	7/17	8/16
Absent	73	30/43	24/40	30/43	30/43	27/46
P		1.000	1.000	0.483	0.341	0.811
Vascular invasion						
Present	26	8/18	8/18	12/14	10/16	7/19
Absent	71	32/39	24/47	30/41	27/44	28/43
P		0.249	1.000	0.818	1.000	0.341
Lymph node permission						
Present	17	4/13	3/14	8/9	6/11	5/12
Absent	80	36/44	29/51	34/46	31/49	30/50
P		0.114	0.116	0.791	1.000	0.59
Intrahepatic metastasis						
Present	32	7/25	5/27	9/23	7/25	6/26
Absent	65	33/32	27/38	33/32	30/35	29/36
P		0.008	0.012	0.049	0.026	0.014
AFP-value						
< 20 ng/dL	28	6/12	13/15	16/12	14/14	15/13
> 20 ng/dL	69	24/45	19/50	26/43	23/46	20/49
P		0.068	0.096	0.113	0.167	0.035

Table 4 Relationship between E-cadherin and catenin expressions in HCCs

		α-catenin		β-catenin		γ-catenin		p120	
		+	-	+	-	+	-	+	-
E-catenin	+	28	12	33	7	29	11	31	9
	-	4	53	9	48	8	49	4	53
P		0.000		0.000		0.000		0.000	

but E-cadherin, was significantly associated with tumor stage and tumor size in our study. The mechanism of these findings was unknown, but they were consistent with tumor clinical features, the small liver cancers have a relatively better clinical course. Interestingly, p120 expression was also correlated with AFP value. It is well known that AFP is only an indicator of malignant liver tumor, and its value is not always related to the classification of tumor cells, so it may be a coincidence and needs to be investigated in the future. However, the findings above may further demonstrate that E-cadherin/catenin complex, at least catenins, correlate with HCC's biological behavior to some degree.

We found some distinct locations for different molecules of complex in HCC, especially for β-, γ-, and p120 catenin. In this study, hepatocytes, endothelial cells of bile ducts, proliferated ductiles and intrahepatic vessels of nontumorous liver tissue strongly expressed all of five molecules at cell membrane, but the staining strength gradually weakened from nontumorous tissue to tumor region. Besides expressing mostly at the membrane, these three members of armidillo protein family were also found in the cytoplasm or nucleus. It has been shown to be important in the development of several tumors that constitutionally activated the Wnt/Wingless signaling pathway by stabilization and accumulation of β-catenin in the nucleus and cytoplasm[24-27]. Like β-catenin, γ-catenin can also bind to APC protein and is able to translocate into the nucleus participating in the Wnt signaling pathway[28].

Several studies demonstrate that p120 can enter the nucleus and the Kaiso may be its receptor in nucleus[29]. Upon loss of E-cadherin, p120 translocates from

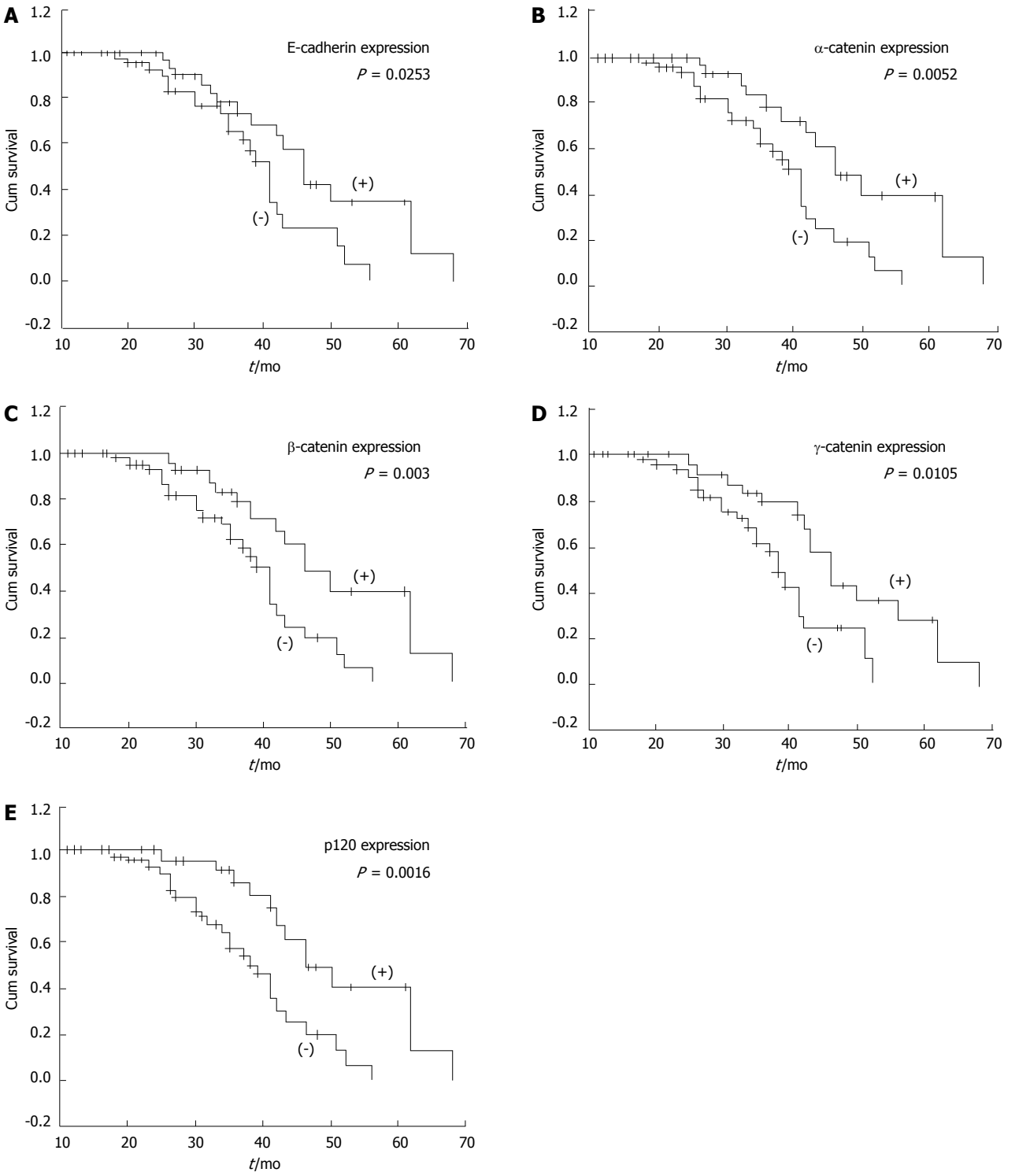


Figure 3 Kaplan-Meier survival curves with normal (pink line) or abnormal (green line), **A**: Expression of E-cadherin (P = 0.0253); **B**: Expression of α-catenin (P = 0.0052); **C**: Expression of β-catenin (P = 0.003); **D**: Expression of γ-catenin (P = 0.0105); **E**: Expression of p120 (P = 0.0016). Abnormal expressions of E-cadherin/catenin complex significantly related with poor survival.

the membrane to the cytoplasm. Alternatively, the cytoplasmic p120 pool may have increased access to the nucleus[30,31]. Therefore, these three molecules may play an important role in cell signal transduction and affect the development and progress of tumor. Unfortunately, there is no further investigation in relationship between p120 nucleus expression and clinicopathologic parameters in HCC due to the small number of cases with positive nucleus expression in our study.

As a focal point, we examined the relationships between expression of E-cadherin/catenin complex and some invasion and metastasis parameters of HCC. The reduced expression of E-cadherin in our experiment was significantly correlated with IM and capsular invasion. There was also a significant correlation between expression of catenins and IM in HCC. Nevertheless, no

statistically significant difference was observed between the expression level of E-cadherin/catenin complex and lymph node permission, vascular invasion and satellite nodules. These results were different from those of the previous studies to some degree. Endo *et al*[16] showed significant positive correlations between γ-catenin high expression and capsular invasion or presence of satellite nodules, and between β-catenin high expression and vascular invasion. Kozyraki *et al*[23] found that E-cadherin and α-catenin expression in HCC was associated with occurrence of capsular and vascular invasion. Unlike most other types of tumors, very few HCC patients in our data had lymph node invasion due to their anatomic characters and complete liver capsule. Because of the small samples in our study (there were only 17 cases of lymph node, 9 cases of complete capsular and 26 cases of vascular invasion among 97 HCC patients), it is difficult to analyze the relationship between these parameters and expression of E-cadherin/catenin complex.

We were astonished that there was a significant correlation between expression of E-cadherin/catenin complex and IM in HCC. The mechanism and pathogenesis of the high frequency of IM in HCC have not yet been elucidated. Mitsunobu *et al*[32] demonstrated that tumor spread in HCC progresses from capsular invasion to intrahepatic invasion and that the portal vein may act as an efferent tumor vessel. Meada *et al*[33] pointed out that the histological features of the intrahepatic metastatic lesions are essentially the same as those of the main nodule. The proliferative activities in the intrahepatic metastatic lesions were generally higher than those in the main nodules. The fact that there are differences in the proliferative activity, despite the similarity in histology between the primary sites and the metastatic lesions, suggests that tumor cells of the metastatic lesions might acquire some characteristic advantages in forming metastasis. Preserved or recovered function of E-cadherin may be one of these advantages. Osada *et al*[34] found that E-cadherin is involved in the IM of HCC. Asayama *et al*[35] had a similar report in HCC-CC patients. These results indicated that E-cadherin/catenin complex might play an important role in the detachment of cancer cells.

To our knowledge, this is the first report of p120 catenin expression in human HCC. p120 was found to strangely correlate with differentiation, IM and patients' survival, and p120 loss was associated with down-regulation of all members of the complex. Thoreson *et al*[30] suggested that it is possible that morphologic and behavioral changes in some tumors are due to p120 loss and consequent destabilization of E-cadherin. The roles for p120 as either tumor suppressor or metastasis promoter during tumor progression differ with the order, in which p120 or E-cadherin was down-regulated first. If p120 is lost first, E-cadherin level will fall significantly[36], which is likely to be paralleled by reduced levels of α - and β -catenins[37]. If E-cadherin is lost first, p120 may directly and actively promote metastasis.

With regard to the relationship between the E-cadherin/catenin complex expression and patients' survival in HCC, we found that patients with low expression of these five molecules had poorer prognosis than those with normal expression. Unlike most previous reports[38-41], which revealed only one or a few members of this complex were associated with patients' survival, our result demonstrated that all molecules of E-cadherin/catenin complex were significantly correlated with HCC patients' survival. However, when E-cadherin/catenin complex status and other clinicopathological parameters were analyzed by the Cox regression model, abnormal expression of the E-cadherin/catenin complex was not found to be an independent prognostic factor. Thus, it may be more exact and valuable to detect the co-expression of E-cadherin/catenin complex than to explore only one of them.

In conclusion, these data indicated that abnormal expression of E-cadherin/catenin complex is common in HCC. The complex expression is mostly located in the cytoplasm of HCCs and correlated with tumor differentiation, IM and patients' survival. E-cadherin/catenin complex may play an important role in development and progression of human HCC.

COMMENTS

Background

The E-cadherin/catenin complex plays a critical role in the control of epithelial differentiation and intercellular adhesion. Altered expression and/or function disruption of its components have been implicated in tumor development and progression.

Research frontiers

The expression of E-cadherin/catenin complex has been shown to associate with tumor histological features in several cancers. E-cadherin gene methylation or β-catenin exon 3 mutation occurred frequently in liver cancer and there was significant correlations between these abnormal biologic behavior and tumor development and progression. Some studies also have explored the relationship between hepatocellular carcinoma (HCC) and the expression of E-cadherin/catenin complex, but the conclusions are still controversial.

Innovations and breakthroughs

The results suggest that down-regulated expressions of E-cadherin, catenins and p120 occurred frequently in HCCs and contributed to the progression and development of tumor. It may be more exact and valuable to detect the co-expression of E-cadherin/catenin complex than to explore only one of them in predicting tumor invasion, metastasis and patient's survival.

Applications

Because down-regulated expression of E-cadherin/catenin complex contributes to the progression and development of HCC, this complex can be used as a valuable biologic marker for predicting the invasion and metastasis of HCC.

Peer review

The authors have shown reduced expression of E-cadherin/catenins in HCC. It is a good study which prognosticates the invasiveness of HCC. The study indicated that E-cadherin/catenin complex may be valuable biological markers for predicting tumor invasion and metastasis. However, its clinical application should be further studied.

REFERENCES

1 **Nollet F**, Kools P, van Roy F. Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members. *J Mol Biol* 2000; **299**: 551-572

2 **Takaishi K**, Sasaki T, Kameyama T, Tsukita S, Tsukita S, Takai Y. Translocation of activated Rho from the cytoplasm

to membrane ruffling area, cell-cell adhesion sites and cleavage furrows. *Oncogene* 1995; **11**: 39-48
3 **Yap AS**. The morphogenetic role of cadherin cell adhesion molecules in human cancer: a thematic review. *Cancer Invest* 1998; **16**: 252-261
4 **Huber O**, Bierkamp C, Kemler R. Cadherins and catenins in development. *Curr Opin Cell Biol* 1996; **8**: 685-691
5 **Pang RW**, Poon RT. From molecular biology to targeted therapies for hepatocellular carcinoma: the future is now. *Oncology* 2007; **72** Suppl 1: 30-44
6 **Tommasi S**, Pinto R, Pilato B, Paradiso A. Molecular pathways and related target therapies in liver carcinoma. *Curr Pharm Des* 2007; **13**: 3279-3287
7 **Boyault S**, Rickman DS, de Reynies A, Balabaud C, Rebouissou S, Jeannot E, Herault A, Saric J, Belghiti J, Franco D, Bioulac-Sage P, Laurent-Puig P, Zucman-Rossi J. Transcriptome classification of HCC is related to gene alterations and to new therapeutic targets. *Hepatology* 2007; **45**: 42-52
8 **Sarrio D**, Moreno-Bueno G, Sanchez-Estevez C, Banon-Rodriguez I, Hernandez-Cortes G, Hardisson D, Palacios J. Expression of cadherins and catenins correlates with distinct histologic types of ovarian carcinomas. *Hum Pathol* 2006; **37**: 1042-1049
9 **Tang WW**, Stelter AA, French S, Shen S, Qiu S, Venegas R, Wen J, Wang HQ, Xie J. Loss of cell-adhesion molecule complexes in solid pseudopapillary tumor of pancreas. *Mod Pathol* 2007; **20**: 509-513
10 **Nozawa N**, Hashimoto S, Nakashima Y, Matsuo Y, Koga T, Sugio K, Niho Y, Harada M, Sueishi K. Immunohistochemical alpha- and beta-catenin and E-cadherin expression and their clinicopathological significance in human lung adenocarcinoma. *Pathol Res Pract* 2006; **202**: 639-650
11 **Bukholm IR**, Nesland JM, Bukholm G. Expression of adhesion proteins E-cadherin, alpha-catenin, beta-catenin and gamma-catenin is different in T1 and T2 breast tumours. *Pathology* 2006; **38**: 403-407
12 **Nakamura E**, Sugihara H, Bamba M, Hattori T. Dynamic alteration of the E-cadherin/catenin complex during cell differentiation and invasion of undifferentiated-type gastric carcinomas. *J Pathol* 2005; **205**: 349-358
13 **Matsumura T**, Makino R, Mitamura K. Frequent down-regulation of E-cadherin by genetic and epigenetic changes in the malignant progression of hepatocellular carcinomas. *Clin Cancer Res* 2001; **7**: 594-599
14 **Lee S**, Kim WH, Jung HY, Yang MH, Kang GH. Aberrant CpG island methylation of multiple genes in intrahepatic cholangiocarcinoma. *Am J Pathol* 2002; **161**: 1015-1022
15 **Yang B**, House MG, Guo M, Herman JG, Clark DP. Promoter methylation profiles of tumor suppressor genes in intrahepatic and extrahepatic cholangiocarcinoma. *Mod Pathol* 2005; **18**: 412-420
16 **Endo K**, Ueda T, Ueyama J, Ohta T, Terada T. Immunoreactive E-cadherin, alpha-catenin, beta-catenin, and gamma-catenin proteins in hepatocellular carcinoma: relationships with tumor grade, clinicopathologic parameters, and patients' survival. *Hum Pathol* 2000; **31**: 558-565
17 **Ihara A**, Koizumi H, Hashizume R, Uchikoshi T. Expression of epithelial cadherin and alpha- and beta-catenins in nontumoral livers and hepatocellular carcinomas. *Hepatology* 1996; **23**: 1441-1447
18 **The general rules for the clinical and pathological study of primary liver cancer**. Liver Cancer Study Group of Japan. *Jpn J Surg* 1989; **19**: 98-129
19 **Hermanek P**, Hutter RV, Sobin LH, Wittekind C. International Union Against Cancer. Classification of isolated tumor cells and micrometastasis. *Cancer* 1999; **86**: 2668-2673
20 **Kumada T**, Nakano S, Takeda I, Sugiyama K, Osada T, Kiriyama S, Sone Y, Toyoda H, Shimada S, Takahashi M, Sassa T. Patterns of recurrence after initial treatment in patients with small hepatocellular carcinoma. *Hepatology* 1997; **25**: 87-92
21 **Gamallo C**, Palacios J, Suarez A, Pizarro A, Navarro P, Quintanilla M, Cano A. Correlation of E-cadherin expression with differentiation grade and histological type in breast carcinoma. *Am J Pathol* 1993; **142**: 987-993
22 **Nuruki K**, Toyoyama H, Ueno S, Hamanoue M, Tanabe G, Aikou T, Ozawa M. E-cadherin but not N-cadherin expression is correlated with the intracellular distribution of catenins in human hepatocellular carcinomas. *Oncol Rep* 1998; **5**: 1109-1114
23 **Kozyraki R**, Scoazec JY, Flejou JF, D'Errico A, Bedossa P, Terris B, Fiorentino M, Bringuier AF, Grigioni WF, Feldmann G. Expression of cadherins and alpha-catenin in primary epithelial tumors of the liver. *Gastroenterology* 1996; **110**: 1137-1149
24 **Ishizaki Y**, Ikeda S, Fujimori M, Shimizu Y, Kurihara T, Itamoto T, Kikuchi A, Okajima M, Asahara T. Immunohistochemical analysis and mutational analyses of beta-catenin, Axin family and APC genes in hepatocellular carcinomas. *Int J Oncol* 2004; **24**: 1077-1083
25 **Park JY**, Park WS, Nam SW, Kim SY, Lee SH, Yoo NJ, Lee JY, Park CK. Mutations of beta-catenin and AXIN I genes are a late event in human hepatocellular carcinogenesis. *Liver Int* 2005; **25**: 70-76
26 **Kim YD**, Park CH, Kim HS, Choi SK, Rew JS, Kim DY, Koh YS, Jeung KW, Lee KH, Lee JS, Juhng SW, Lee JH. Genetic alterations of Wnt signaling pathway-associated genes in hepatocellular carcinoma. *J Gastroenterol Hepatol* 2008; **23**: 110-118
27 **de La Coste A**, Romagnolo B, Billuart P, Renard CA, Buendia MA, Soubrane O, Fabre M, Chelly J, Beldjord C, Kahn A, Perret C. Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas. *Proc Natl Acad Sci USA* 1998; **95**: 8847-8851
28 **Su LK**, Vogelstein B, Kinzler KW. Association of the APC tumor suppressor protein with catenins. *Science* 1993; **262**: 1734-1737
29 **Daniel JM**, Reynolds AB. The catenin p120(ctn) interacts with Kaiso, a novel BTB/POZ domain zinc finger transcription factor. *Mol Cell Biol* 1999; **19**: 3614-3623
30 **Thoreson MA**, Reynolds AB. Altered expression of the catenin p120 in human cancer: implications for tumor progression. *Differentiation* 2002; **70**: 583-589
31 **Thoreson MA**, Anastasiadis PZ, Daniel JM, Ireton RC, Wheelock MJ, Johnson KR, Hummingbird DK, Reynolds AB. Selective uncoupling of p120(ctn) from E-cadherin disrupts strong adhesion. *J Cell Biol* 2000; **148**: 189-202
32 **Mitsunobu M**, Toyosaka A, Oriyama T, Okamoto E, Nakao N. Intrahepatic metastases in hepatocellular carcinoma: the role of the portal vein as an efferent vessel. *Clin Exp Metastasis* 1996; **14**: 520-529
33 **Maeda T**, Adachi E, Kajiyama K, Takenaka K, Sugimachi K, Tsuneyoshi M. Proliferative activity in intrahepatic metastasis of hepatocellular carcinoma. *J Gastroenterol Hepatol* 1996; **11**: 752-757
34 **Osada T**, Sakamoto M, Ino Y, Iwamatsu A, Matsuno Y, Muto T, Hirohashi S. E-cadherin is involved in the intrahepatic metastasis of hepatocellular carcinoma. *Hepatology* 1996; **24**: 1460-1467
35 **Asayama Y**, Taguchi Ki K, Aishima Si S, Nishi H, Masuda K, Tsuneyoshi M. The mode of tumour progression in combined hepatocellular carcinoma and cholangiocarcinoma: an immunohistochemical analysis of E-cadherin, alpha-catenin and beta-catenin. *Liver* 2002; **22**: 43-50
36 **Ireton RC**, Davis MA, van Hengel J, Mariner DJ, Barnes K, Thoreson MA, Anastasiadis PZ, Matrisian L, Bundy LM, Sealy L, Gilbert B, van Roy F, Reynolds AB. A novel role for p120 catenin in E-cadherin function. *J Cell Biol* 2002; **159**: 465-476

37 **Papkoff J**. Regulation of complexed and free catenin pools by distinct mechanisms. Differential effects of Wnt-1 and v-Src. *J Biol Chem* 1997; **272**: 4536-4543
38 **Han SA**, Chun H, Park CM, Kang SJ, Kim SH, Sohn D, Yun SH, Lee WY. Prognostic significance of beta-catenin in colorectal cancer with liver metastasis. *Clin Oncol* (R Coll Radiol) 2006; **18**: 761-767
39 **Lugli A**, Zlobec I, Minoo P, Baker K, Tornillo L, Terracciano L, Jass JR. Prognostic significance of the wnt signalling pathway molecules APC, beta-catenin and E-cadherin in colorectal cancer: a tissue microarray-based analysis. *Histopathology* 2007; **50**: 453-464
40 **Faleiro-Rodrigues C**, Macedo-Pinto I, Pereira D, Lopes CS. Loss of beta-catenin is associated with poor survival in ovarian carcinomas. *Int J Gynecol Pathol* 2004; **23**: 337-346
41 **Paredes J**, Correia AL, Ribeiro AS, Milanezi F, Cameselle-Teijeiro J, Schmitt FC. Breast carcinomas that co-express E- and P-cadherin are associated with p120-catenin cytoplasmic localisation and poor patient survival. *J Clin Pathol* 2008; **61**: 856-862

S- Editor Li DL **L- Editor** Ma JY **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5674

World J Gastroenterol 2008 October 7; 14(37): 5674-5682
World Journal of Gastroenterology ISSN 1007-9327

Virologic characteristics of hepatitis B virus in patients infected *via* maternal-fetal transmission

Tao Shen, Xin-Min Yan, Yun-Lian Zou, Jian-Mei Gao, Hong Dong

Tao Shen, Xin-Min Yan, Yun-Lian Zou, Jian-Mei Gao, Hong Dong, The Institute of Basic Medicine of the First People's Hospital of Yunnan Province, Kunming 650032, Yunnan Province, China

Author contributions: Shen T and Yan XM designed research; Shen T, Zou YL, Gao JM and Dong H performed research; Shen T, Zou YL and Gao JM performed sequencing; Shen T analyzed data and wrote the paper. Yan XM provided advice and review. All authors read and approved the final manuscript.

Supported by The Natural Science Foundation of Yunnan Province, No.200300172; Hospital Science Foundation of The First People's Hospital of Yunnan Province (2004)

Correspondence to: Xin-Min Yan, Institute of Basic Medicine of the First People's Hospital of Yunnan Province, 157 Jinbi Road, Kunming 650032, Yunnan Province, China. yxmin08@163.com
Telephone: +86-871-3638453 Fax: +86-871-3648772

Received: July 16, 2008 Revised: August 22, 2008
Accepted: August 29, 2008
Published online: October 7, 2008

Abstract

AIM: To determine whether HBV with the same characteristics causes dissimilar mutations in different hosts.

METHODS: Full-length HBV genome was amplified and linked with pMD T18 vector. Positive clones were selected by double-restriction endonuclease digestion (*Eco*R Ⅰ and *Hin*dⅢ) and PCR. Twenty seven clones were randomly selected from an asymptomatic mother [at two time points: 602 (1 d) and 6022 (6 mo)] and her son [602 (S)], and the phylogenetic and mutational analysis was performed using BioEditor, Clustal X and MEGA software. Potential immune epitopes were determined by the Stabilized Matrix Method (SMM), SMM-Align Method and Emini Surface Accessibility Prediction.

RESULTS: All of the 27 sequences were genotype C, the divergence between the mother and son was 0%-0.8%. Compared with another 50 complete sequences of genotype C, the mother and her son each had 13 specific nucleotides that differed from the other genotype C isolates. AA 1-11 deletion in preS1 was the dominant mutation in the mother (14/18). The 1762T/1764A double mutation existed in all clones of the mother, 3 of them were also coupled with G1896A mutation, but none were found in the son. 17 bp deletion starting at nucleotide 2330 was the major mutation (5/9) in the son, which caused seven potential HLA class Ⅰ epitopes and one B cell epitope deletion, and produced a presumptive new start codon, downstream from the original one of the P gene.

CONCLUSION: The HBV strain in the son came from his mother, and discrepant mutation occurred in the mother and her son during infection.

Key words: Hepatic B virus; Vertical transmission; Full genome; Mutation; Phylogenetic; Deletion

Peer reviewer: David R Gretch, MD, PhD, Viral Hepatitis Laboratory, Room 706, UW Research and Training Building, Harborview Medical Center, Box 359690, 325 Ninth Avenue, Seattle, Washington 98104, United States

Shen T, Yan XM, Zou YL, Gao JM, Dong H. Virologic characteristics of hepatitis B virus in patients infected *via* maternal-fetal transmission. *World J Gastroenterol* 2008; 14(37): 5674-5682 Available from: URL: http://www.wjgnet.com/1007-9327/14/5674.asp DOI: http://dx.doi.org/10.3748/wjg.14.5674

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a significant global public health problem. Approximately 3-4 billion people worldwide have been infected with HBV, and 350-400 million persons have chronic HBV infection. Vertical transmission, especially mother-to-infant transmission is the primary pathway of hepatitis B virus infection, in approximately 90% of the infants born to HBeAg positive HBsAg carrier mothers, if no immunoprophylaxis is given. HBV with the same characteristics in the mother and child interacts with the immune system and may result in different mutations to escape immune pressure.

In the present study, we isolated the full genomic sequence of HBV in an asymptomatic mother and her son by direct sequencing. Phylogenetic and mutational analysis were performed to obtain evidence of mother to infant HBV transmission, and to elucidate distinctive HBV mutations in the different hosts.

MATERIALS AND METHODS

Patient

A 31-year-old female who was first seen at the First People's Hospital of Yunnan Province, Kunming, Yunnan, China in 2005 because of allergic purpura was reevaluated. She was HBsAg and HBeAg positive since at least 1999. Her sera were obtained from two time points: 602 (1 d) and 6022 (6 mo later). When seen again, 6 mo after her initial visit, her 7-year old son's serum [602 (S)] was collected. The alanine aminotransferase (ALT) level and abdominal ultrasound examination of the mother and son were normal. There was no history of alcohol abuse, parenteral drug use and hepatotoxin exposure. Neither the mother nor her son had received immunoprophylaxis or immunotherapy.

Serological markers and HBV DNA

HBV serological markers were evaluated using commercially available enzyme-linked immunosorbent assay (ELISA) kit (Kehua Bio-Engineering Co. LTD, Shanghai, China). HBV DNA in the sera was determined using a commercially available real-time fluorescence quantitative PCR (FQ-PCR) kit (Da An Gene Diagnostic Center, Shenzheng, China). Both subjects were also tested for hepatitis C virus (HCV), hepatitis A virus (HAV) and hepatitis E virus (HEV) infection.

Amplification of full-length HBV genome

HBV DNA was extracted from the sera using proteinase K digestion followed by phenol/chloroform extraction. The complete HBV genome was amplified by polymerase chain reaction (PCR) as described previously[1]. Amplification was performed in a 96-well cycler (Bio-RAD, USA), and 25 μL PCR mixture containing 2.5 mmol $MgCl_2$, 200 μmol/L dNTP, 400 nmol/L of each primer, and 2 U of Ex Taq polymerase (Takara BioTech). The PCR reaction was performed using the following cycles: 94℃ pre-denature for 5 min, 30 cycles of 94℃ for 1 min, 56℃ for 1 min, 72℃ for 2 min; and 72℃ for 10 min as a final extension step. The full-length amplicon were purified using a gel extraction kit (HuaShun Bio-Engineering).

Cloning and sequencing of the full-length HBV genome

Purified full-length HBV-DNA was directly linked with pMD18-T vector (TaKaRa Bio-Tech), using the standard cloning techniques. White colonies were picked and the genomic length of insertions was confirmed with PCR and double-restriction endonuclease digestion with *Eco*R I and *Hin*dⅢ. DNA sequencing analysis of the correct recombinants was performed with BigDye Terminator v3.1 and 3130 Genetic Analyzer (Applied Biosystems). For each patient and time point, 9 recombinants were randomly selected and sequenced for the full-length genome of HBV.

Sequence analysis

Thirty three HBV complete genome sequences (genotypes A to H) were used in as reference[2]. Additionally, 40 complete HBV genome sequences of genotype C from GenBank were used for phylogenetic analysis. The complete genome sequences were assembled from the sequencing data, using the BioEdit sequence alignment editor software, version 7.0.5.2[3]. Alignment was performed using Clustal X software, version 1.83[4]. Phylogenetic and molecular evolutionary analysis and nucleotide differences between the isolate sequences were carried out by the MEGA program, version 3.1[5]. The genetic distance was estimated using the Kimura two-parameter algorithm[6]. The phylogenetic trees were constructed using the neighbor-joining method[7]. Bootstrap re-sampling and reconstruction was carried out 1000 times to confirm the reliability of the phylogenetic trees[8]. The HBV genotype was assigned according to the classification system reported previously.

Potential immune epitopes prediction

For a new deletion fragment (17 bp deletion in Core gene region from nt 2330), Stabilized Matrix Method (SMM) and SMM-Align method were used to predict the potential major histocompatibility classes Ⅰ/Ⅱ[9,10] (Predicted IC50 < 200), and the Emini Surface Accessibility Prediction was used to predict the potential B cell epitope[11].

RESULTS

Serological markers and HBV DNA quantification

Both the mother and son were positive for HBsAg, HBeAg and anti-HBc. They were seronegative for HAV, HCV and HEV. The HBV viral load was 2.4×10^8 copy/mL in the mother (6022) and 4.2×10^8 copy/mL in the son [602(S)]. The 602 sera were not quantified due to insufficient material.

Genotypic and serotypic relatedness

The 27 sequenced clones (Gene Bank accession No: DQ377159-377165, EU306713- EU306729, EU439005-439007) ranged in length from 3036bp to 3254 bp. The divergence of the complete genome of 18 clones for the mother was 0%-0.8%, while the divergence of the 9 clones for the son was 0.1%-0.6 %, and the divergence of the 27 clones for the mother and child was 0%-0.8%. For the 27 clones, the homogenous of S gene was 98.5%-100%. The 27 clones were 51H, 54E, 62A, 73G, 125T and 227S in the large S gene, 136A, 142K, 233R, 236S, 248P, 252R, 304H, and 354H in the P gene, and 122K,160R and 159A in the small S gene. Compared with the 33 global complete genome sequences of HBV from GenBank, phylogenetic analysis showed that with bootstrap values of 100%, the clones were classified into genotype C and C2 subgroup (Figure 1)[12]. The serotype was classified as $adrq^+$[13].

Characterization of the nucleotides and the deduced amino acid sequence

Compared with the other 50 complete sequences of

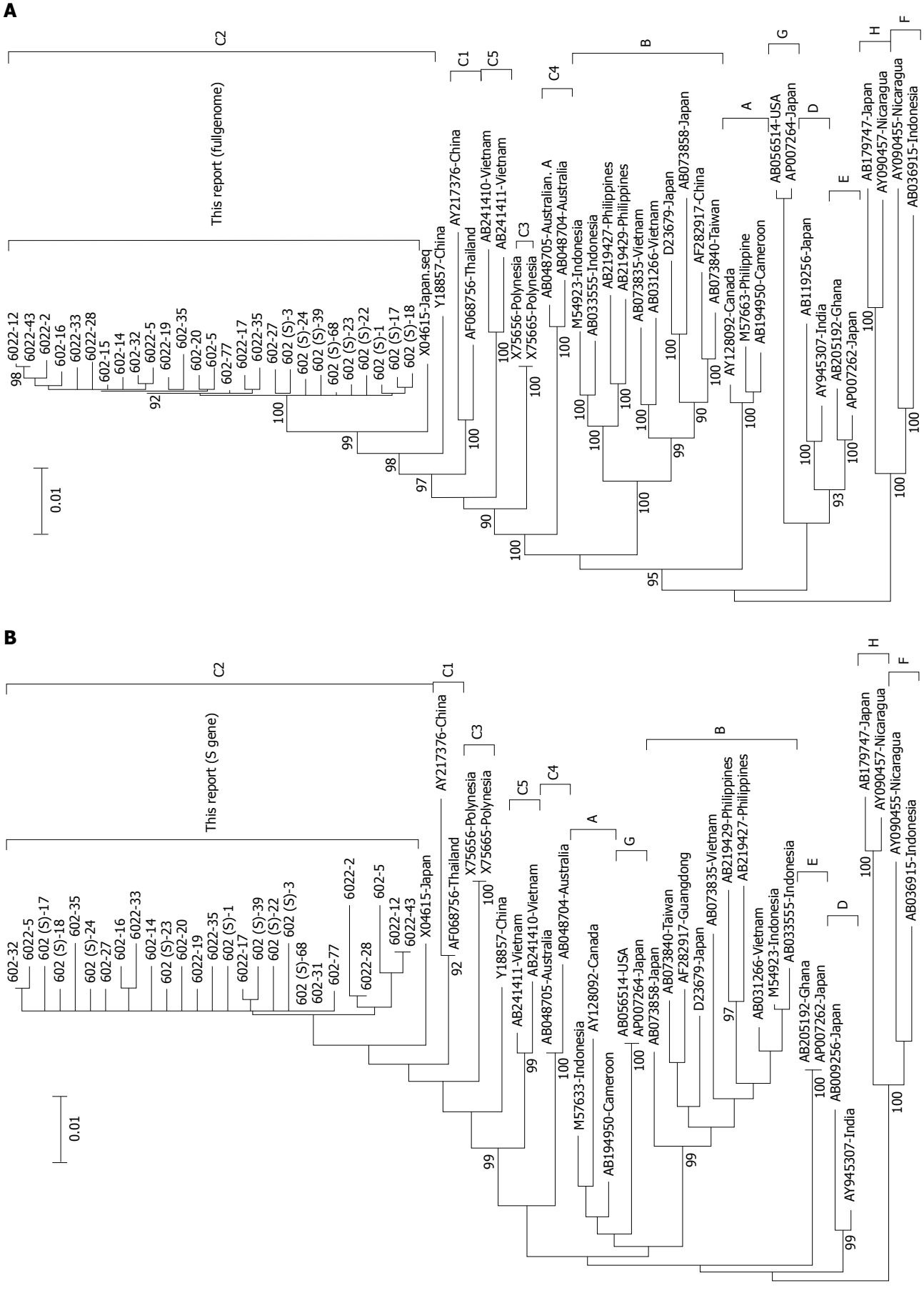


Figure 1 Phylogenetic tree constructed on the complete genome (**A**) and S gene (**B**) [Mother: 602 (1 d), 6022 (6 mo); Son: 602 (S)]. Bootstrap values are shown at the beginning of each main node. The length of the horizontal bar indicates the number of nucleotide substitutions per site. The origin of each strain has also been shown.

Table 1 MHC-I binding predictions of 144-183 residue of HbcAg, processed by the SMM method

Allele	Position (aa)	Pep length	Sequence	IC_{50} (nm)[1]
HLA A*3101	148-157	10	VVRRRGRSPR	30.9
HLA A*3101	150-159	10	RRRGRSPRRR	13.7
HLA A*3101	157-166	10	RRRTPSPRRR	19.2
HLA A*3101	159-167	9	RTPSPRRRR	38.4
HLA A*3101	165-174	10	RRRSQSPRRR	19.2
HLA A*3101	166-175	10	RRSQSPRRRR	35.4
HLA A*3101	167-175	9	RSQSPRRRR	13.7
HLA A*3201	159-167	9	RTPSPRRRR	48.6
HLA A*6801	145-154	9	ETTVVRRRGR	23
HLA A*6801	146-154	9	TTVVRRRGR	22.3
HLA B*0702	162-170	9	SPRRRRSQS	21.3
HLA B*0702	170-178	9	SPRRRRSQS	21.3
HLA B*0702	155-163	9	SPRRRTPSP	40.4
HLA B*0801	162-170	9	SPRRRRSQS	39.5
HLA B*0801	170-178	9	SPRRRRSQS	39.5
HLA B*2705	150-159	10	RRRGRSPRRR	49.5
HLA B*2705	164-173	10	RRRRSQSPRR	14.7
HLA B*2705	166-175	10	RRSQSPRRRR	33.9

[1]IC_{50} (nM) < 50 was considered as high affinity. Only IC_{50} (nM) has been shown in the Table.

genotype C, there were 13 specific point mutations in the 27 clones, the nucleotide positions were at 105T, 346C, 855C, 861T, 930G, 951T, 1110T, 1341C, 1972C, 2215C, 2246C, 2480T and 3102A. Except for C105T and G3102A which caused mutation of amino acid A158V in the large S gene and D266N in the P gene, respectively, the other substitutions resulted in the same sense mutation. Within the pre-S1 region, 14 clones of the mother had 18bp deletion from the start codon, which resulted in deletion at codons 1-11. The C-terminal truncated S protein was found in both the mother and son, which caused random point mutation in or near the hydrophilic region of S gene (nt 200, nt361, nt455, and nt565, respectively). Within the X region, 1762T/1764A double mutation was observed in all clones in the mother, but none was found in the son. The hypervariable region (HVR) sequence at positions 1751 to 1755, and the AT-rich region sequence at 1789 to 1795 in the X-ORF was conserved in all of the clones. In the precore and core regions, 3 clones of the mother had G1896A mutation and one clone had 19bp deletion, resulting in a novel stop codon of the C gene. In the P region, the YMDD motif was conserved in all the clones, 5 clones of the son had a 17 bp deletion from nt2330, and produced a 43 aa residue in the N-terminal of polymerase (Figure 2). Other random mutations occurred throughout the sequenced genomes, like a "G" insertion at nt 761 [602 (S)-39] was observed in one clone of the son and a "TT" deletion at nt2012 in one clone of the mother.

Potential immune epitopes caused by 17 bp deletion from nt2330

The 17bp deletion mutant from nt2330 in 5 clones of the son led to the deletion of aa144-183 of the HBcAg, as reported in this study after performing Blastn. By the immune epitope prediction method, potential clustal immune epitopes were located in the deletion region of Core gene, including 7 HLA class I epitopes and one B cell epitope (PRRRRSQ; Table 1, Figures 3 and 4).

DISCUSSION

Using phylogenetic and mutational analysis, clones of the mother obtained at two time points were conserved. The dominant mutants in the mother obtained at two time points were the same (18 bp deletion in preS1 region and BCP double mutation), except for the scattering mutation. Although, the son was infected from the mother, the divergence of the mother and the son was 0%-0.8%, and the dominant mutants in the son were different from the mother.

Universal infant immunization has reduced HBV infection rate in highly endemic countries with a relatively homogenous population. However, HBV transmission continues to occur *via* vertical (mother-to-child) and horizontal (sexual, parenteral and household) routes. In Asia, where genotypes B and C predominate, vertical transmission is common. In the present study, we isolated HBV strains from an asymptomatic mother and her son and analyzed the sequences phylogenetically. All the 27 clones were of the C2 subtype and adrq$^+$ serotype. Compared with 50 other complete sequences of genotype C, there were 13 specific nucleotides belonging to the mother and her son. It should be noted that there was no history of alcohol abuse, parenteral drug use or hepatotoxin exposure, and no immunoprophylaxis or immunotherapy was given to the mother and her son. The son's father was seronegative for HBV markers. Based on these findings combined with the data of divergence in the complete viral genome and homogeneity in the S gene, it can be concluded that the son became infected *via* a mother-to-infant transmission.

The pre-S1 domain is the essential binding site for hepatocyte receptors, and mutations at this region may directly influence HBV infection, and progression of liver disease. In the present study, 14 clones of the mother had aa 1-11 deletion in preS1 caused by a 18bp deletion from the original start codon of preS1. This deletion mutant has previously been reported in different clinical conditions, especially hepatocellular carcinoma (HCC) and chronic hepatitis B (CHB) infection with genotypes C and D, and fulminant hepatitis B (FHB) with genotype A in Africa. It has also been observed in isolates obtained from nonhuman primates (Table 2)[14-32]. Interestingly, there are no reports of this deletion mutation in genotypes B, E, F and G. In a heart transplant recipient who died from fulminate hepatitis B transmitted by the donor, the 18bp deletion was detected in the recipient, but not in the donor[20]. In our case, the 18bp deletion mutants in preS1 were present in 14 clones of the mother (78%), but in none of the sequenced clones in the son (0%). This phenomenon may imply that host immune pressure was the primary cause of aa 1-11 deletion in preS1. In a subsequent study (2 years later), we observed that the

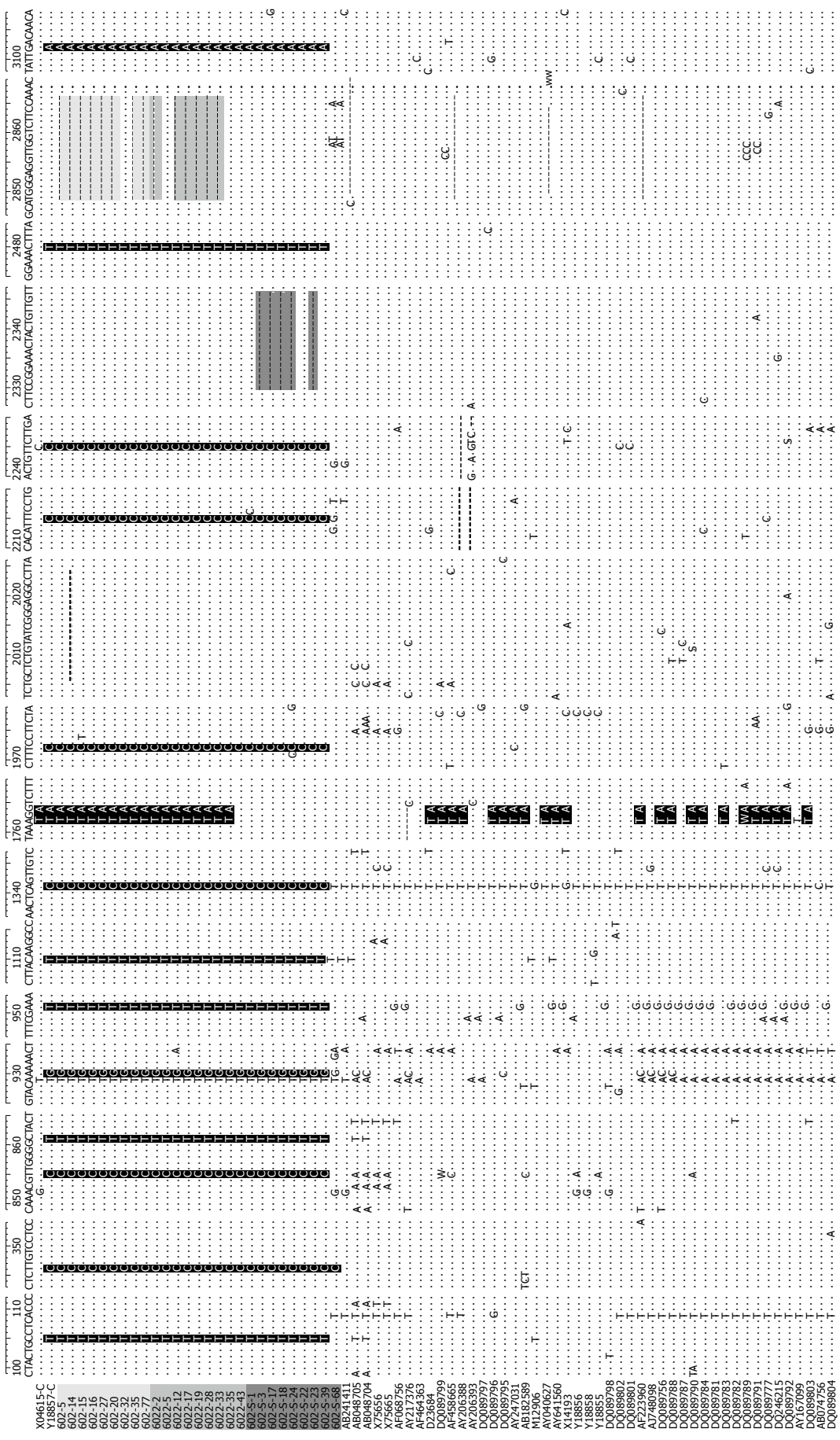


Figure 2 Nucleotide sequences of the 27 HBV/C isolates and 50 other complete genome sequences of genotype C [light gray, Mother: 602 (1 d); Gray, 6022 (6 mo); Dark gray, Son: 602-S; Sequences: Gray (deletion), black (substitution of nucleotides)]. Compared with the 50 complete genome sequences of genotype C, nucleotide positions at 105T, 346C, 855C,861T, 930G, 951T, 1110T, 1341C, 1972C, 2215C, 2246C,2480T and 3102A were specific for the 27 samples. 14 clones of the mother had an 18bp deletion from start codon; 5 clones of the son had a 17nt deletion from nt2330; 1762T/1764A double mutations were found in all clones of the mother, but none in the sequenced clones of the son. Nucleotides A1762A and G1764A double mutation comprised 54.5% of the total genotype C (42/77).

18bp deletion in preS1 had a tendency for substitution by large fragment deletions in preS1, and laboratory tests in the mother showed abnormal values (ALT > 2 times of reference values) (detailed discussion reported in a

Table 2 Deletion of aa 1-11 in preS1 in the different HBV genotypes

Genotype	GenBank No.	Clinic status	Countries/district	Ref.
Human				
Genotype C	AF223959	CHB	Vietnam	Erik *et al*, 2001
	AY217372	CHB	China	Luo *et al*, 2004
	AB195945	CHB	Japan	Horiike *et al*, 2007
	EF533714	HCC	China	[1]Gao *et al*, 2007
	AY206389	HCC	China	[1]Lin *et al*, 2002
	AB014395	HCC	Japan	Takahashik *et al*, 1998
	AY641563	HCC	South Korea	Song *et al*, 2005
	D1666	AHB	Japan	Uchida *et al*, 1995
	X98076	FHB	Switzerland	Pult *et al*, 1997
Genotype D	AB033559	CHB	Papua New Guinea	Okamoto *et al*, 1988
	AB119256	CHB	Japan	Michitaka *et al*, 2006
	M32138	Anti-HBe	France	Tong *et al*, 1990
	AY902777	FHB	Montana	Garfein *et al*, 2004
Genotype A	AF297621	FHB	Africa	Owiredu *et al*, 2001
	U87743	AHB	South Africa	Bowyer *et al*, 1997
	X69458	AHB	Zimbabwe	Chirara *et al*, 1994
Ape				
Gibbon	AB037928		East Asia	Aiba *et al*, 2003
Chimpanzee	D00220	HBsAg (+)	London	Vaudin *et al*, 1988
Gorila	AJ131567	HBsAg (+)	Japan	Grethe *et al*, 2000
Orangutan	AF193864	CHB	Bornean	Verschoor *et al*, 2001
Recombination				
Human-Gibbon	AB048705	HBsAg (+)	Australian Aborigines	Sugauchi *et al*, 2001

CHB: Chronic hepatitis B; AHB: Acute hepatitis B; FHB: Fulminant hepatitis B. [1]Sequences were submitted directly to the GenBank.

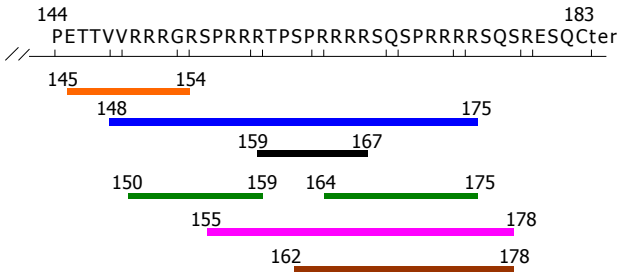


Figure 3 Potential HLA class Ⅰ epitopes of the deletion fragment in HBcAg (aa residue144-183), determined by the SMM method [only IC_{50} (nM) < 50]. Blue line: HLA A*3101 (aa 148-175); Black line: HLA A*3201(aa 159-167); Orange line: HLA A*6801 (aa 145-154); Pink line: HLA B*0702 (aa 155-178); Brown line: HLA B*0801 (aa 162-178); Green line: HLA B*2705 (aa 150-159, 164-175).

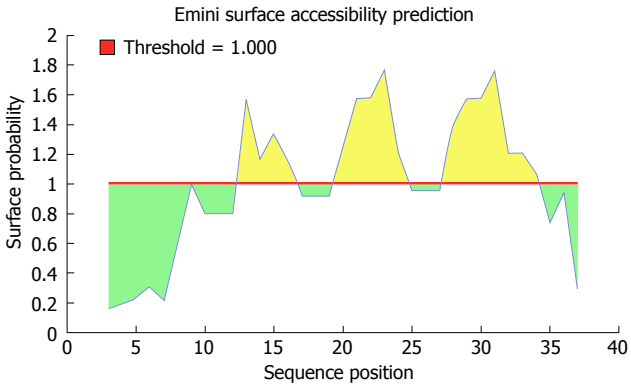


Figure 4 Predicted peptides of the potential B cell epitope of the deleted fragment in HBcAg (aa residue 144-183). A potential sequence of B cell epotide in aa residue 144-183 of HBcAg was PRRRRSQ, start position at aa 171, end position at aa 177.

separate publication). However, whether the deletion of aa 1-11 mutation in preS1 was a precursor of large fragment deletion mutation or was an isolated event under immune pressure remains to be determined. To our knowledge, no previous study has reported such deletion mutants. This deletion, which disrupts the preS1 start codon, may play an important role in enhancing the progression of chronic liver disease.

The 18 bp deletion mutants in preS1, located in the overlapping region of S/P gene region, caused six amino acid deletions, from 183 to 187 of the HBV polymerase. In the present study, all of these deletion mutants coexisted with the wild-type full-length genome. Transfection experiments in human hepatocells (HuH-7) demonstrated that the polymerase gene function was not affected by the large pre-S1 deletions, and mutant viral genomes were capable of replication[33]. This finding may explain the above observation, although deletion mutation of pre S1 was the major mutation in the mother, and the viral load in the serum remained high.

Cloning and sequence analysis showed that 3 clones of the mother and one clone of the son had premature S protein, caused by random points mutation. These mutations could affect not only the spatial structure, but also the immune reactivity. Some reports have found that C-terminal truncated S proteins exist in patients with chronic hepatitis B infection and hepatocellular carcinoma[34]. The shorted S proteins can stimulate gene expression from the endogenous HBV enhancer I and can also deregulate the expression of oncogenes such as c-myc[35], which may help clarify the tumor development mechanism of HBV. Whether the truncated S proteins

transactivate the oncogenes and/or worsen the clinical status of the subjects remains to be established.

The double mutation of nucleotide A1762T/G1764A in basal core promoter (BCP), and G1986A in the precore region are frequently observed in HBV sequences isolated from patients with chronic HBV infection, fulminant hepatitis, HCC, and in reactivation of HBV with a fulminant course[36-38], which results in mutations at two codons in the carboxyl functional region of X protein (K130M and V131I), and a stop codon at aa 28 of HBeAg, respectively. At present, there are conflicting opinions regarding 1762T/1764A hotspot mutations. Some studies suggest that these mutations decrease HBeAg expression and slightly increase viral DNA replication, and are mostly found in patients who seroconvert to anti-HBe[39,40]. By contrast, other studies indicate that this mutation is not associated with HBeAg/anti-HBe status or HBV DNA[41,42]. The lack of influence of BCP mutation on HBeAg expression was mainly indicated by its higher prevalence in genotype C than genotype B patients. In the present study, all clones of the mother from two time points had these double mutations (Figure 2); genotype C has a tendency for higher prevalence of 1762T/1764A double mutation (42 *vs* 35, 54.5%). Based on our data from two time points, we did not observe any HBeAg/anti-HBeAg seroconversion, and we did not detect the impact of BCP mutation on HBV DNA load, due to insufficient quantity of 602 sera. Besides, the 1762T/1764A double mutation was present in all clones of the mother, 3 of whom were also coupled with G1896A. The G1896A mutation is supposed to increase the stability of the stem-loop structure. Recent reports suggest that HBV subgenotypes Ba, C1, and C2 have an intermediate frequency of 1896A mutation[43]. Interestingly, although the son was infected *via* the mother, none of the BCP and 1896A mutants were found in the son (0/9). This finding is consistent with previous studies in which mutants with 1896A were seldom transmitted to the infant *via* the mother[44]. Whether the BCP and precore mutations in the mother were related to infection with genotype C, or whether she was seroconverting to anti-HBeAg, or may develop severe disease exacerbations remains to be determined.

A heterogeneous population of core antigen internal deletions (CID) has been found to be highly prevalent in chronic HBV carriers[45,46], HCC patients[47] and immune-suppressed patients[48]. Normally, CID coincides with a potent T/B-cell epitope, and is almost always found in the presence of HBV with an apparent full-length core gene[49-51]. HBcAg has been shown to be a major target of T-cell immunity[52]. However, deletion type HBc did not show any antibody response[53].

In the present study, the wild type precore/core protein and deletion type precore/core protein co-existed in the sera of both the mother and son. Out of the 27 clones, one clone in the mother had 19nt deletion at position nt2006, and 5 clones in the son had 17bp deletion at position nt2330, which caused in-frame shift and resulted in a C-terminal truncated preCore/Core protein. To the best of our knowledge, this is the first report on 17bp deletion mutant, resulting in immune epitope deletion based on the immune epitope prediction tools. Interestingly, none of these mutants were found in the mother. Therefore, we concluded that under host immune pressure, due to the loss of HLA class and B cell I epitopes, this deletion variant may evade the immune system and became dominant in the host.

The 17 bp deletion mutation occurred in the overlapping region of P/C gene, and produced 43 aa peptides of HBV polymerase, possibly resulting in a new start codon, downstream of the original one. P-ORF encodes the terminal protein (TP), polymerase (pol) and RNase H. Previous studies have identified that in a protein-primed reaction, the minimal domain of TP ranged from amino acid 20 to 175[54]. In the present report, these 5 clones had incomplete TP, based on the minimal function domain of TP. These mutants have little or no activity. Interestingly, the viral load in the son was high (4.2×10^8 copy/mL), and no similar deletion mutants were found in his mother. Whether this phenomenon coexisted with the wild-type genome, or was the function domain of TP remains to be examined.

In addition, there were other point mutations and deletion/insertion mutations scattered in the complete genome, many of these were located in the T and B lymphocytes epitope, such as P46L, C69R and F73L in X gene[55], and P54T and M132T in C gene[56], while others resulted in truncated surface or preCore/Core proteins (T361A, TT deletion at nt2012, *etc.*). Because these mutants were dissimilar in different clones, they may have been caused by spontaneous mutation or immune pressure during evolution.

In summary, the HBV strain in the son was transmitted *via* maternal-fetal transmission, based on the presence of 13 specific and common nucleotides in both the mother and son. The dominant HBV mutants were different in the mother and son, because of different immune-environments. These mutants were capable of escaping immune-pressure and were present in HBV carriers. These newly discovered variants were located within or close to the functional domains of viral proteins. Whether they can exist "silently" in the hosts, or will cause liver disease remains to be determined.

COMMENTS

Background

Mother-to-infant transmission is the main pathway of hepatitis B virus (HBV) infection. Approximately 90% infants of HBeAg positive HBsAg carrier mothers become carriers, if no immunoprophylaxis is given. HBV with the same characteristics in the child and the mother interacts with the immune system, resulting in the development of different mutations designed to escape the immune pressure.

Research frontiers

Biosoftwares are powerful tools designed to perform virologic and phylogenetic analysis, and to predict immune epitopes. Combining this knowledge with clinical data on HBV-infected patients, may provide new information on the prevention and treatment of HBV infection.

Innovations and breakthroughs

The results of our study suggest that the preS1 deletion of HBV may be

associated with disease progression. Using immune epitope prediction tools, the immune epitope deletion was found to occur in the C gene region.

Applications

Data obtained by virologic and phylogenetic analysis may provide clues to the prevention and treatment of chronic HBV infection.

Peer review

Virologic and phylogenetic analysis provides evidence of mother to infant transmission, and indicates that HBV with the same characteristics has dissimilar mutations in different hosts. Follow-up studies should greatly enhance the significance of the present report.

REFERENCES

1 **Gunther S**, Sommer G, Von Breunig F, Iwanska A, Kalinina T, Sterneck M, Will H. Amplification of full-length hepatitis B virus genomes from samples from patients with low levels of viremia: frequency and functional consequences of PCR-introduced mutations. *J Clin Microbiol* 1998; **36**: 531-538
2 **Schaefer S**. Hepatitis B virus taxonomy and hepatitis B virus genotypes. *World J Gastroenterol* 2007; **13**: 14-21
3 **BioEdit**. Available from URL: http://www.mbio.ncsu.edu/BioEdit/BioEdit.html
4 **Thompson JD**, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 1997; **25**: 4876-4882
5 **Kumar S**, Tamura K, Jakobsen IB, Nei M. MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* 2001; **17**: 1244-1245
6 **Kimura M**. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 1980; **16**: 111-120
7 **Saitou N**, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987; **4**: 406-425
8 **Felsenstein J**. Estimating effective population size from samples of sequences: a bootstrap Monte Carlo integration method. *Genet Res* 1992; **60**: 209-220
9 **Nielsen M**, Lundegaard C, Lund O. Prediction of MHC class II binding affinity using SMM-align, a novel stabilization matrix alignment method. *BMC Bioinformatics* 2007; **8**: 238
10 **IEDB Analysis Resource**. Available from URL: http://tools.immuneepitope.org/main/jsp/menu.jsp
11 **Emini EA**, Hughes JV, Perlow DS, Boger J. Induction of hepatitis A virus-neutralizing antibody by a virus-specific synthetic peptide. *J Virol* 1985; **55**: 836-839
12 **Huy TT**, Ushijima H, Quang VX, Win KM, Luengrojanakul P, Kikuchi K, Sata T, Abe K. Genotype C of hepatitis B virus can be classified into at least two subgroups. *J Gen Virol* 2004; **85**: 283-292
13 **Margeridon S**, Lachaux A, Trepo C, Zoulim F, Kay A. A quasi-monoclonal anti-HBs response can lead to immune escape of 'wild-type' hepatitis B virus. *J Gen Virol* 2005; **86**: 1687-1693
14 **Alestig E**, Hannoun C, Horal P, Lindh M. Phylogenetic origin of hepatitis B virus strains with precore C-1858 variant. *J Clin Microbiol* 2001; **39**: 3200-3203
15 **Luo K**, Liu Z, He H, Peng J, Liang W, Dai W, Hou J. The putative recombination of hepatitis B virus genotype B with pre-C/C region of genotype C. *Virus Genes* 2004; **29**: 31-41
16 **Horiike N**, Duong TN, Michitaka K, Joko K, Hiasa Y, Konishi I, Yano M, Onji M. Characteristics of lamivudine-resistant hepatitis B virus (HBV) strains with and without breakthrough hepatitis in patients with chronic hepatitis B evaluated by serial HBV full-genome sequences. *J Med Virol* 2007; **79**: 911-918
17 **Takahashi K**, Akahane Y, Hino K, Ohta Y, Mishiro S. Hepatitis B virus genomic sequence in the circulation of hepatocellular carcinoma patients: comparative analysis of 40 full-length isolates. *Arch Virol* 1998; **143**: 2313-2326
18 **Song BC**, Kim H, Kim SH, Cha CY, Kook YH, Kim BJ. Comparison of full length sequences of hepatitis B virus isolates in hepatocellular carcinoma patients and asymptomatic carriers of Korea. *J Med Virol* 2005; **75**: 13-19
19 **Uchida T**, Gotoh K, Shikata T. Complete nucleotide sequences and the characteristics of two hepatitis B virus mutants causing serologically negative acute or chronic hepatitis B. *J Med Virol* 1995; **45**: 247-252
20 **Pult I**, Chouard T, Wieland S, Klemenz R, Yaniv M, Blum HE. A hepatitis B virus mutant with a new hepatocyte nuclear factor 1 binding site emerging in transplant-transmitted fulminant hepatitis B. *Hepatology* 1997; **25**: 1507-1515
21 **Okamoto H**, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, Mayumi M. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* 1988; **69** (Pt 10): 2575-2583
22 **Michitaka K**, Tanaka Y, Horiike N, Duong TN, Chen Y, Matsuura K, Hiasa Y, Mizokami M, Onji M. Tracing the history of hepatitis B virus genotype D in western Japan. *J Med Virol* 2006; **78**: 44-52
23 **Tong SP**, Li JS, Vitvitski L, Trepo C. Active hepatitis B virus replication in the presence of anti-HBe is associated with viral variants containing an inactive pre-C region. *Virology* 1990; **176**: 596-603
24 **Garfein RS**, Bower WA, Loney CM, Hutin YJ, Xia GL, Jawanda J, Groom AV, Nainan OV, Murphy JS, Bell BP. Factors associated with fulminant liver failure during an outbreak among injection drug users with acute hepatitis B. *Hepatology* 2004; **40**: 865-873
25 **Owiredu WK**, Kramvis A, Kew MC. Molecular analysis of hepatitis B virus genomes isolated from black African patients with fulminant hepatitis B. *J Med Virol* 2001; **65**: 485-492
26 **Bowyer SM**, van Staden L, Kew MC, Sim JG. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. *J Gen Virol* 1997; **78** (Pt 7): 1719-1729
27 **Chirara MM**, Chetsanga CJ. Variant of hepatitis B virus isolated in Zimbabwe. *J Med Virol* 1994; **42**: 73-78
28 **Aiba N**, Nishimura H, Arakawa Y, Abe K. Complete nucleotide sequence and phylogenetic analyses of hepatitis B virus isolated from two pileated gibbons. *Virus Genes* 2003; **27**: 219-226
29 **Vaudin M**, Wolstenholme AJ, Tsiquaye KN, Zuckerman AJ, Harrison TJ. The complete nucleotide sequence of the genome of a hepatitis B virus isolated from a naturally infected chimpanzee. *J Gen Virol* 1988; **69** (Pt 6): 1383-1389
30 **Grethe S**, Heckel JO, Rietschel W, Hufert FT. Molecular epidemiology of hepatitis B virus variants in nonhuman primates. *J Virol* 2000; **74**: 5377-5381
31 **Verschoor EJ**, Warren KS, Langenhuijzen S, Heriyanto, Swan RA, Heeney JL. Analysis of two genomic variants of orang-utan hepadnavirus and their relationship to other primate hepatitis B-like viruses. *J Gen Virol* 2001; **82**: 893-897
32 **Sugauchi F**, Mizokami M, Orito E, Ohno T, Kato H, Suzuki S, Kimura Y, Ueda R, Butterworth LA, Cooksley WG. A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. *J Gen Virol* 2001; **82**: 883-892
33 **Melegari M**, Bruno S, Wands JR. Properties of hepatitis B virus pre-S1 deletion mutants. *Virology* 1994; **199**: 292-300
34 **Dong J**, Cheng J, Wang QH, Wang G, Shi SS, Liu Y, Xia XB, Si CW. Cloning and sequence analysis of truncated S gene from circulation of patients with chronic hepatitis B virus infection. *Zhonghua Ganzangbing Zazhi* 2001; **9**: 163-165
35 **Banerjee A**, Datta S, Chandra PK, Roychowdhury S, Panda CK, Chakravarty R. Distribution of hepatitis B virus genotypes: phylogenetic analysis and virological characteristics of genotype C circulating among HBV carriers in Kolkata, Eastern India. *World J Gastroenterol* 2006; **12**: 5964-5971

36 **Chauhan R**, Kazim SN, Bhattacharjee J, Sakhuja P, Sarin SK. Basal core promoter, precore region mutations of HBV and their association with e antigen, genotype, and severity of liver disease in patients with chronic hepatitis B in India. *J Med Virol* 2006; **78**: 1047-1054

37 **Dai MS**, Lu JJ, Chen YC, Perng CL, Chao TY. Reactivation of precore mutant hepatitis B virus in chemotherapy-treated patients. *Cancer* 2001; **92**: 2927-2932

38 **Huang YH**, Wu JC, Chang TT, Sheen IJ, Huo TI, Lee PC, Su CW, Lee SD. Association of core promoter/precore mutations and viral load in e antigen-negative chronic hepatitis B patients. *J Viral Hepat* 2006; **13**: 336-342

39 **Lin CL**, Liao LY, Liu CJ, Chen PJ, Lai MY, Kao JH, Chen DS. Hepatitis B genotypes and precore/basal core promoter mutants in HBeAg-negative chronic hepatitis B. *J Gastroenterol* 2002; **37**: 283-287

40 **Preikschat P**, Gunther S, Reinhold S, Will H, Budde K, Neumayer HH, Kruger DH, Meisel H. Complex HBV populations with mutations in core promoter, C gene, and pre-S region are associated with development of cirrhosis in long-term renal transplant recipients. *Hepatology* 2002; **35**: 466-477

41 **Yoo BC**, Park JW, Kim HJ, Lee DH, Cha YJ, Park SM. Precore and core promoter mutations of hepatitis B virus and hepatitis B e antigen-negative chronic hepatitis B in Korea. *J Hepatol* 2003; **38**: 98-103

42 **Orito E**, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, Okanoue T, Yotsuyanagi H, Iino S. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. *Hepatology* 2001; **33**: 218-223

43 **Wang Z**, Tanaka Y, Huang Y, Kurbanov F, Chen J, Zeng G, Zhou B, Mizokami M, Hou J. Clinical and virological characteristics of hepatitis B virus subgenotypes Ba, C1, and C2 in China. *J Clin Microbiol* 2007; **45**: 1491-1496

44 **Candotti D**, Danso K, Allain JP. Maternofetal transmission of hepatitis B virus genotype E in Ghana, west Africa. *J Gen Virol* 2007; **88**: 2686-2695

45 **Okamoto H**, Tsuda F, Mayumi M. Defective mutants of hepatitis B virus in the circulation of symptom-free carriers. *Jpn J Exp Med* 1987; **57**: 217-221

46 **Melegari M**, Scaglioni PP, Wands JR. The small envelope protein is required for secretion of a naturally occurring hepatitis B virus mutant with pre-S1 deleted. *J Virol* 1997; **71**: 5449-5454

47 **Hosono S**, Tai PC, Wang W, Ambrose M, Hwang DG, Yuan TT, Peng BH, Yang CS, Lee CS, Shih C. Core antigen mutations of human hepatitis B virus in hepatomas accumulate in MHC class II-restricted T cell epitopes. *Virology* 1995; **212**: 151-162

48 **Gunther S**, Li BC, Miska S, Kruger DH, Meisel H, Will H. A novel method for efficient amplification of whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutants in immunosuppressed patients. *J Virol* 1995; **69**: 5437-5444

49 **Yuan TT**, Lin MH, Chen DS, Shih C. A defective interference-like phenomenon of human hepatitis B virus in chronic carriers. *J Virol* 1998; **72**: 578-584

50 **Yuan TT**, Lin MH, Qiu SM, Shih C. Functional characterization of naturally occurring variants of human hepatitis B virus containing the core internal deletion mutation. *J Virol* 1998; **72**: 2168-2176

51 **Milich DR**, McLachlan A, Moriarty A, Thornton GB. Immune response to hepatitis B virus core antigen (HBcAg): localization of T cell recognition sites within HBcAg/HBeAg. *J Immunol* 1987; **139**: 1223-1231

52 **Lee YS**, Yoon SJ, Kwon TK, Kim YH, Woo JH, Suh MH, Suh SI, Baek WK, Kim HJ, Ahn SY, Choe BK, Park JW. Immune response induced by immunization with Hepatitis B virus core DNA isolated from chronic active hepatitis patients. *Immunol Lett* 2001; **78**: 13-20

53 **Peters B**, Sette A. Generating quantitative models describing the sequence specificity of biological processes with the stabilized matrix method. *BMC Bioinformatics* 2005; **6**: 132

54 **Lanford RE**, Kim YH, Lee H, Notvall L, Beames B. Mapping of the hepatitis B virus reverse transcriptase TP and RT domains by transcomplementation for nucleotide priming and by protein-protein interaction. *J Virol* 1999; **73**: 1885-1893

55 **Stemler M**, Weimer T, Tu ZX, Wan DF, Levrero M, Jung C, Pape GR, Will H. Mapping of B-cell epitopes of the human hepatitis B virus X protein. *J Virol* 1990; **64**: 2802-2809

56 **Heathcote J**, McHutchison J, Lee S, Tong M, Benner K, Minuk G, Wright T, Fikes J, Livingston B, Sette A, Chestnut R. A pilot study of the CY-1899 T-cell vaccine in subjects chronically infected with hepatitis B virus. The CY1899 T Cell Vaccine Study Group. *Hepatology* 1999; **30**: 531-536

S- Editor Zhong XY **L- Editor** Anand BS **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5683

World J Gastroenterol 2008 October 7; 14(37): 5683-5688
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Protective effect of ilomastat on trinitrobenzenesulfonic acid-induced ulcerative colitis in rats

Ying-De Wang, Wei Wang

Ying-De Wang, Department of Gastroenterology, the First Affiliated Hospital of Dalian Medical University, Dalian 116011, Liaoning Province, China
Wei Wang, Department of Gastroenterology, the Affiliated Zhongshan Hospital of Dalian University, Dalian 116023, Liaoning Province, China
Author contributions: Wang YD designed the research; Wang W performed the research; Wang YD and Wang W analyzed the data; Wang YD wrote the paper.
Correspondence to: Dr. Ying-De Wang, Department of Gastroenterology, the First Affiliated Hospital of Dalian Medical University, Dalian 116011, Liaoning Province, China. albertwyd@yahoo.com.cn
Telephone: +86-411-83635963-3162 Fax: +86-411-83632844
Received: April 9, 2008 Revised: August 8, 2008
Accepted: August 15, 2008
Published online: October 7, 2008

Abstract

AIM: To evaluate the protective effects of ilomastat, an exogenous matrix metalloproteinase (MMP) inhibitor, on trinitrobenzenesulfonic acid (TNB)-induced ulcerative colitis (UC) in rats.

METHODS: Male SD rats were randomly divided into model group, protective groups A and B, and normal control group. Rats in the model group received only intra-colonic TNB. Rats in the protective groups A and B received intra-peritoneal ilomastat of 10 mg/kg and 20 mg/kg, respectively, beside TNB. Rats in the normal control group received only intra-colonic normal saline. After 3 wk, segments of colon were obtained**.** RT-PCR and immunohistochemistry were used to examine the expression of MMP-1 and TIMP-1. Hematoxylin-eosin (HE) staining was used for pathological study.

RESULTS: The model of UC was successfully induced in rats. Inflammation of colonic mucosa greatly improved in protective groups A and B. Expression of MMP-1 and TIMP-1 in the model group, protective groups A and B was significantly higher than that in the normal control group ($P < 0.0001$) with MMP-1 expression increased more significantly than TIMP-1 expression. Expression of MMP-1 in protective groups A and B was significantly lower than that in the model group ($P < 0.0001$) . Expression of MMP-1 in protective group B was significantly lower than that in protective group A ($P < 0.0001$).

CONCLUSION: Ilomastat improves TNB-induced UC in rats by inhibiting the MMP-1 activity.

Key words: Ulcerative colitis; Trinitrobenzenesulfonic acid; Matrix metalloproteinase-1; Tissue inhibitors of matrix metalloproteinase-1; Ilomastat

Peer reviewer: Alastair John Watson, Professor, The Henry Wellcome Laboratory, Nuffield Building, University of Liverpool, Crown St., Liverpool, L69 3GE, United Kingdom

Wang YD, Wang W. Protective effect of ilomastat on trinitrobenzenesulfonic acid-induced ulcerative colitis in rats. *World J Gastroenterol* 2008; 14(37): 5683-5688 Available from: URL: http://www.wjgnet.com/1007-9327/14/5683.asp DOI: http://dx.doi.org/10.3748/wjg.14.5683

INTRODUCTION

Ulcerative colitis (UC) is a chronic nonspecific inflammatory disease of the colon characterized clinically by abdominal pain, diarrhea and mucus-pus stool. It is believed that factors such as heredity, immune disturbance, environment, and inflammatory cytokines, *etc.* play an important role in the development of UC[1]. Pathologically, UC invades mainly mucosa and sub-mucosa of colon with excessive degradation of extracellular matrix (ECM) by matrix metalloproteinases (MMPs) secreted by interstitial cells and activated by various cytokines[2-4]. MMPs possess many important physiological and pathophysiological functions in the gastrointestinal tract, such as implantation of T cells in mucosa, regeneration of epithelium, aggregation of lymphocytes and production of cytokines in the process of inflammation[5,6]. MMPs are regulated by their specific tissue inhibitors at transcription, pre-enzyme activation, ECM, and inhibition levels[7]. Tissue inhibitors of matrix metalloproteinases (TIMPs) are natural inhibitors of MMPs. TIMP-1, extensively present in the human body, mainly inhibits MMP-1. Mckaig BC *et al*[8] found that expression of MMP-1 is significantly higher while expression of TIMP-1 is relatively lower in colonic tissues. Therefore, it is quite possible that exogenous MMP inhibitors exert their inhibitory effects on expression of MMPs and subsequently improve

colonic tissue damages. Disebatiano *et al*[9] utilized batimastat (BB-94), an artificial MMPI, in a rat model of UC to study its protective effect on colonic tissue and found that batimastat is water insoluble with a low bio-availability. Ilomastat is thought to be the most powerful MMPI with a promising future and has been used in trauma healing, tumor inhibition and cornea repair[10-12]. However, no study on its protective effect on UC is available. Therefore, in this study, we used ilomastat to study its protective effect on trinitrobenzenesulfonic acid (TNB)-induced UC in rats.

MATERIALS AND METHODS

Establishment of animal model

Thirty two male Sprague-Dawley (SD) rats, weighing 180-220 g, were used to establish a Morris model of UC. Briefly, the animals were fasted for 48 h before experiment. A 2 mm catheter was inserted into the anus of rats. One hundred mg/kg TNB dissolved in 0.25 mL of 50% alcohol was injected into the colon through the catheter. The animals were inverted for about 3-5 min to prevent backflow of the solution.

Animal grouping

The rats were divided randomly into four groups: UC model group, protective groups A and B, and control group (n = 8). Rats in the model group were given only the mixed solution of TNB and alcohol, rats in protective groups A and B were intra-peritoneally given 10 mg/kg and 20 mg/kg of ilomastat, twice daily beside administration of TNB and alcohol, rats in the control group were given only normal saline. Ilomastat was administered 30 min before enema. All the animals were sacrificed 3 wk after enema.

Treatment of samples

An abdominal midline incision was made to open the abdomen and separate the colon. The colon was cut open to take the diseased colon samples. The samples were washed in 4℃ normal saline to remove stool residues. Then the colon samples were snap frozen in liquid nitrogen for isolation of RNA and RT-PCR, and fixed in 10% formalin and embedded in paraffin for HE staining and immunohistochemistry.

Pathological observation

Paraffin-embedded and formalin-fixed colon samples were cut into 4-μm thick sections for HE staining and pathological observation.

RT-PCR for MMP-1 and TIMP-1

RT-PCR for MMP-1 and TIMP-1 was performed using a TaKaRa RNA PCR kit 3.0 (AMV, supplied by Dalian Baosheng Biotechnology Company) following the manufacturer's instructions. Five μL of PCR product was run on 2% agarose gel electrophoresis. The sequences of primers[13,14] used are as followings: MMP-1 (639 bp): forward TTGTTGCTGCCCATGAGCTT and reverse ACTTTGTCGCCAATTCCAGG; TIMP-1 (495 bp): forward TTTGCATCTCTGGCCTCTG and reverse AATGACTGTCACTCTCCAG; β-actin (357 bp): forward TAAAGACCTCTATGCCAACAC and reverse TAAAGCCATGCCTAATGTCTC.

Immunohistochemistry

Briefly, sample sections were washed 3 times with PBS, 3 min each time after initial treatment. Primary antibodies, mouse anti-human MMP-1 and TIMP-1 monoclonal antibodies (Santa Cruz, USA) were added and incubated at room temperature for about 1.5 h, washed and incubated with peroxidase-conjugated secondary antibody for 15 min and washed. A brown product was developed in diaminobenzidine (DAB) for 10 min.

Result determination and statistical analysis

A bio-imaging system (PALL Company, USA) was employed to analyze the density of PCR product bands. MMP-1 and TIMP-1 mRNAs were semi-quantitatively expressed as the ratio of MMP-1, TIMP-1 and β-actin OD values. All values were expressed as mean ± SD.

Positive results of immunohistochemistry were determined by the appearance of brown substances in cells after DAB staining. An image-pro-plus 4.5 microscopic image analyzing system (Media Cybernetics Company, USA) was utilized to measure the density of brown substances. Five fields in each section were randomly chosen, and the total density and total area in the fields were measured. The mean density was calculated by the ratio of the total density and total area. A bigger ratio indicates a greater expression of the corresponding proteins.

Student-Neuman-Keuls test was used to compare the expressions of MMP-1 mRNA, TIMP-1 mRNA and their corresponding proteins. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS 11.5 for windows.

RESULTS

Pathological change under light microscope (HE staining)

As shown in Figure 1, the colonic mucosa structure in the normal control group was intact. Mucosa and sub-mucosa defects could be clearly seen with infiltrations of inflammatory neutrophils and lymphocytes in the lamina properia of the model group. Tissue necrosis was obvious. Lobed neutrophils were observed in protective group A and proliferation of new blood vessels was found in protective group B. Regenerated epithelia covered the surface of ulcer and the mucosa tended to be complete. Necrotic tissue was absorbed. The base of ulcer was filled with tissues and scars.

Results of RT-PCR for MMP-1 and TIMP-1

As shown in Table 1 and Figure 2, expression of MMP-1 in the model group, protective groups A and B was significantly higher than that in the normal control group

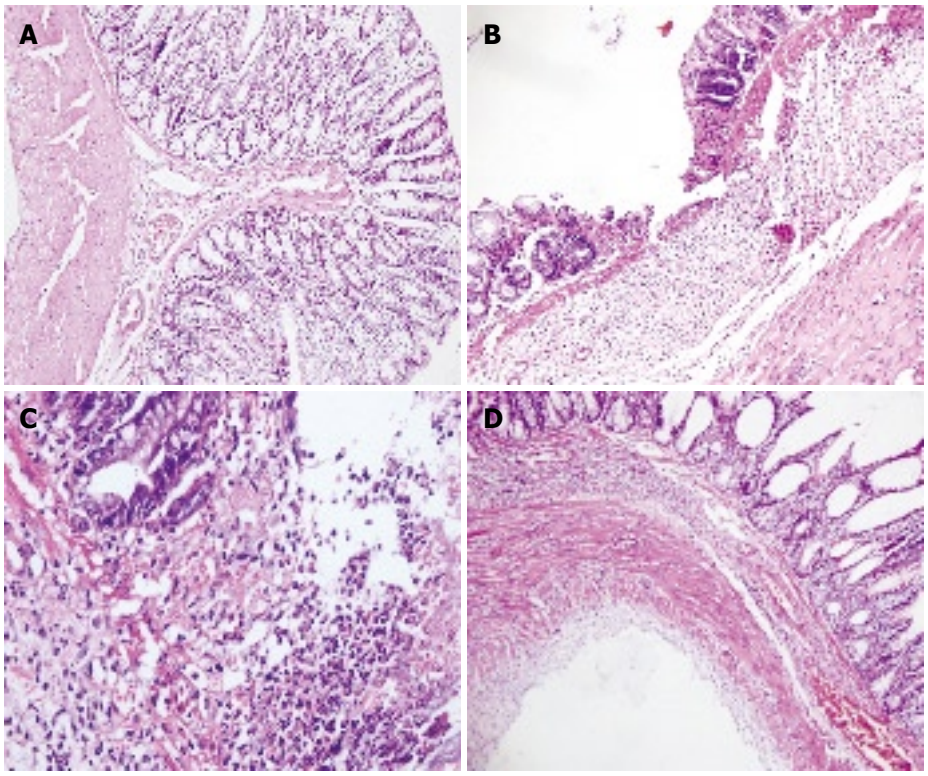


Figure 1 Pathological change under light microscopy (HE staining, × 400) in normal control group (**A**), model group (**B**), protective group A (**C**), and protective group B (**D**).

Table 1 Expression of MMP-1 and TIMP-1 transcripts in different groups of UC in rats (mean ± SD)

Groups	MMP-1	TIMP-1
UC Model group	0.9698 ± 0.0583[a,c,e]	0.8741 ± 0.0807[a]
Protective group A	0.6395 ± 0.0419[a,c]	0.7933 ± 0.0771[a]
Protective group B	0.1115 ± 0.0508[a]	0.7095 ± 0.0978
Normal controls	0.0109 ± 0.0048	0.4147 ± 0.0756

[a]$P < 0.05$ *vs* normal control group, [c]$P < 0.05$ *vs* protective group B, [e]$P < 0.05$ *vs* protective group A.

Table 2 Biosoftwares analysis of MMP-1 and TIMP-1 proteins in different groups of UC in rats (mean ± SD)

Groups	MMP-1	TIMP-1
UC model group	0.0877 ± 0.0088[a,c,e]	0.0841 ± 0.0081[a]
Protective group A	0.0779 ± 0.0059[a,c]	0.0794 ± 0.0058[a]
Protective group B	0.0158 ± 0.0034	0.0705 ± 0.0069
Normal controls	0.0041 ± 0.0009	0.0344 ± 0.0082

[a]$P < 0.05$ *vs* normal control group, [c]$P < 0.05$ *vs* protective group B, [e]$P < 0.05$ *vs* protective group A.

($P < 0.0001$) and the expression of MMP-1 was more significant than that of TIMP-1. Expression of MMP-1 in protective groups A and B was significantly lower than that in the model group ($P < 0.0001$). Expression of MMP-1 in protective group B was significantly lower than that in protective group A ($P < 0.0001$).

Expression of TIMP-1 in the model group was significantly higher than that in protective group B and in the normal control group ($P = 0.004$). There was no significant difference between protective groups A and B ($P > 0.05$).

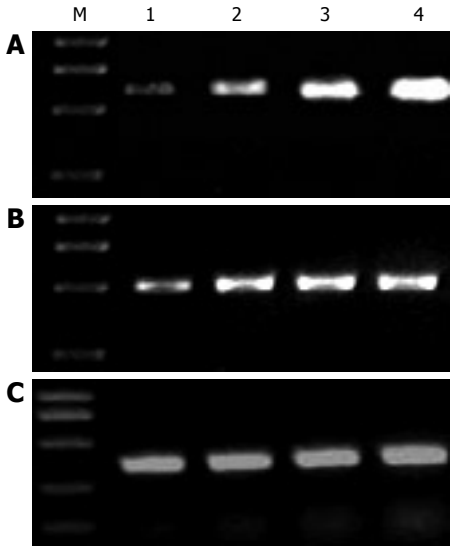


Figure 2 RT-PCR for MMP-1 (**A**), TIMP-1 (**B**) and β-actin (**C**). M: marker; lane1: normal controls; lane 2: protective group B; lane 3: protective group A; lane 4: UC model group.

Results of immunohistochemistry

Immunohistochemistry showed that the expression of MMP-1 and TIMP-1 proteins was basically identical to that at transcription level. The expression of MMP-1 and TIMP-1 in the model group, protective groups A and B was significantly higher than that in the normal control group ($P < 0.0001$, Table 2, Figure 3).

DISCUSSION

Establishment of an appropriate animal model of UC is essential to the study of pathogenesis and treatment of UC. In this study, we utilized the Morris model of UC induced by TNB[15]. The pathological changes in this model which has been extensively used in recent years mimic those of human UC, revealing dynamic changes at

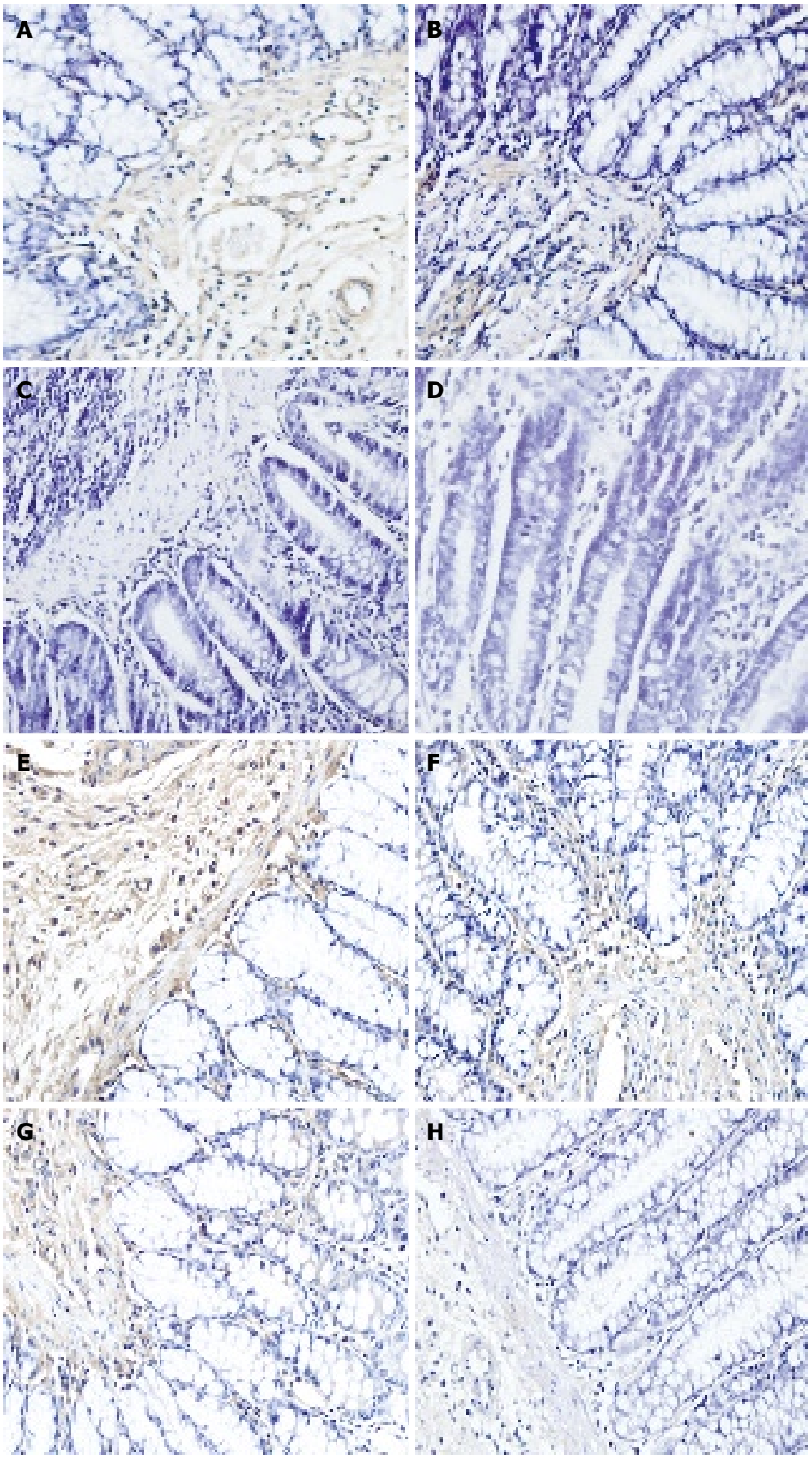


Figure 3 Immunohistochemistry showing MMP-1 in model group (**A**, **E**), protective group A (**B**, **F**), protective group B (**C**, **G**), normal control group (**D**, **H**).

acute and chronic phases. The possible mechanism is that TNB may act as a chemical semi-antigen which combines with keratin protein of the colonic mucosa, thus leading to local cellular immune responses and inflammatory changes when ethanol, an organic solvent, damages the colonic mucosa barrier. Neither TNB nor ethanol is able to produce typical UC[16]. In this study, HE staining revealed erosion and ulceration in the mucosa and submucosa of colon with infiltrations of massive neutrophils and lymphocytes under light microscope. These changes

were correspondent to the features of acute pathological lesions in UC. Allgayer *et al*[17] also suggested that the dividing line between acute and chronic phases in this model is approximately 3 wk after the initiation of TNB administration.

MMPs are a group of zinc-dependent proteases that play an important role in ECM remodeling, connective tissue injury, formation of new blood vessels, and proliferation of inflammatory cells[18]. An excessive degradation of ECM by MMPs is the basic pathophysiological event leading to mucosa ulceration in UC. The activity of MMPs can be inhibited specifically by their natural inhibitor, TIMP. TIMP-1 is the main member of TIMPs inhibiting nearly all MMPs, particularly MMP-1. In this study, we detected the expression of MMP-1 and TIMP-1 at transcription level using RT-PCR. The results showed that expression of both MMP-1 and TIMP-1 was significantly higher than that in the control group and the expression of MMP-1 was significantly increased. Salmela *et al*[19] have also observed similar results using *in situ* hybridization and gene sequence analysis. Our immunohistochemistry results showed that expression of MMP-1 and TIMP-1 was identical to that at transcription level, showing that the expression level of MMP-1 is higher than that of TIMP-1. MMP-1/TIMP-1 ratio was 0.02631 in the control group and 1.1164 in the model group, implying that imbalance between MMP-1 and TIMP-1 is closely related to the ulceration of colonic mucosa in UC patients[20]. Therefore, we speculate that exogenous MMP inhibitors may be able to inhibit expression of MMP-1, thus balancing the expression of MMP-1 and TIMP-1 and relieving, at least partially, the pathological changes in the colonic mucosa of UC patients. Sykers *et al*[21] used marimastat, another artificial MMPI, to treat TNB-induced ulcerative colitis in rats and found that marimastat is capable of relieving colonic injury and decreasing colonic inflammatory score. Medina *et al*[22] showed that inflammation of the colonic mucosa improves to some extent in rat models of inflammatory bowel disease. Disebatiano *et al*[9] found that intraperitoneal injection of batimastat can decrease the activity of MPO and improve colonic tissue damage in TNB-induced ulcerative colitis.

In this study, we used ilomastat, a known most potent artificial MMPI, to observe its protective role in TNB-induced ulcerative colitis in rats. Its intraperitoneal dosage used was 10 mg/kg in protective group A and 20 mg/kg in protective group B as previously described[9,12]. The pathological changes in the protective groups A and B under light microscope much improved compared with the model group. Infiltration of neutrophils and lymphocytes was greatly lessened and the mucosa tended to be perfect in the protective groups A and B, suggesting that ilomastat is able to relieve ulceration of colonic mucosa in UC patients. RT-PCR and immunohistochemistry showed that expression of MMP-1 in the protective groups A and B was obviously decreased when compared with the model group, suggesting that ilomastat is able to inhibit expression of MMP-1. Meanwhile, there was no significant difference in expression of TIMP-1 between protective groups A and B and the model group, indicating that ilomastat does not influence TIMP-1 expression. Therefore, we speculate that ilomastat exerts its protective effects by inhibiting MMP-1 expression and by restoring the balance between MMP-1 and TIMP-1. The possible mechanisms underlying its protective effect on MMP-1 expression and balance between MMP-1 and TIMP-1 may be as follows: (1) ilomastat inhibits the catalytic activity and hydrolysis of ECM by combining the active site of zinc in MMP-1 molecule; (2) ilomastat inhibits hydrolysis of collagens in a dose-dependent manner in the presence of plasminogen; (3) ilomastat inhibits formation of active MMP-1 by suppressing transfer of MMP-1 precursor.

Comparing protective groups A and B, we found that the morphological improvement was different in different dosage groups. In protective group B receiving a bigger dosage of ilomastat, mucosa ulcer was much shallower and infiltration of neutrophils and lymphocytes was fewer than that in protective group A, with regenerated epithelia cells covering the ulcer and granulation and scar tissues filling the base. Furthermore, expression of MMP-1 was also different. The expression level of MMP-1 was obviously lower in protective group B than in protective group A. These results indicate that the protective effects of ilomastat are dose dependent, which is consistent with the reported findings[21].

The protective effects of ilomastat on experimental rat model of UC observed in our study indicate that exogenous MMPI may be used in treatment of UC. Nevertheless, intervention on up-regulation of endogenous TIMP-1 to restore the balance between MMP-1 and TIMP-1 might be another treatment modality for UC.

In conclusion, expression of MMP-1 and TIMP-1 is significantly increased in TNB-induced ulcerative colitis of rats. Both MMP-1 and TIMP-1 play an important role in the pathogenesis of UC. Ilomastat is able to relieve colonic mucosa damage in TNB-nduced ulcerative colitis of rats in a dose-dependent manner by inhibiting expression of MMP-1. Therefore, ilomastat can be used in treatment of UC.

COMMENTS

Background

Matrix metalloproteinase-1 (MMP-1) has been implicated in the development of ulcerative colitis (UC) by the fact that it is over-expressed in this inflammatory bowel disease. Tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), a natural inhibitor of MMP-1, is also believed to be over-expressed, but not parallel to that of MMP-1. Therefore, in this study, ilomastat, an artificial MMP-1 inhibitor (MMPI), was employed to verify its protective effect on the rat model of UC.

Research frontiers

In recent years, intense and extensive studies have shown that MMPs, TIMPs and other inflammatory cytokines play an important role in the development of UC, while studies on the effects of exogenous MMPI on UC are not well documented. Therefore, more studies both in animals and in humans are badly needed to verify the protective and therapeutic effects of artificial MMPI on UC.

Innovations and breakthroughs

Ilomastat, a most powerful artificial MMPI, has been used in experimental and

clinical treatment of tumors of animal and human beings. Ilomastat can inhibit tumor transfer due to its powerful inhibitory effect of on MMPs, especially on MMP-1. However, its protective effects on UC remain largely unknown. Therefore, in this study, we verified the protective effects of ilomastat on UC in rats and provided a new therapeutic approach to UC.

Applications

Up to now, no satisfactory therapy for UC is available. MMPI targeting MMPs may become a new and effective treatment modality for UC.

Peer review

The authors showed that ilomastat could improve the pathological injuries of colonic mucosa in TNB-induced UC in rats by inhibiting the MMP-1 activity, thus providing a new and effective treatment modality for UC. The study is well designed and interesting.

REFERENCES

1 **Kho YH**, Pool MO, Jansman FG, Harting JW. Pharmacotherapeutic options in inflammatory bowel disease: an update. *Pharm World Sci* 2001; **23**: 17-21

2 **Pender SL**, Tickle SP, Docherty AJ, Howie D, Wathen NC, MacDonald TT. A major role for matrix metalloproteinases in T cell injury in the gut. *J Immunol* 1997; **158**: 1582-1590

3 **Heuschkel RB**, MacDonald TT, Monteleone G, Bajaj-Elliott M, Smith JA, Pender SL. Imbalance of stromelysin-1 and TIMP-1 in the mucosal lesions of children with inflammatory bowel disease. *Gut* 2000; **47**: 57-62

4 **von Lampe B**, Barthel B, Coupland SE, Riecken EO, Rosewicz S. Differential expression of matrix metalloproteinases and their tissue inhibitors in colon mucosa of patients with inflammatory bowel disease. *Gut* 2000; **47**: 63-73

5 **Naito Y**, Yoshikawa T. Role of matrix metalloproteinases in inflammatory bowel disease. *Mol Aspects Med* 2005; **26**: 379-390

6 **Pender SL**, MacDonald TT. Matrix metalloproteinases and the gut - new roles for old enzymes. *Curr Opin Pharmacol* 2004; **4**: 546-550

7 **Brew K**, Dinakarpandian D, Nagase H. Tissue inhibitors of metalloproteinases: evolution, structure and function. *Biochim Biophys Acta* 2000; **1477**: 267-283

8 **McKaig BC**, McWilliams D, Watson SA, Mahida YR. Expression and regulation of tissue inhibitor of metalloproteinase-1 and matrix metalloproteinases by intestinal myofibroblasts in inflammatory bowel disease. *Am J Pathol* 2003; **162**: 1355-1360

9 **Di Sebastiano P**, di Mola FF, Artese L, Rossi C, Mascetta G, Pernthaler H, Innocenti P. Beneficial effects of Batimastat (BB-94), a matrix metalloproteinase inhibitor, in rat experimental colitis. *Digestion* 2001; **63**: 234-239

10 **Winding B**, NicAmhlaoibh R, Misander H, Hoegh-Andersen P, Andersen TL, Holst-Hansen C, Heegaard AM, Foged NT, Brunner N, Delaisse JM. Synthetic matrix metalloproteinase inhibitors inhibit growth of established breast cancer osteolytic lesions and prolong survival in mice. *Clin Cancer Res* 2002; **8**: 1932-1939

11 **Garton KJ**, Gough PJ, Blobel CP, Murphy G, Greaves DR, Dempsey PJ, Raines EW. Tumor necrosis factor-alpha-converting enzyme (ADAM17) mediates the cleavage and shedding of fractalkine (CX3CL1). *J Biol Chem* 2001; **276**: 37993-38001

12 **Witte MB**, Thornton FJ, Kiyama T, Efron DT, Schulz GS, Moldawer LL, Barbul A. Metalloproteinase inhibitors and wound healing: a novel enhancer of wound strength. *Surgery* 1998; **124**: 464-470

13 **Chen H**, Li D, Saldeen T, Mehta JL. TGF-beta 1 attenuates myocardial ischemia-reperfusion injury via inhibition of upregulation of MMP-1. *Am J Physiol Heart Circ Physiol* 2003; **284**: H1612-H1617

14 **McLennan SV**, Kelly DJ, Cox AJ, Cao Z, Lyons JG, Yue DK, Gilbert RE. Decreased matrix degradation in diabetic nephropathy: effects of ACE inhibition on the expression and activities of matrix metalloproteinases. *Diabetologia* 2002; **45**: 268-275

15 **Morris GP**, Beck PL, Herridge MS, Depew WT, Szewczuk MR, Wallace JL. Hapten-induced model of chronic inflammation and ulceration in the rat colon. *Gastroenterology* 1989; **96**: 795-803

16 **Little JR**, Eisen HN. Preparation and characterization of antibodies specific for the 2,4,6-trinitrophenyl group. *Biochemistry* 1966; **5**: 3385-3395

17 **Allgayer H**, Deschryver K, Stenson WF. Treatment with 16,16′-dimethyl prostaglandin E2 before and after induction of colitis with trinitrobenzenesulfonic acid in rats decreases inflammation. *Gastroenterology* 1989; **96**: 1290-1300

18 **Woessner JF Jr**. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J* 1991; **5**: 2145-2154

19 **Salmela MT**, MacDonald TT, Black D, Irvine B, Zhuma T, Saarialho-Kere U, Pender SL. Upregulation of matrix metalloproteinases in a model of T cell mediated tissue injury in the gut: analysis by gene array and in situ hybridisation. *Gut* 2002; **51**: 540-547

20 **Wang YD**, Yan PY. Expression of matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 in ulcerative colitis. *World J Gastroenterol* 2006; **12**: 6050-6053

21 **Sykes AP**, Bhogal R, Brampton C, Chander C, Whelan C, Parsons ME, Bird J. The effect of an inhibitor of matrix metalloproteinases on colonic inflammation in a trinitrobenzenesulphonic acid rat model of inflammatory bowel disease. *Aliment Pharmacol Ther* 1999; **13**: 1535-1542

22 **Medina C**, Videla S, Radomski A, Radomski M, Antolin M, Guarner F, Vilaseca J, Salas A, Malagelada JR. Therapeutic effect of phenantroline in two rat models of inflammatory bowel disease. *Scand J Gastroenterol* 2001; **36**: 1314-1319

S- Editor Zhong XY **L- Editor** Wang XL **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5689

World J Gastroenterol 2008 October 7; 14(37): 5689-5694
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Accuracy of the automated cell counters for management of spontaneous bacterial peritonitis

Oliviero Riggio, Stefania Angeloni, Antonella Parente, Cinzia Leboffe, Giorgio Pinto, Teresa Aronne, Manuela Merli

Oliviero Riggio, Stefania Angeloni, Antonella Parente, Cinzia Leboffe, Giorgio Pinto, Teresa Aronne, Manuela Merli, Department of Clinical Medicine, "Sapienza" University of Rome, Rome 00185, Italy
Author contributions: Angeloni S, Leboffe C and Parente A enrolled the patients and collected and analysed the data; Pinto G and Aronne T performed the PMN cell count in the ascitic fluid by the manual method and the automatic cell counter, respectively; Merli M and Riggio O designed the study and wrote the paper.
Correspondence to: Oliviero Riggio, Professor, II Gastroenterologia, Dipartimento di Medicina Clinica, "Sapienza" Università di Roma, Viale dell'Università 37, Rome 00185, Italy. oliviero.riggio@uniroma1.it
Telephone: +39-6-49972001 Fax: +39-6-49972001
Received: March 28, 2008 Revised: September 16, 2008
Accepted: September 23, 2008
Published online: October 7, 2008

Abstract

AIM: To evaluate the accuracy of automated blood cell counters for ascitic polymorphonuclear (PMN) determination for: (1) diagnosis, (2) efficacy of the ongoing antibiotic therapy, and (3) resolution of spontaneous bacterial peritonitis (SBP).

METHODS: One hundred and twelve ascitic fluid samples were collected from 52 consecutive cirrhotic patients, 16 of them with SBP. The agreement between the manual and the automated method for PMN count was assessed. The sensitivity/specificity and the positive/negative predictive value of the automated blood cell counter were also calculated by considering the manual method as the "gold standard".

RESULTS: The mean ± SD of the difference between manual and automated measurements was 7.8 ± 58 cells/mm^3, while the limits of agreement were +124 cells/mm^3 [95% confidence interval (CI): +145 to +103] and -108 cells/mm^3 (95% CI: -87 to -129). The automated cell counter had a sensitivity of 100% and a specificity of 97.7% in diagnosing SBP, and a sensitivity of 91% and a specificity of 100% for the efficacy of the ongoing antibiotic therapy. The two methods showed a complete agreement for the resolution of infection.

CONCLUSION: Automated cell counters not only have a good diagnostic accuracy, but are also very effective in monitoring the antibiotic treatment in patients with SBP. Because of their quicker performance, they should replace the manual counting for PMN determination in the ascitic fluid of patients with SBP.

Key words: Spontaneous bacterial peritonitis; Ascites; Polymorphonuclear cell count; Automated cell counter; Paracentesis; Cirrhosis

Peer reviewer: Takayuki Yamamoto, MD, Inflammatory Bowel Disease Center, Yokkaichi Social Insurance Hospital, 10-8 Hazuyamacho, Yokkaichi 510-0016, Japan

Riggio O, Angeloni S, Parente A, Leboffe C, Pinto G, Aronne T, Merli M. Accuracy of the automated cell counters for management of spontaneous bacterial peritonitis. *World J Gastroenterol* 2008; 14(37): 5689-5694 Available from: URL: http://www.wjgnet.com/1007-9327/14/5689.asp DOI: http://dx.doi.org/10.3748/wjg.14.5689

INTRODUCTION

Spontaneous bacterial peritonitis (SBP) is a well-recognized and potentially fatal complication in cirrhotic patients with ascites[1-3]. The prevalence of SBP in hospitalized patients has been reported to range between 10% and 30%[4-6]. The mortality rate related to this complication remains high, approximately 20%, despite the recent improvements achieved in the management of this complication[7-9]. A rapid diagnosis and a prompt treatment are essential for the survival of these patients. SBP symptoms, however, are not always present and may be insidious; in addition, the ascitic fluid cultures require several days to grow and, in the clinical practice, they are negative in more than 60% of patients with SBP[10].

For these reasons, the current guidelines[10] recommend the use of polymorphonuclear (PMN) cell count in the ascitic fluid for diagnosing SBP, suggesting that a PMN cell count greater than 250 cells/mm^3 should be considered highly suspicious for SBP, thus providing an indication to empirically initiate the antibiotic treatment.

To date, PMN cell count is routinely performed

by using the traditional hematological method with a light microscope in a manual counting chamber (Burker chamber). The manual laboratory counting of ascitic PMN is however laborious, time-consuming, and costly. Moreover, it is not always timely available in all hospitals, especially in those small patient care units with limited laboratory facilities, and it cannot be frequently performed on an emergency basis (at night or on weekends). The manual system, therefore, too often delays the initiation of the important sequence of events that should lead to a rapid diagnosis and treatment of this infection.

In the last years, a series of reports proposed the use of urinary reagent strips to achieve an "instant" bedside diagnosis of SBP[11-14] with promising results[15-17]. A recent prospective multicenter study[18] in which 2123 paracenteses were performed in 1041 patients, however, reports the lack of any diagnostic efficacy of the strip-test. The authors concluded that a routine cytological examination remains mandatory for the diagnosis of SBP. Moreover, the urine screening test has never demonstrated to be useful in the monitoring of PMN cell count at follow-up paracenteses performed 48 h after the beginning of the treatment for SBP. According to current guidelines[10], in fact, the antibiotic treatment empirically administered should be changed if a decrease in the PMN count of less than 25% of the pre-treatment value is not obtained. A qualitative method providing only a negative/trace/positive score needs therefore to be always confirmed by standard cytology of the ascitic fluid.

A valid alternative to manual PNM counting is represented by automated blood cell counters, commonly and largely used in all laboratories for blood cell counting; they offer accurate and rapid differential counts of leukocytes, there including PMN. Our previous study[19], published in 2003, demonstrated that automated blood cell counters are a reliable tool for the rapid diagnosis of SBP and our experience was also confirmed by Cereto *et al*[20]. Moreover, automated cell counters, as a quantitative method able to provide a reliable PMN value not requiring further confirmation, could be useful not only for diagnostic purposes, but also for determining the effectiveness of the ongoing empiric antibiotic therapy.

The aim of the present study was therefore to evaluate the validity of the automated blood cell counter not only for SBP diagnosis, but also for monitoring the responsiveness to the ongoing antibiotic treatment. For this purpose, we compared the determination of PMN count in the ascitic fluid obtained by the manual and the automated methods at basal and follow-up diagnostic paracenteses in a group of cirrhotic patients with SBP.

MATERIALS AND METHODS

Patients

A total of 112 ascitic fluid samples was collected from 52 consecutive cirrhotic patients with ascites (36 men and 16 women, mean age 65.3 ± 11.7 years) hospitalized at our Gastroenterology Unit. The diagnosis of liver cirrhosis was based on clinical, biochemical, and/or histopathological data. The severity of the liver disease was classified in each patient at entry, according to the Child-Pugh[21] scores.

Methods

All the patients underwent a routine abdominal paracentesis at the time of hospitalization. Paracentesis was repeated if, during hospitalization, the patient had signs or symptoms compatible with infection (i.e. fever, change in the mental status, abdominal pain, peripheral leukocytosis, development of renal failure, hypotension, *etc.*). In some patients re-admitted for recurrent ascites, a diagnostic paracentesis was also repeated. Two samples of ascitic fluid for each patient were collected under aseptic conditions in tubes containing ethylenediamine tetraacetic acid (EDTA) as anticoagulant. White blood cells (WBC) and PMN counts were determined by both the traditional method with a light microscope in a manual counting chamber, and the automated cell blood counter (Technicon System H*1; Bayer Diagnostics, Milan, Italy), as previously described[19]. The specimens were analyzed within 1 h. Additional samples of ascitic fluid were collected for the determination of albumin and total protein concentrations. Moreover, 10 mL of ascitic fluid were directly inoculated at the patient's bedside into aerobic and anaerobic blood culture bottles for bacteriological examination[22].

For traditional manual WBC and PMN counts, ascitic fluids were collected in tubes containing 0.084 mL of 15% EDTA. Ten milliliters were centrifuged at 1500 r/min for 10 min; 9 mL of the supernatant were discharged and 40 μL of the remaining ascitic fluid were diluted with 800 μL of Turk's fluid and gently shaked; 20 μL were used to fill the counting chamber. The cells were counted (× 40) in one of the nine large squares, and the number of WBC per cubic millimeter was calculated. Another sample of 10 mL of ascitic fluid was used for the PMN percentage determination (× 100), after centrifugation and May-Grünwald-Giemsa staining.

For WBC and PMN cell counts by the automated method, 100 μL of ascitic fluid, collected in tubes containing 0.054 mL of 15% EDTA anticoagulant, were directly injected into the analyzer.

Diagnosis and treatment of patients with SBP

SBP was diagnosed when the PMN cell count in the ascitic fluid was greater than 250 cells/mm^3 and an antibiotic treatment with i.v. cefotaxime (2 g/8 h, for a minimum of 5 d) was empirically initiated in all the patients with these values, regardless of the positivity of the culture. The antibiotic dosage was adjusted to the renal function throughout the treatment period and the efficacy was evaluated by further diagnostic paracenteses 2 and 5 d after the beginning of the treatment. A further paracentesis was performed in the patients with no resolution at 5 d.

Table 1 Demographic, clinical characteristics and outcome of the 16 cirrhotic patients with SBP (mean ± SD)

Variables	Cirrhotic patients with SBP
n	16
Sex (M/F)	11/5
Age (yr)	63.8 ± 10.3
Child-Pugh class (A/B/C)	0/8/8
Alcoholic origin (No/Yes)	10/6
Treatment efficacy at 48 h (No/Yes)	11/5
Resolution of infection (No/Yes)	12/4

In those cases not responding to the initial antibiotic regimen, the therapy was appropriately changed, either according to the in vitro susceptibility of the isolated bacteria, or empirically. For this purpose, a further paracentesis was always performed 2 d after the beginning of the antibiotic treatment. Treatment failure was established when the condition of the patients rapidly deteriorated within the first hours of the antibiotic therapy (i.e. with development of shock), or when no significant decrease in the ascitic PMN count was observed in the follow-up paracentesis. A reduction in the PMN count of less than 25% of the pre-treatment value was considered as suggestive of failure of the antibiotic treatment[10].

At the time of the 48-h paracentesis, as well as at the following paracentesis, WBC and PMN counts were performed by both manual method and automated cell counter.

SBP was considered resolved when PMN count in the ascitic fluid had decreased to less than 250 cells/mm^3.

Statistical analysis

Results are expressed as mean ± SD. The values of PMN count determined by the two methods were compared using the Student's *t* test. The agreement between the two techniques was assessed by using the method suggested by Bland and Altman[23]. The differences between the results of the manual counting and the automated blood cell counter in each patient were plotted against the mean of the two readings observed in each patient. The mean and SD of the differences were calculated. The limits of agreement, defined as the mean ± 2 SDs of the difference, and their 95% confidence intervals (CI) were then calculated.

By considering the PMN count determined by the traditional manual method as the "gold standard", the sensitivity/specificity and the positive/negative predictive value of the automated blood cell counter were calculated according to Ransohoff and Feinstein[24] for the following end-points: (1) diagnosis of SBP defined as a PMN count of more than 250 cells/mm^3; (2) treatment efficacy defined as a decrease in the PMN count of more than 25% of the pre-treatment value at the 48-h diagnostic paracentesis; (3) resolution of the infection defined as a reduction of PMN count to less than 250 cells/mm^3.

The statistical significance was established at a $P < 0.05$. Calculations were performed by using a statistical software program (Number Cruncher Statistical System 97).

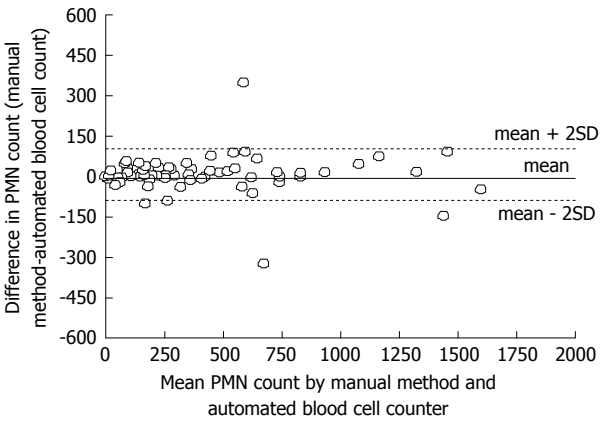


Figure 1 PMN cell count-Scatter plot of the differences between the manual method and the automated blood cell counter against the mean of the two measurements, showing the limits of agreement defined as the mean of the difference ± 2SD.

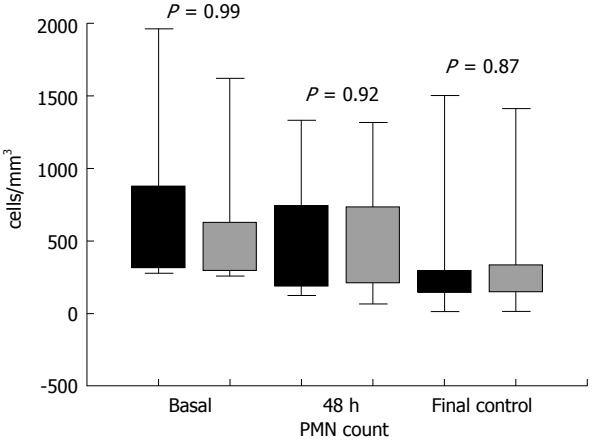


Figure 2 Comparison between PMN cell counts determined with the automated blood cell counters (black bars) or the manual method (gray bars) in the patients with SBP.

RESULTS

A total of 112 samples of ascitic fluid were collected from 52 consecutive cirrhotic patients (36 male/16 female; age: 65.3 ± 11.7 years; Child-Pugh class: 24 B/28 C; alcoholic origin: 29%) with ascites. The degree of agreement between the measurements of PMN count in the ascitic fluid, using the manual method or the automated blood cell counter, is reported in Figure 1. The mean ± SD of the difference between the manual and the automated measurements was 7.8 ± 58 cells/mm^3, while the limits of agreement were +124 cells/mm^3 (95% CI: +145 to +103) and -108 cells/mm^3 (95% CI: -129 to -87).

SBP, as indicated by a PMN count > 250 cells/mm^3 with the traditional manual method, was diagnosed in 16 patients. Demographic, clinical characteristics, and outcome of these 16 patients with SBP are reported in Table 1. No significant differences were observed when PMN counts were determined by using both methods (Figure 2).

As far as the diagnosis of SBP is concerned, the

agreement between the two methods was observed in all the patients but one, who had a PMN count of 270 cells/mm^3 at the automated blood cell counter and of 249 cells/mm^3 at the manual method (false positive result). By considering a PMN count > 250 cells/mm^3 determined by the manual method as the "gold standard" for the SBP diagnosis, the automated blood cell counter had a sensitivity of 100% and a specificity of 97.7%, whereas positive and negative predictive values were 94.1% and 100%, respectively.

As far as the efficacy of the antibiotic treatment is concerned, the agreement between the two methods was obtained in all the patients but one, in whom the therapy was not considered effective by the automated counter only (false negative result). By considering the treatment efficacy as a reduction in the PMN count > 25% of the pre-treatment value (determined by the traditional manual method) as the "gold standard", the automated blood cell counter had a sensitivity of 91% and a specificity of 100%, whereas positive and negative predictive values were 100% and 83.3%, respectively.

By considering the resolution of infection as a reduction of PMN count to less than 250 cells/mm^3 (determined by the traditional manual method) at the final paracentesis as the "gold standard", the automated blood cell counter showed a complete agreement (sensitivity 100%, specificity 100%, positive and negative predictive values 100%).

DISCUSSION

In the guidelines for the diagnosis and treatment of SBP in cirrhotic patients[10] published in the year 2000, the International Ascites Club suggested that a PMN cell count greater than 250 cells/mm^3 should be considered highly suspicious for SBP, thus providing an indication to promptly initiate an empiric antibiotic treatment. Forty eight hours after the initiation of antibiotics, a repeat diagnostic paracentesis is also recommended, either to document the response by a greater-than-25% decrease in the ascitic fluid neutrophil count, or to induce a change in the antibiotic therapy. A further diagnostic paracentesis showing that PMN cell count is below 250 cells/mm^3 is finally indicated to confirm the infection resolution and stop the antibiotic treatment.

To date, PMN cell count is routinely performed by using the traditional hematological method with a light microscope in a manual counting chamber. Urinary reagent strips have been used to make an "instant" bedside diagnosis of SBP with controversial results[12,18]. This screening test, however, is a qualitative method providing only a negative/trace/positive score; it is therefore, by definition, unable to monitor the PMN cell count at follow-up paracentesis (performed 48 h after the beginning of the treatment). In the management of SBP, the cytological examination remains then mandatory. Manual laboratory counting of ascitic PMN is however laborious, time-consuming, costly, and not always timely available in all the hospitals. Automated blood cell counters, commonly and largely used in laboratories for blood cell counting, offer instead an accurate and rapid count of PMN in the ascitic fluid and has proven to be a reliable tool for the rapid diagnosis of SBP[19,20]. This finding is confirmed by the present study in which we analyzed with both methods 112 samples of ascitic fluid collected from 52 consecutive cirrhotic patients with ascites. Although the agreement limits (as reported in Figure 1) may in fact range between +124 and -108 cells/mm^3, SBP was correctly diagnosed by automated blood cell counter in all the cases but one (a false positive result). The use of the automated method as the only diagnostic tool, therefore, would have erroneously submitted this patient to an antibiotic treatment: a less significant error than that of no treating a patient who actually needed to be treated.

However, in order to suggest the use of automated blood cell counters as an alternative to manual counting, its usefulness for the optimization of the antibiotic treatment should be demonstrated. The agreement between the two methods should therefore be obtained not only in the first diagnostic paracentesis, but also in those performed for monitoring the antibiotic treatment. The paracentesis performed after 48 h is in fact particularly significant, since the efficacy of the antibiotic treatment is usually established in this phase. As a matter of fact, in case of SBP, in the large majority of the patients antibiotics are chosen empirically-that is, without the support of the result of the ascitic fluid culture. This is because antibiotics should be started immediately after the result of the PMN cell count[10] (before the result of the culture), and because the ascitic fluid culture outcome-by using conventional culture techniques-may be negative in up to 60% of patients with SBP. The reduction of more than 25% of the initial PMN cell count value is the criterion to establish the efficacy of the antibiotic treatment. The present study showed that, at the diagnostic paracentesis performed 48 h after the start of the antibiotic treatment, the two methods agreed in all the patients but one, in whom a false negative result was obtained. By using the automated cell counter, this patient would then be erroneously considered as a non-responder to the ongoing antibiotic treatment. This would have led to a switch in the antibiotic treatment, a less significant mistake than that of erroneously considering the patient as a responder to the ongoing antibiotic treatment. As far as the infection resolution assessed at the final diagnostic paracentesis is concerned (based on a PMN count below 250 cell/mm^3), an agreement between the two methods was achieved in all the patients. These results suggest that automated cell counters should be considered a reliable tool not only for the diagnosis of SBP, but also in its optimal management; automated methods could therefore definitely replace manual counting. The benefits of a quicker and precise method in the evaluation of the ascitic fluid have been clearly stressed[25].

Another, although less important, advantage of the automated cell counting method over both reagent strips and the manual method is the possibility to precisely

assess the amount of PMN in a bloody (for a traumatic tap or a condition inducing bleeding) ascitic fluid. With the last two methods, the amount of PMN deriving directly from the blood spilled over into the ascitic fluid cannot be differentiated from the amount of PMN due to the infection. A correction factor of 1 PMN per 250 red blood cells has been proposed[10], since this is the maximum expected ratio of PMN to red cell normally present in the peripheral blood. With automated cell counters, a measure of the amount of red blood cells and PMN in both the peripheral blood and the ascitic fluid can be simultaneously obtained. The amount of ascitic PMN due to the infection can be therefore calculated by the real PMN-to-red blood cells ratio in the blood and by the real erythrocytes and PMN number in the ascitic fluid. The simultaneous count of PMN and erythrocytes in the same sample of ascitic fluid cannot be made by manual count, since to obtain a reliable PMN count in a manual counting chamber, erythrocytes should be previously hemolyzed by an acidic solution.

In conclusion, the manual and the automated methods have a good agreement in the determination of PMN in the ascitic fluid. Automated cell counters have a good diagnostic accuracy, not only for the diagnosis, but also for the monitoring of the antibiotic treatment in patients with SBP. Automated cell counters, which offer an easier and quicker PMN count, should therefore replace the manual counting for PMN determination in the ascitic fluid analysis.

COMMENTS

Background

Spontaneous bacterial peritonitis (SBP), a severe complication of ascites, is managed on the results of polymorphonuclear (PMN) cell count in the ascitic fluid, which are used both to test the efficacy of the ongoing antibiotic treatment and to establish the resolution of infection.

Research frontiers

Manual PMN counting is time-consuming, costly, and not always timely available. Automated blood cell counters, commonly used in all laboratories for blood cell counting, may be a valid alternative.

Innovations and breakthroughs

Automated cell counters provide a reliable ascitic PMN count and have a good diagnostic accuracy, not only for the diagnosis, but also for monitoring the effectiveness of the ongoing antibiotic treatment in patients with SBP.

Applications

Automated cell counters, which offer an easier and quicker PMN count, should therefore replace the manual counting for PMN determination in the ascitic fluid analysis.

Peer review

This is a well-written paper. Authors demonstrated that automated cell counters not only have a good diagnostic accuracy, but are also very effective in monitoring the antibiotic treatment in patients with SBP.

REFERENCES

1 **Caly WR**, Strauss E. A prospective study of bacterial infections in patients with cirrhosis. *J Hepatol* 1993; **18**: 353-358

2 **Pinzello G**, Simonetti RG, Craxi A, Di Piazza S, Spano C, Pagliaro L. Spontaneous bacterial peritonitis: a prospective investigation in predominantly nonalcoholic cirrhotic patients. *Hepatology* 1983; **3**: 545-549

3 **Almdal TP**, Skinhoj P. Spontaneous bacterial peritonitis in cirrhosis. Incidence, diagnosis, and prognosis. *Scand J Gastroenterol* 1987; **22**: 295-300

4 **Llach J**, Rimola A, Navasa M, Gines P, Salmeron JM, Gines A, Arroyo V, Rodes J. Incidence and predictive factors of first episode of spontaneous bacterial peritonitis in cirrhosis with ascites: relevance of ascitic fluid protein concentration. *Hepatology* 1992; **16**: 724-727

5 **Gilbert JA**, Kamath PS. Spontaneous bacterial peritonitis: an update. *Mayo Clin Proc* 1995; **70**: 365-370

6 **Garcia-Tsao G**. Bacterial infections in cirrhosis: treatment and prophylaxis. *J Hepatol* 2005; **42** Suppl: S85-S92

7 **Thuluvath PJ**, Morss S, Thompson R. Spontaneous bacterial peritonitis--in-hospital mortality, predictors of survival, and health care costs from 1988 to 1998. *Am J Gastroenterol* 2001; **96**: 1232-1236

8 **Llovet JM**, Planas R, Morillas R, Quer JC, Cabre E, Boix J, Humbert P, Guilera M, Domenech E, Bertran X. Short-term prognosis of cirrhotics with spontaneous bacterial peritonitis: multivariate study. *Am J Gastroenterol* 1993; **88**: 388-392

9 **Toledo C**, Salmeron JM, Rimola A, Navasa M, Arroyo V, Llach J, Gines A, Gines P, Rodes J. Spontaneous bacterial peritonitis in cirrhosis: predictive factors of infection resolution and survival in patients treated with cefotaxime. *Hepatology* 1993; **17**: 251-257

10 **Rimola A**, Garcia-Tsao G, Navasa M, Piddock LJ, Planas R, Bernard B, Inadomi JM. Diagnosis, treatment and prophylaxis of spontaneous bacterial peritonitis: a consensus document. International Ascites Club. *J Hepatol* 2000; **32**: 142-153

11 **Sapey T**, Mena E, Fort E, Laurin C, Kabissa D, Runyon BA, Mendler MH. Rapid diagnosis of spontaneous bacterial peritonitis with leukocyte esterase reagent strips in a European and in an American center. *J Gastroenterol Hepatol* 2005; **20**: 187-192

12 **Castellote J**, Lopez C, Gornals J, Tremosa G, Farina ER, Baliellas C, Domingo A, Xiol X. Rapid diagnosis of spontaneous bacterial peritonitis by use of reagent strips. *Hepatology* 2003; **37**: 893-896

13 **Sapey T**, Kabissa D, Fort E, Laurin C, Mendler MH. Instant diagnosis of spontaneous bacterial peritonitis using leukocyte esterase reagent strips: Nephur-Test vs. MultistixSG. *Liver Int* 2005; **25**: 343-348

14 **Kim DY**, Kim JH, Chon CY, Han KH, Ahn SH, Kim JK, Paik YH, Lee KS, Moon YM. Usefulness of urine strip test in the rapid diagnosis of spontaneous bacterial peritonitis. *Liver Int* 2005; **25**: 1197-1201

15 **Campillo B**, Richardet JP, Dupeyron C. Diagnostic value of two reagent strips (Multistix 8 SG and Combur 2 LN) in cirrhotic patients with spontaneous bacterial peritonitis and symptomatic bacterascites. *Gastroenterol Clin Biol* 2006; **30**: 446-452

16 **Rerknimitr R**, Rungsangmanoon W, Kongkam P, Kullavanijaya P. Efficacy of leukocyte esterase dipstick test as a rapid test in diagnosis of spontaneous bacterial peritonitis. *World J Gastroenterol* 2006; **12**: 7183-7187

17 **Thevenot T**, Cadranel JF, Nguyen-Khac E, Tilmant L, Tiry C, Welty S, Merzoug N. Diagnosis of spontaneous bacterial peritonitis in cirrhotic patients by use of two reagent strips. *Eur J Gastroenterol Hepatol* 2004; **16**: 579-583

18 **Nousbaum JB**, Cadranel JF, Nahon P, Khac EN, Moreau R, Thevenot T, Silvain C, Bureau C, Nouel O, Pilette C, Paupard T, Vanbiervliet G, Oberti F, Davion T, Jouannaud V, Roche B, Bernard PH, Beaulieu S, Danne O, Thabut D, Chagneau-Derrode C, de Ledinghen V, Mathurin P, Pauwels A, Bronowicki JP, Habersetzer F, Abergel A, Audigier JC, Sapey T, Grange JD, Tran A. Diagnostic accuracy of the Multistix 8 SG reagent strip in diagnosis of spontaneous bacterial peritonitis. *Hepatology* 2007; **45**: 1275-1281

19 **Angeloni S**, Nicolini G, Merli M, Nicolao F, Pinto G, Aronne T, Attili AF, Riggio O. Validation of automated blood cell

counter for the determination of polymorphonuclear cell count in the ascitic fluid of cirrhotic patients with or without spontaneous bacterial peritonitis. *Am J Gastroenterol* 2003; **98**: 1844-1848
20 **Cereto F**, Genesca J, Segura R. Validation of automated blood cell counters for the diagnosis of spontaneous bacterial peritonitis. *Am J Gastroenterol* 2004; **99**: 1400
21 **Pugh RN**, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. *Br J Surg* 1973; **60**: 646-649
22 **Runyon BA**, Canawati HN, Akriviadis EA. Optimization of ascitic fluid culture technique. *Gastroenterology* 1988; **95**: 1351-1355
23 **Bland JM**, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986; **1**: 307-310
24 **Ransohoff DF**, Feinstein AR. Problems of spectrum and bias in evaluating the efficacy of diagnostic tests. *N Engl J Med* 1978; **299**: 926-930
25 **Runyon BA**. The evolution of ascitic fluid analysis in the diagnosis of spontaneous bacterial peritonitis. *Am J Gastroenterol* 2003; **98**: 1675-1677

S- Editor Li DL **L- Editor** Megro F **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5695

World J Gastroenterol 2008 October 7; 14(37): 5695-5700
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Association between hepatocellular carcinoma and type 2 diabetes mellitus in Italy: Potential role of insulin

Valter Donadon, Massimiliano Balbi, Pietro Casarin, Alessandro Vario, Alfredo Alberti

Valter Donadon, Massimiliano Balbi, Pietro Casarin, Alessandro Vario, Department of Medicine and Internal Medicine 3rd, Pordenone Hospital, Pordenone 33170, Italy
Alfredo Alberti, Department of Clinical and Experimental Medicine, University of Padova, Padova 35100, Italy
Author contributions: Donadon V conceived and designed research; Balbi M, Casarin P, Donadon V performed research, diagnosis and patient follow-up, Vario A analyzed the data; Donadon V, Alberti A wrote the paper; Alberti A revised the paper.
Correspondence to: Dr. Valter Donadon, Internal Medicine 3rd, Pordenone Hospital, Via Montereale 24, Pordenone 33170, Italy. valter.donadon@aopn.fvg.it
Telephone: +39-434-399330 Fax: +39-434-399559
Received: July 29, 2008 Revised: September 1, 2008
Accepted: September 8, 2008
Published online: October 7, 2008

Abstract

AIM: To investigate the relationships between Type 2 diabetes mellitus (DM2) and the risk of hepatocellular carcinoma (HCC).

METHODS: We studied the association between DM2 and HCC in a large case-control study that enrolled 465 consecutive Caucasian patients with HCC (78.3% males, mean age 68.5 ± 8.9 years) compared with an age and sex matched control group of 490 subjects.

RESULTS: Prevalence of DM2 was significantly higher in HCC patients (31.2% *vs* 12.7%; OR = 3.12, 95% CI: 2.22-4.43) and in HCC cases with alcohol abuse. DM2 has been diagnosed before the appearance of HCC in 84.1% of diabetic HCC subjects with mean duration of 141.5 mo, higher in cases treated with insulin than in those with oral antidiabetic agents (171.5 *vs* 118.7 mo). Compared to controls, males DM2 with HCC were more frequently treated with insulin (38.1% *vs* 17.6%, $P = 0.009$) and with sulfonylurea with or without metformin than with diet with or without metformin (84% *vs* 68.3%, $P = 0.049$).

CONCLUSION: DM2 in our patients is associated with a 3-fold increase risk of HCC. In most of our cases DM2 pre-existed to HCC. Patients with DM2 and chronic liver disease, particularly insulin treated males, should be considered for HCC close surveillance programs.

Key words: Hepatocellular carcinoma; Type 2 diabetes mellitus; Hepatitis virus B and C; Insulin; Antidiabetic therapy

Peer reviewers: Deepak Narayan Amarapurkar, PhD, Department of Gastroenterology, Bombay Hospital & Medical Research Centre, D 401 Ameya Soc, New Prabhadevi Road, Prabhadevi, Mumbai 400025, India; Ned Snyder, Professor, University of Texas Medical Branch, 301 University, University of Texas Medical Branch, Galveston, Texas 77555-0764, United States

Donadon V, Balbi M, Casarin P, Vario A, Alberti A. Association between hepatocellular carcinoma and type 2 diabetes mellitus in Italy: Potential role of insulin. *World J Gastroenterol* 2008; 14(37): 5695-5700 Available from: URL: http://www.wjgnet.com/1007-9327/14/5695.asp DOI: http://dx.doi.org/10.3748/wjg.14.5695

INTRODUCTION

The incidence of hepatocellular carcinoma (HCC) has increased significantly over the past decades in many parts of the Western world, including Italy. The reasons for this increase are only partially understood. The hepatitis C virus (HCV) epidemics certainly play a role due to the cohort effect of individuals infected in pre-serological age[1,2]. However, approximately 15%-50% of HCC cases are not associated with HCV or hepatitis B virus (HBV), suggesting that other risk factors are responsible for this increase[3]. Diabetes has been suggested to be a risk factor for HCC. During the past two decades the prevalence of Type 2 diabetes mellitus (DM2) has dramatically increased in most developed countries and several epidemiologic studies indicate that it is nowadays epidemic, mostly because of the exponential explosion of obesity[4]. A recent study reported that the prevalence of known diabetes mellitus has increased in Italy from 3.6% to 4.3% during the past 10 years[5]. DM2 is a compensatory high insulin state caused by insulin resistance in fat, muscle tissue and liver[6], associated with an insulin-secretory defect that varies in severity and may lead to a relative insulin deficiency during the patients' lifetime. Therefore, DM2 is initially treated with diet and antidiabetic oral agents; after years, to control glucose metabolism, many patients

are required exogenous insulin treatment. Several studies have shown a relationship between diabetes mellitus and liver diseases[7]. Indeed, diabetes has been recognized as a risk factor for non-alcoholic fatty liver disease (NAFLD), while chronic hepatitis C has been associated with an increased risk of diabetes[8-12]. Several studies have investigated the association of diabetes mellitus and solid malignancies[13-15], and particularly with HCC[3,16-20]. While early studies reported no association[21,22], more recent data have clearly identified DM2 as a risk factor for HCC[20,23]. On this line, a high concentration of insulin could stimulate the IGF pathway in DM2 and molecular studies have shown that insulin and insulin-like growth factor 1 (IGF-1) may have carcinogenetic effects on liver and other tissues[24-28]. Moreover, chronic hyperglycaemia may cause oxidative stress and cellular damage[29]. Although DM2 has been associated in the development of HCC, only few studies have considered the confounding role of HBV and of HCV, two of the major risk factors for HCC. Moreover, diabetes may be secondary to HCC and to the underlying cirrhosis. To our knowledge, there are no studies that have investigated the temporal relationship between the onset of diabetes and the development of HCC. The purpose of the present study was to explore the association between HCC and diabetes in a large cohort of Italian patients with HCC and to describe the temporal relationship between onset of diabetes and development of HCC, and the clinical and metabolic characteristics of patients with DM2 and HCC.

PATIENTS AND METHODS

HCC group

We conducted a population based case-control study recruiting a consecutive cohort of 465 Caucasian patients with HCC seen at the Liver Unit of the Division of Internal Medicine of the Pordenone General Hospital (Pordenone, Italy) between January, 1994 and June, 2006. For the diagnosis of HCC, histological or cytological confirmation was available from the majority (85.6%) of HCC cases. In the remaining HCC cases, the diagnosis was established by coincidental finding of two dynamic imaging techniques [computer tomograghy (CT) scan, magnetic resonance imaging (MRI) and Contrast Enhanced Ultrasound examination in the last 3 years] showing a nodule with arterial hypervascularization followed by portal wash-out, or with a single positive imaging technique associated with alpha-fetoprotein > 400 ng/mL. Patients were divided in two groups: the first group included 305 cases derived from a surveillance program of HCC in cirrhotic patients, consisting of periodical (every 3-6 mo) ultrasound and AFP monitoring (follow-up group, FU); the second group consisted of 160 cases presenting with clinically overt and advanced HCC (clinically overt group, CO).

Control group

A control group of 490 cases was chosen by matching age, sex and time of admission among 28 740 patients seen in our Division in the same period of enrollment of patients with HCC. Patients were excluded if admitted for malignancies, alcohol-related diseases (neuropathy, gastric ulcer), virus-related liver diseases, and diabetes mellitus. However, co-morbidity with these conditions was not considered exclusion criteria. The admission diagnosis of the selected 490 control cases was heart failure (34.9%), hypertension (21.4%), chronic obstructive broncho-pneumopathies or pneumonia (16.5%), atrial fibrillation (7.8%), deep venous thrombosis (6.5%), fever of unknown origin (5.3%), benign tumours (4.1%), and gastritis (3.5%). In order to ensure that the selected control group was indeed well representative of the general population of our region, we considered two main parameters: prevalence of chronic HCV infection, the main risk factor for HCC, and that of DM2. The prevalence of HCV infection in the control group was 5.3%, similar to that reported in our region for individuals aged more than 65 years[30,31]. The prevalence of DM2 by age groups was compared to that reported in Italy[32]. Thus, for the purposes of the present study, our control group could be indeed considered representative of the general population in our region.

Methods

For all HCC patients and control cases the following demographic, clinical and biochemical features were registered in a computerized database: age, gender, race, glycated hemoglobin (HbA1c). Biochemical parameters were determined in the Central Laboratory of our Hospital by standard and validated methods. Anti-HBV surface antigen (anti-HBs), anti-HBV core antigen (anti-HBc), hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg) were determined using commercial assays (Abbott Diagnostic Division, Wiesbaden; Germany). Sera were also screened for antibodies against HCV (anti-HCV) using a third-generation microparticle enzyme immunoassay (AxSYM HCV version 3.0, Abbott Diagnostic Division)[33]. Positive samples were tested for anti-HCV using a third generation line immunoassay (Innogenetics, Gent, Belgium) and for serum HCV RNA using the Roche Amplicor version 2.0 (Roche Molecular System, Pleasenton, CA). The diagnosis of DM2 was based on the American Diabetes Association guidelines[6,34]. Alcohol abuse was defined as a daily consumption of more than 30 g in males and of more than 20 g in females, considering an average alcohol content of 5% for beer, 12% for wine and 40% for superalcoholics[35].

Statistical analysis

Descriptive results were expressed as mean ± SD or number (percentage) of patients with a condition. The *t*-test or non parametric Mann-Whitney test was used to compare quantitative data and the chi-square test was applied for comparison of frequency data. $P < 0.05$ was considered significant. Odds ratios (OR) and their corresponding 95% confidence intervals (CI) were calculated using simple logistic-regression analysis.

Table 1 Frequency of DM2 in HCC patients and in controls

	Number of subjects	DM2 absent (%)	DM2 present (%)	OR (95% CI)	*P*	*RR*
HCC	465	320 (68.8)	145 (31.2)	3.12 (2.22-4.43)	< 0.001	2.46
Controls	490	428 (87.3)	62 (12.7)			
Males				3.14 (2.14-4.63)	< 0.001	2.45
HCC	364	246 (67.6)	118 (32.4)			
Controls	385	334 (86.7)	51 (13.3)			
Females				3.11 (1.38-7.4)	0.002	2.55
HCC	101	74 (73.3)	27 (26.7)			
Controls	105	94 (89.5)	11 (10.5)			

HCC: Hepatocellular carcinoma; DM2: Type 2 diabetes mellitus; OR: Odds ratio; CI: Confidence interval; RR: Relative risk.

Table 2 Multivariate analysis of variables associated with HCC

	Odds ratio (95% CI)	*P*
Diabetes		
Absent	1	0.01
Present	2.2 (1.2-4)	
HBV		
Absent	1	⩽ 0.001
Present	252.1 (53.7-1183.9)	
HCV		
Absent	1	⩽ 0.001
Present	106.5 (58.2-194.9)	
Alcohol		
Absent	1	⩽ 0.001
Present	121.2 (61.9-233.7)	

Multivariate logistic regression analysis was used to assess the independent role of different variables.

RESULTS

Among the 465 patients with HCC, mean age was 68.5 ± 8.9 years and 364 (78.3%) were males. The corresponding figures for the control group were 69.4 ± 13.8 years and a male prevalence of 78.6%.

Prevalence of DM2

With regard to the type and frequency of diabetes mellitus in our patients[6,34], we found that every HCC patient of our population with abnormal glucose tolerance has the clinical and metabolic characteristics of DM2 and, therefore, nobody of our HCC patients was found affected by insulin dependent DM1. Overall, 145 (31.2%) HCC patients and 62 (12.7%) of control cases had DM2 (Table 1). This difference was statistically significant with an odd ratio of 3.12 (CI: 2.22-4.43). This odd ratio was higher for male than for female patients (3.14 *vs* 3.11, *P* = 0.632). Among DM2 patients, glycated haemoglobin was significantly higher in male HCC cases than in control males (7.8% *vs* 6.9%, *P* = 0.02). Multivariate analysis (Table 2) identified HBV infection, HCV infection, alcohol abuse and also DM2 as independent variables, all associated with an increased risk of HCC. In regards to the duration of DM2 in HCC patients, diabetes had been diagnosed at least 6 mo before the appearance of HCC in 122 of the 145 cases (84.1%). In 89 of these 122 cases, the time interval between diagnosis of DM2 and HCC could be precisely calculated and the mean time interval was 141.5 ± 9.4 mo. Moreover, in the subgroup of patients with pre-existing DM2, mean duration of diabetes was higher in patients treated with insulin than in those treated with oral antidiabetic agents (171.5 ± 87.6 mo *vs* 118.7 ± 95.2 mo, *P* = 0.05). The prevalence of DM2 in the different etiologic groups of HCC is described in Table 3. Patients classified as having cryptogenic cirrhosis had a somehow higher prevalence of DM2 compared to the other groups, but the difference did not reach statistical significance. On the other hand, alcohol related HCC had a significantly higher prevalence of DM2 compared to the HCV-related group of HCC (36.9% *vs* 26.6%, *P* = 0.048). Males HCC patients with DM2 were more frequently treated with insulin than control cases (38.1% *vs* 17.6%, *P* = 0.009). Among HCC cases, those using antidiabetic oral agents were more frequently treated with insulin secretagogues (sulfonylureas), with or without metformin (insulin sensitizer), than with simple diet, with or without metformin (Table 4).

Table 3 Etiology in the 465 HCC patients and prevalence of DM2 [mean ± SD, *n* (%)]

Etiology	N° HCC (%)	Age (yr)	Prevalence of DM2 (%)
HBV	20 (4.3)	63.3 ± 10.3[1]	3 (15.0)
HCV	177 (38.1)	71.5 ± 7.3[1]	47 (26.6)[2]
Alcohol	141 (30.4)	66.7 ± 8.5[1]	52 (36.9) [2]
HBV + HCV	8 (1.7)	60.8 ± 12.8[1]	2 (25.0)
HBV + alcohol	9 (1.9)	62.9 ± 9.3[1]	2 (22.2)
HCV + alcohol	81 (17.4)	67.7 ± 9.3[1]	27 (33.3)
HBV + HCV + alcohol	2 (0.4)	68.4 ± 10.3	0
Cryptogenetic	27 (5.8)	68.6 ± 9.3	11 (40.7)

[1]HCV *vs* HBV + HCV, *P* < 0.001; HCV *vs* HBV, *P* < 0.001; HCV *vs* HBV + alcohol, *P* < 0.001; HCV *vs* HCV + alcohol; HCV *vs* alcohol, *P* < 0.001; [2]HCV *vs* alcohol, *P* = 0.048.

Table 4 Type of therapy with oral antidiabetic agents in HCC patients and controls with DM2

	Number of subjects	Diet with or without metformin N° cases (%)	Sulfonylureas with or without metformin N° cases (%)	*P*
Total				
HCC	88	14 (15.9)	74 (84.1)	0.04
Controls	48	15 (31.2)	33 (68.8)	
Males				
HCC	75	12 (16.0)	63 (84.0)	0.049
Controls	41	13 (31.7)	28 (68.3)	
Females				
HCC	13	2 (15.4)	11 (84.6)	0.5
Controls	7	2 (28.6)	5 (71.4)	

Features of DM2 in FU and CO groups with HCC

The clinical features of DM2 in HCC cases of the FU and CO groups are compared in Table 5. The prevalence of diabetes was similar (30.2% *vs* 33.1%, *P* = 0.51). Mean HbA1c was somehow higher in FU group, but the difference was not statistically significant (8.2% ± 2.78% *vs* 7.1% ± 2.12%, *P* = 0.1). The mean duration of

Table 5 Clinical features of DM2 in HCC patients of the FU and CO groups (%)

	FU group	CO group	*P*
Prevalence of DM2	92 (30.2)	53 (33.1)	0.51
HbA1c (mean% ± SD)	8.2 ± 2.78	7.1 ± 2.12	0.1
Insulin therapy	45 (48.9)	15 (28.3)	0.01
Antidiabetic oral agents	47 (51.1)	38 (71.7)	0.01
Diet with/without metformin	7 (14.9)	4 (10.5)	0.55
Sulfonylureas with/ without metformin	40 (85.1)	34 (89.5)	0.55
Duration of DM2 (mean ± SD, mo)	127.8 ± 80.1	167.1 ± 114.3	0.03
Duration of insulin therapy (mean ± SD, mo)	50.0 ± 50.5	77.8 ± 86.4	0.12

FU: Follow-up group; CO: Clinically overt group; HbA1c: Glycated haemoglobin.

DM2 was higher in CO cases (167.1 ± 114.3 mo *vs* 127.8 ± 80.1 mo, $P = 0.03$). FU cases with DM2 were more frequently treated with insulin compared to CO cases with DM2 (48.9% *vs* 28.3%, $P = 0.01$).

DISCUSSION

The association of type 2 diabetes with solid tumours[36,37] has been long suspected and several studies have reported increased mortality rates for neoplastic diseases in patients with DM2[13,38-41]. Recent studies would suggest that treatments with anti-diabetic drugs, prone to increase circulating insulin levels, might favour tumour development, as shown with sulfonylurea and insulin[42], while treatment with drugs that contrast hyperinsulinemia may in fact be protective, as in the case of metformin[43]. Many reports have described an increased risk for HCC in patients with DM2, particularly males[13]. However, most of these studies did not assess the individual role of DM2 in relation to confounding cofactors such as HBV and HCV infection, particularly in patients with chronic liver disease. In this clinical setting, DM2 may be the consequence rather than the cause of HCC developing in a cirrhotic liver. Thus, precise definition in our study of the temporal relationship between onset of DM2 and of HCC is of major importance, and this information has been lacking in most previous surveys[3,13,19].

Our results confirm that patients with DM2 have a significantly increased risk of HCC, independently of cofactors such as HBV and HCV infection and alcohol intake, and demonstrate that DM2 pre-exists to the development of HCC in most cases, suggesting that DM2 is more likely a concourse rather than merely a consequence of the liver tumour. This conclusion is also supported by the finding of a similar frequency and severity of DM2 in patients with small HCC detected during follow-up of cirrhosis and in those with more advanced and diffuse cancers detected outside of a surveillance program.

Because diabetes may be due to HCC or to the underlying cirrhosis and the liver cirrhosis may be caused by diabetes[7,10], our data cannot fully explain the reciprocal connections between them. Therefore, further studies, including cirrhotic patients, must be planned in the future to evaluate whether the diabetes itself has a direct carcinogenetic effect.

The observation that patients with DM2, particularly males, treated with insulin had an increased frequency of HCC is intriguing and clinically relevant. These patients are those often showing the highest insulin blood levels[44], and this might have contributed to facilitate the development of HCC. It is well known that patients with DM2 treated with insulin are those with more severe hyperinsulinaemia and more complications, including microalbuminuria and ischemic heart disease[45,46]. Our results indicate the need for close surveillance for HCC in patients with chronic liver disease and DM2, particularly when males and treated with insulin. They also suggest that in these patients strategies to improve the metabolic control should be directed primarily against hyperinsulinaemia by avoiding, as much as possible, the use of oral secretogogue drugs and of insulin treatment, giving preference to insulin-sensitizers such as metformin and glitazones.

COMMENTS

Background

The association of type 2 diabetes mellitus (DM2) with solid tumours, and particularly with hepatocellular carcinoma (HCC), has been long suspected and several studies have reported increased mortality rates for neoplastic diseases in patients with DM2. However, the temporal relationship between onset of diabetes and development of HCC, and the clinical and metabolic characteristics of patients with DM2 and HCC are not been well examined.

Research frontiers

Whether the diabetes itself has a direct carcinogenetic effect remains unknown.

Innovations and breakthroughs

This study shown that DM2 is associated with a 3-fold increase risk of HCC. In most of patients DM2 pre-existed to HCC. Patients with DM2 and chronic liver disease, particularly insulin treated males, should be considered for HCC close surveillance programs.

Applications

Further studies, including cirrhotic patients, must be planned to evaluate the complex relationships between DM2, liver cirrhosis and HCC.

Terminology

DM2 is a compensatory high insulin state caused by impaired insulin action in fat, muscle tissue and liver. HCC is the most frequent cancer of the liver that occurs mainly in patients with chronic liver disease.

Peer review

This is a good paper that is well organized. The data shows the association between diabetes and HCC.

REFERENCES

1 **Deuffic S**, Buffat L, Poynard T, Valleron AJ. Modeling the hepatitis C virus epidemic in France. *Hepatology* 1999; **29**: 1596-1601

2 **Armstrong GL**, Alter MJ, McQuillan GM, Margolis HS. The past incidence of hepatitis C virus infection: implications for the future burden of chronic liver disease in the United States. *Hepatology* 2000; **31**: 777-782

3 **Davila JA**, Morgan RO, Shaib Y, McGlynn KA, El-Serag HB. Diabetes increases the risk of hepatocellular carcinoma in the United States: a population based case control study. *Gut* 2005; **54**: 533-539

4 **King H**, Aubert RE, Herman WH. Global burden of

diabetes, 1995-2025: prevalence, numerical estimates, and projections. *Diabetes Care* 1998; **21**: 1414-1431
5 **Bonadonna RC**, Cucinotta D, Fedele D, Riccardi G, Tiengo A. The metabolic syndrome is a risk indicator of microvascular and macrovascular complications in diabetes: results from Metascreen, a multicenter diabetes clinic-based survey. *Diabetes Care* 2006; **29**: 2701-2707
6 **American Diabetes Association**. Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 2004; **27** Suppl 1: S5-S10
7 **Petrides AS**. [New aspects of the regulation of glucose metabolism in chronic liver diseases] *Z Gastroenterol* 1993; **31** Suppl 5: 53-55
8 **Bugianesi E**, Leone N, Vanni E, Marchesini G, Brunello F, Carucci P, Musso A, De Paolis P, Capussotti L, Salizzoni M, Rizzetto M. Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. *Gastroenterology* 2002; **123**: 134-140
9 **Lecube A**, Hernandez C, Genesca J, Esteban JI, Jardi R, Simo R. High prevalence of glucose abnormalities in patients with hepatitis C virus infection: a multivariate analysis considering the liver injury. *Diabetes Care* 2004; **27**: 1171-1175
10 **Smedile A**, Bugianesi E. Steatosis and hepatocellular carcinoma risk. *Eur Rev Med Pharmacol Sci* 2005; **9**: 291-293
11 **Picardi A**, D'Avola D, Gentilucci UV, Galati G, Fiori E, Spataro S, Afeltra A. Diabetes in chronic liver disease: from old concepts to new evidence. *Diabetes Metab Res Rev* 2006; **22**: 274-283
12 **Moscatiello S**, Manini R, Marchesini G. Diabetes and liver disease: an ominous association. *Nutr Metab Cardiovasc Dis* 2007; **17**: 63-70
13 **Coughlin SS**, Calle EE, Teras LR, Petrelli J, Thun MJ. Diabetes mellitus as a predictor of cancer mortality in a large cohort of US adults. *Am J Epidemiol* 2004; **159**: 1160-1167
14 **Adami HO**, Chow WH, Nyren O, Berne C, Linet MS, Ekbom A, Wolk A, McLaughlin JK, Fraumeni JF Jr. Excess risk of primary liver cancer in patients with diabetes mellitus. *J Natl Cancer Inst* 1996; **88**: 1472-1477
15 **Braga C**, La Vecchia C, Negri E, Franceschi S. Attributable risks for hepatocellular carcinoma in northern Italy. *Eur J Cancer* 1997; **33**: 629-634
16 **La Vecchia C**, Negri E, Decarli A, Franceschi S. Diabetes mellitus and the risk of primary liver cancer. *Int J Cancer* 1997; **73**: 204-207
17 **Lagiou P**, Kuper H, Stuver SO, Tzonou A, Trichopoulos D, Adami HO. Role of diabetes mellitus in the etiology of hepatocellular carcinoma. *J Natl Cancer Inst* 2000; **92**: 1096-1099
18 **Hassan MM**, Hwang LY, Hatten CJ, Swaim M, Li D, Abbruzzese JL, Beasley P, Patt YZ. Risk factors for hepatocellular carcinoma: synergism of alcohol with viral hepatitis and diabetes mellitus. *Hepatology* 2002; **36**: 1206-1213
19 **Verlato G**, Zoppini G, Bonora E, Muggeo M. Mortality from site-specific malignancies in type 2 diabetic patients from Verona. *Diabetes Care* 2003; **26**: 1047-1051
20 **Yuan JM**, Govindarajan S, Arakawa K, Yu MC. Synergism of alcohol, diabetes, and viral hepatitis on the risk of hepatocellular carcinoma in blacks and whites in the U.S. *Cancer* 2004; **101**: 1009-1017
21 **Green A**, Jensen OM. Frequency of cancer among insulin-treated diabetic patients in Denmark. *Diabetologia* 1985; **28**: 128-130
22 **O'Mara BA**, Byers T, Schoenfeld E. Diabetes mellitus and cancer risk: a multisite case-control study. *J Chronic Dis* 1985; **38**: 435-441
23 **Schiel R**, Beltschikow W, Steiner T, Stein G. Diabetes, insulin, and risk of cancer. *Methods Find Exp Clin Pharmacol* 2006; **28**: 169-175
24 **Rosenfeld RG**. Insulin-like growth factors and the basis of growth. *N Engl J Med* 2003; **349**: 2184-2186
25 **Calle EE**, Kaaks R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nat Rev Cancer* 2004; **4**: 579-591
26 **Ish-Shalom D**, Christoffersen CT, Vorwerk P, Sacerdoti-Sierra N, Shymko RM, Naor D, De Meyts P. Mitogenic properties of insulin and insulin analogues mediated by the insulin receptor. *Diabetologia* 1997; **40** Suppl 2: S25-S31
27 **Chou CK**, Ho LT, Ting LP, Hu CP, Su TS, Chang WC, Suen CS, Huang MY, Chang CM. Selective suppression of insulin-induced proliferation of cultured human hepatoma cells by somatostatin. *J Clin Invest* 1987; **79**: 175-178
28 **Niedernhofer LJ**, Daniels JS, Rouzer CA, Greene RE, Marnett LJ. Malondialdehyde, a product of lipid peroxidation, is mutagenic in human cells. *J Biol Chem* 2003; **278**: 31426-31433
29 **Ford ES**, Cogswell ME. Diabetes and serum ferritin concentration among U.S. adults. *Diabetes Care* 1999; **22**: 1978-1983
30 **Bellentani S**, Tiribelli C, Saccoccio G, Sodde M, Fratti N, De Martin C, Cristianini G. Prevalence of chronic liver disease in the general population of northern Italy: the Dionysos Study. *Hepatology* 1994; **20**: 1442-1449
31 **Bellentani S**, Miglioli L, Bedogni G, Croce LS, Tiribelli C. Epidemiology of hepatitis C virus infection. *Minerva Gastroenterol Dietol* 2005; **51**: 15-29
32 **Rosso D**, Campagna S, Di Stefano F, Romano G, Maugeri D, Maggi S, Motta M, Catanzaro S, Carnazzo G. Prevalence of diabetes mellitus in a sample of the elderly population of the city of Catania. *Arch Gerontol Geriatr* 1998; **27**: 223-235
33 **Colin C**, Lanoir D, Touzet S, Meyaud-Kraemer L, Bailly F, Trepo C. Sensitivity and specificity of third-generation hepatitis C virus antibody detection assays: an analysis of the literature. *J Viral Hepat* 2001; **8**: 87-95
34 **American Diabetes Association**. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 1997; **20**: 1183-1197
35 **Corrao G**, Lepore AR, Torchio P, Valenti M, Galatola G, D'Amicis A, Arico S, di Orio F. The effect of drinking coffee and smoking cigarettes on the risk of cirrhosis associated with alcohol consumption. A case-control study. Provincial Group for the Study of Chronic Liver Disease. *Eur J Epidemiol* 1994; **10**: 657-664
36 **Wideroff L**, Gridley G, Mellemkjaer L, Chow WH, Linet M, Keehn S, Borch-Johnsen K, Olsen JH. Cancer incidence in a population-based cohort of patients hospitalized with diabetes mellitus in Denmark. *J Natl Cancer Inst* 1997; **89**: 1360-1365
37 **Hu FB**, Manson JE, Liu S, Hunter D, Colditz GA, Michels KB, Speizer FE, Giovannucci E. Prospective study of adult onset diabetes mellitus (type 2) and risk of colorectal cancer in women. *J Natl Cancer Inst* 1999; **91**: 542-547
38 **Silverman DT**, Schiffman M, Everhart J, Goldstein A, Lillemoe KD, Swanson GM, Schwartz AG, Brown LM, Greenberg RS, Schoenberg JB, Pottern LM, Hoover RN, Fraumeni JF Jr. Diabetes mellitus, other medical conditions and familial history of cancer as risk factors for pancreatic cancer. *Br J Cancer* 1999; **80**: 1830-1837
39 **Lindblad P**, Chow WH, Chan J, Bergstrom A, Wolk A, Gridley G, McLaughlin JK, Nyren O, Adami HO. The role of diabetes mellitus in the aetiology of renal cell cancer. *Diabetologia* 1999; **42**: 107-112
40 **Saydah SH**, Loria CM, Eberhardt MS, Brancati FL. Abnormal glucose tolerance and the risk of cancer death in the United States. *Am J Epidemiol* 2003; **157**: 1092-1100
41 **Caldwell SH**, Crespo DM, Kang HS, Al-Osaimi AM. Obesity and hepatocellular carcinoma. *Gastroenterology* 2004;

127: S97-S103
42 **Bowker SL**, Majumdar SR, Veugelers P, Johnson JA. Increased cancer-related mortality for patients with type 2 diabetes who use sulfonylureas or insulin. *Diabetes Care* 2006; **29**: 254-258
43 **Evans JM**, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD. Metformin and reduced risk of cancer in diabetic patients. *BMJ* 2005; **330**: 1304-1305
44 **Dailey G**. New strategies for basal insulin treatment in type 2 diabetes mellitus. *Clin Ther* 2004; **26**: 889-901
45 **Inchiostro S**, Bertoli G, Zanette G, Donada C, Maccioni A, Tommasi G, Citroni N, Castagna S, Donadon V. Increased urinary albumin excretion is associated with a cluster of metabolic alterations in type 2 diabetes mellitus. *Acta Diabetol* 1992; **29**: 240-245
46 **Inchiostro S**, Bertoli G, Zanette G, Donadon V. Evidence of higher insulin resistance in NIDDM patients with ischaemic heart disease. *Diabetologia* 1994; **37**: 597-603

S- Editor Li DL **L- Editor** Rippe RA **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5701

World J Gastroenterol 2008 October 7; 14(37): 5701-5706
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Estrogen and progesterone receptor isoforms expression in the stomach of Mongolian gerbils

Milena Saqui-Salces, Teresa Neri-Gómez, Armando Gamboa-Dominguez, Guillermo Ruiz-Palacios, Ignacio Camacho-Arroyo

Milena Saqui-Salces, Teresa Neri-Gómez, Ignacio Camacho-Arroyo, Facultad de Química, Departamento de Biología, Universidad Nacional Autónoma de México, México DF 04510, México
Armando Gamboa-Dominguez, Departamento de Patología, INCMNSZ, México DF14000, México
Guillermo Ruiz-Palacios, Departamento de Infectología, INCMNSZ, México DF 14000, México
Author contributions: Saqui-Salces M, and Camacho-Arroyo I designed research; Saqui-Salces M, and Neri-Gómez T performed research; Gamboa-Dominguez A, and Ruiz-Palacios G provided Mongolian gerbils; Saqui-Salces M analyzed data; and Saqui-Salces M, and Camacho-Arroyo I wrote the paper.
Correspondence to: Dr. Ignacio Camacho-Arroyo, Facultad de Química, Departamento de Biología, Universidad Nacional Autónoma de México, Ciudad Universitaria, Coyoacán, México DF04510, México. camachoarroyo@gmail.com
Telephone: +52-55-56223869 Fax: +52-55-56162010
Received: May 23, 2008 Revised: July 15, 2008
Accepted: July 22, 2008
Published online: October 7, 2008

Abstract

AIM: We studied the estrogen receptor (ER) and progesterone receptor (PR) isoforms expression in gastric antrum and corpus of female gerbils and their regulation by estradiol (E2) and progesterone (P4).

METHODS: Ovariectomized adult female gerbils were subcutaneously treated with E2, and E2 + P4. Uteri and stomachs were removed, the latter were cut along the greater curvature, and antrum and corpus were excised. Proteins were immunoblotted using antibodies that recognize ER-alpha, ER-beta, and PR-A and PR-B receptor isoforms. Tissues from rats treated in the same way were used as controls.

RESULTS: Specific bands were detected for ER-alpha (68 KDa), and PR isoforms (85 and 120 KDa for PR-A and PR-B isoforms, respectively) in uteri, gastric antrum and corpus. We could not detect ER-beta isoform. PR isoforms were not regulated by E2 or P4 in uterus and gastric tissues of gerbils. ER-alpha isoform content was significantly down-regulated by E2 in the corpus, but not affected by hormones in uterus and gastric antrum.

CONCLUSION: The presence of ER-alpha and PR isoforms in gerbils stomach suggests that E2 and P4 actions in this organ are in part mediated by their nuclear receptors.

Key words: Estrogen receptor; Progesterone receptor isoforms; Gerbil; Stomach; Sex steroid hormones

Peer reviewers: Shingo Tsuji, Professor, Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine(A8), 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan; Julio H Carri, Professor, Internal Medicine – Gastroenterology, Universidad Nacional de Córdoba, Av.Estrada 160-P 5-Department D, Córdoba 5000, Argentina

Saqui-Salces M, Neri-Gómez T, Gamboa-Dominguez A, Ruiz-Palacios G, Camacho-Arroyo I. Estrogen and progesterone receptor isoforms expression in the stomach of Mongolian gerbils. *World J Gastroenterol* 2008; 14(37): 5701-5706 Available from: URL: http://www.wjgnet.com/1007-9327/14/5701.asp DOI: http://dx.doi.org/10.3748/wjg.14.5701

INTRODUCTION

Mongolian gerbils (*Meriones unguiculatus*) have been used in scientific research for a long time, especially in neuronal protection studies and as a model of human *H pylori* infection. In spite of the numerous publications using Mongolian gerbils, and the characterization of their estrous cycle, which is 4 d to 6 d in duration[1,2], their sexual hormonal levels and receptors have not been characterized to date.

Estradiol (E2) and progesterone (P4) participate in numerous cellular functions, such as proliferation and differentiation in normal and cancer cells. Most of these functions are mediated by their nuclear receptors, ER and PR[3-5].

There are two well characterized isoforms or ER, alpha and beta, that are encoded by different genes[6]. The ER-alpha protein has 595 aminoacids, with a molecular weight of 66 KDa[7]. ER-beta is constituted by 485 amino acids, and has a molecular weight of 54 KDa. The ER-beta isoform is homologous to ER-alpha at the DNA binding domain (95%), and the heat shock protein binding domain (55%). Both isoforms bind estrogens

with high affinity, but regulate different functions[8].

PR has two main isoforms, PR-A (72-94 kDa) and PR-B (108-120 kDa), encoded by the same gene, but regulated by distinct promoters. PR-A is a truncated form of PR-B, lacking 128-164 aminoacids, depending on the species, in the amino terminal end[9,10].

We have previously shown that E2 and P4 have clear and distinct effects on inflammatory response and gastric epithelial changes during early *H pylori* infection[11]. Recently, Ohtani *et al* have shown a protective role of E2 administration in *H pylori*-infected InGas mice[12]. Other studies have demonstrated that E2 and P4 have anti-ulcerative effects in murine gastric mucosa[13-16].

ER and PR have been reported in human, mouse and rat stomach. However, there is no information to date of their expression and regulation in the Mongolian gerbil stomach. Here we demonstrated the presence of ER-alpha, PR-A and PR-B isoforms in gerbil uterine and gastric tissues by Western blot, as well as a differential regulation of ER-alpha by E2 in gastric corpus.

MATERIALS AND METHODS

Animals

Mature (16-week-old) female Mongolian gerbils (*Meriones unguiculatus*) kept in micro isolators under a 12:12 h light-dark cycle, with food and water *ad libitum* were ovariectomized and after 10 d of recovery, subcutaneous injections of vehicle (corn oil/10% ethanol), 17-beta-estradiol (5 μg/100 μL) or E2 + P4 (1 mg/100 μL) where applied as previously reported[17]. Briefly, gerbils were injected with E2 in two consecutive days and then euthanized after 24 h, while E2 + P4 group received an additional P4 injection on the third day and euthanized 24 h later. Adult Sprague Dawley rats, ovariectomized and treated in the same manner, were used as positive controls.

Gerbils and rats were euthanized by exsanguination under anesthesia and uteri and stomach were removed. The stomachs were cut along the greater curvature, antrum and corpus mucosa were excised. All tissues were snap frozen in liquid nitrogen and kept at -80℃ until protein extraction was performed. The experiments were carried out under the guidelines of the Committee for the use of Animals for Research of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán.

Protein extraction and Western Blotting

Uteri were homogenized in TDG lysis buffer with protease inhibitors (10 mmol/L Tris-HCl, 1 mmol/L dithiotreitol, 30% glycerol, 1% Triton X-100, 15 mmol/L sodium azide, 1 mmol/L EDTA, 4 μg/mL leupeptin, 22 μg/mL aprotinin, 1 mmol/L PMSF, 1 mmol/L sodium orthovanadate). Gastric samples were homogenized using TDG lysis buffer without EDTA. Supernatants were clarified by centrifugation at 15000 r/min, 4℃ for 15 min, and proteins quantified by the method of Bradford (Biorad, CA USA)[18]. Proteins (150 μg) were separated by electrophoresis on SDS-PAGE gels at 80 V. Prestained and ECL markers (Biorad, CA) were included for size determination. Gels were transferred to nitrocellulose membranes (Amersham, NJ, USA) at 20 V, room temperature, for 120 min, and were blocked at room temperature with 5% non-fat dry milk and 0.5% bovine serum albumin for 2 h. They were incubated at 4℃ overnight with a mix of rabbit polyclonal anti-PR (Neo Markers, Fremont CA) and mouse monoclonal anti-PR (AB-52, sc-810, Santa Cruz, CA, USA) (1 μg/mL each), both antibodies recognize PR-A and PR-B isoforms with similar affinity[19]; 1 μg/mL rabbit polyclonal anti-RE-alpha (Santa Cruz sc-542), 1 μg/mL goat polyclonal anti ER-beta (Y-19, sc-6821, Santa Cruz) or goat polyclonal anti ER-beta (N-19, sc-6820, Santa Cruz) antibodies. Blots were then incubated with a 1:1500 dilution of goat anti-mouse IgG, donkey anti-rabbit IgG or donkey anti-goat IgG antibodies conjugated to horseradish peroxidase (Santa Cruz) for 1 hour at room temperature and detected by enhanced chemiluminescence (ECL, Amersham, NJ, USA).

To correct for differences in the amount of total protein loaded in each lane, blots were stripped with glycine (0.1 mol/L, pH 2.5, 0.5% SDS) overnight at 4℃, and 30 min at 37℃, and reprobed with 1 μg/mL of mouse anti-alpha-tubulin antibody (Santa Cruz) at room temperature for 2 h. Blots were incubated with a 1:1500 dilution goat anti-mouse IgG conjugated to horseradish peroxidase (Santa Cruz) for 1 h. Signals were detected by ECL. The intensity of ER and PR isoforms and alpha-tubulin signals was quantified by densitometry using the Scion Image software (Scion Corp., Maryland, USA).

Statistical analysis

Data were analyzed using one-way ANOVA and Student's-*t* test for group comparisons, after homogeneity of variances was tested. A $P < 0.05$ value was considered as statistically significant. The analysis was performed using SPSS 12.0 for Windows.

RESULTS

We were able to distinguish two bands of 120 and 85 KDa corresponding to PR-B and PR-A isoforms, respectively, and a 66 KDa band corresponding to ER-alpha isoform in the uteri and stomach of the female gerbil (Figure 1), even when ER and PR have not been sequenced or identified before in gerbils tissues and there are no specific antibodies against these receptors in this species. Unfortunately, we were unable to detect the ER-beta isoform with the antibodies tested in this study in any gerbil tissue.

Gerbil uterine samples presented PR-A and PR-B in similar amounts. Interestingly, their expression was not regulated by E2 or P4 (Figure 2A). ER-alpha isoform in uteri (Figure 2B) was detected and, although there is a tendency to an increase after E2 treatment, this was not statistically significant.

In gastric antrum, PR isoforms were present in similar amounts and were not regulated by hormonal

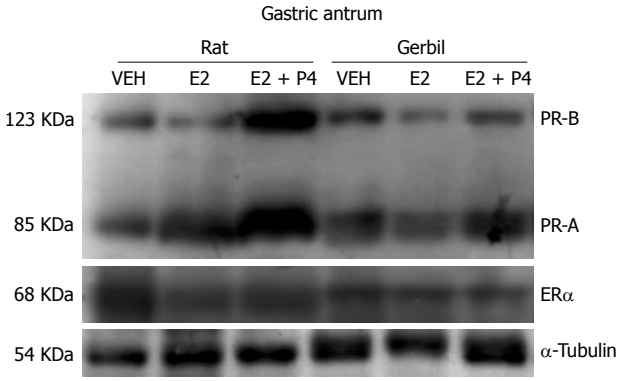


Figure 1 Estrogen and progesterone receptor isoforms expression in gastric tissue. PR isoforms and ER-alpha expressed in gerbil tissues are similar to those present in rats. Ovariectomized adult female gerbils were treated with subcutaneous injections of vehicle (VEH), 17-beta-estradiol (E2) alone and E2+ progesterone (E2 + P4). Gastric antrum lysates were immunoblotted with commercially available antibodies for ER-alpha, and PR. Equally treated rat tissues were used as positive control. The images show representative blots of gastric antrum of rat and gerbil.

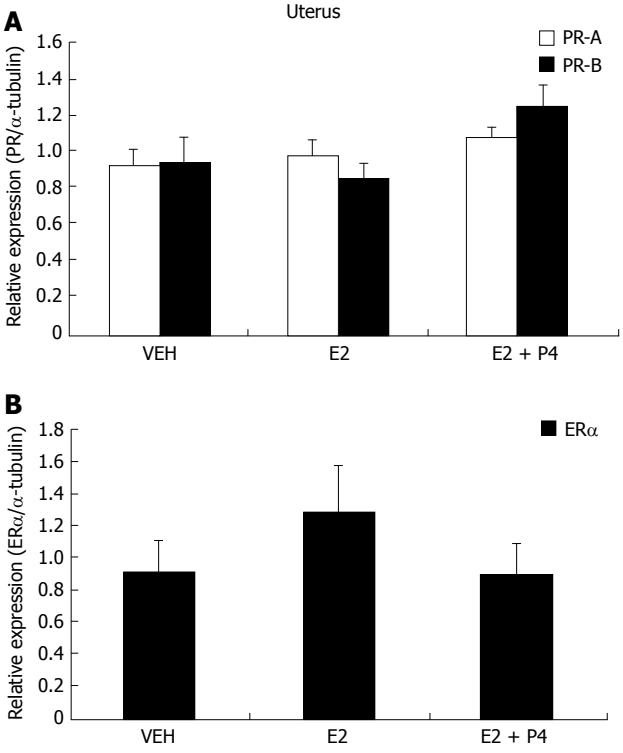


Figure 2 Sex steroid receptors content in gerbil uterus. PR isoforms (**A**) and ERα (**B**) in gerbil uterine lysates is shown. Ovariectomized adult female gerbils were treated with subcutaneous injections of VEH, E2 and E2 + P4. Receptors were detected by immunoblotting and content was adjusted to alpha-tubulin expression (n = 5). Data are mean ± SE.

treatments (Figure 3A). ER-alpha isoform was also present in antrum and showed a tendency to be increased under E2 and E2 + P4 treatments, however, this was not significant (Figure 3B).

Similar results were obtained for PR isoforms in gastric corpus (Figure 4A). Interestingly, ER-alpha isoform in this tissue was significantly diminished after E2 treatment, and this effect was blocked by P4 (Figure 4B).

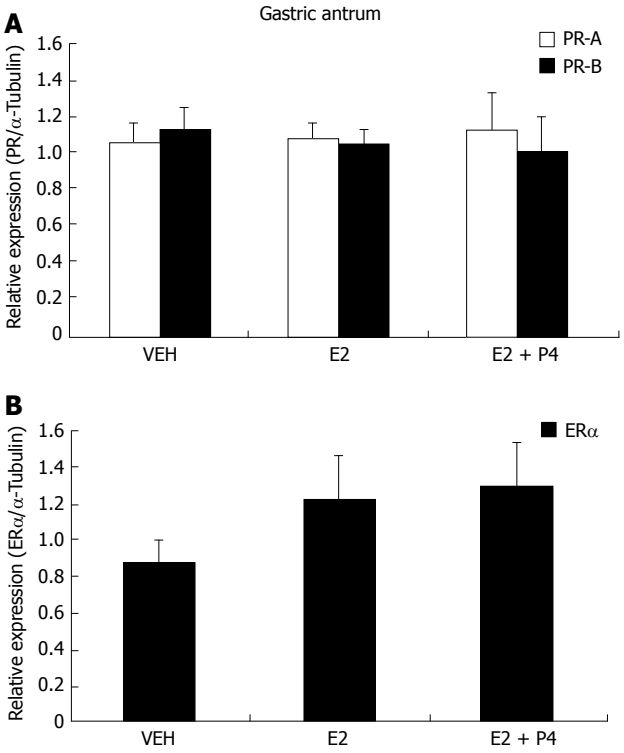


Figure 3 Sex steroid receptors content in gerbil gastric antrum. PR isoforms (**A**) and ER-alpha (**B**) in gerbil gastric antrum lysates is shown. Ovariectomized adult female gerbils were treated with subcutaneous injections of VEH, E2 and E2 + P4. Receptors were detected by immunoblotting and content was adjusted to alpha-tubulin expression (n = 5). Data are mean ± SE.

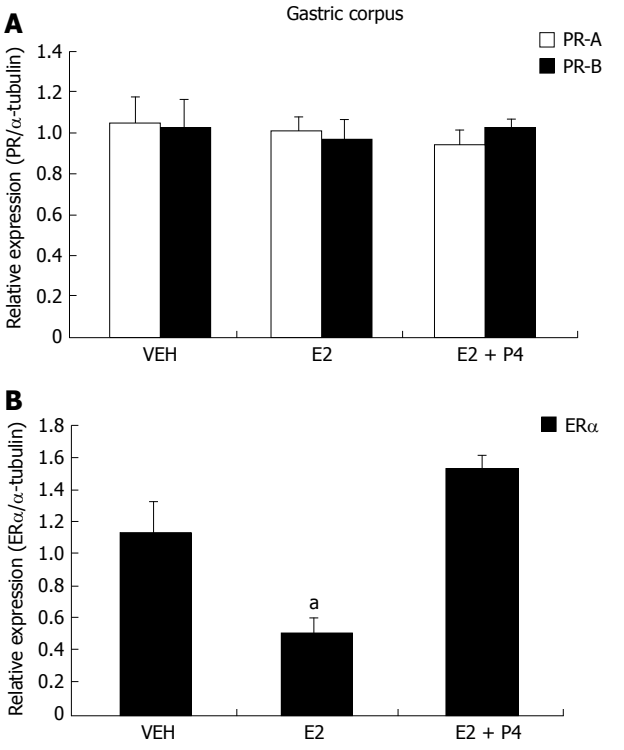


Figure 4 Sex steroid receptors content in gerbil gastric corpus. PR-A (**A**) and PR-B (**B**) ER-alpha in gerbil gastric corpus lysates is shown. Ovariectomized adult female gerbils were treated with subcutaneous injections of VEH, E2 and E2 + P4. Receptors were detected by immunoblotting and content was adjusted to alpha-tubulin expression (n = 5). Data are mean ± SE. $^{a}P < 0.05$ *vs* VEH and E2 + P4.

The antibodies used for Western blot analysis were tested for immunohistochemical staining in both uterine and gastric gerbil tissues, but we were not able to detect ER and PR using this approach (data not shown).

DISCUSSION

Mongolian gerbils (*Meriones unguiculatus*) have become a regular laboratory species, mainly employed in neuronal damage models. However, since 1998, when the *H pylori* infection model was described[12,20], showing persistent *H pylori* infection, and similar gastric damage to that observed in human stomach, their use in research has been constantly increasing.

To our knowledge, this is the first study in evaluating the presence of ER and PR in gerbils. We demonstrated the presence of both PR (PR-A and PR-B) isoforms and ER-alpha in gerbils uteri and stomach. These receptors are similar enough to rat receptors since they can be recognized by commercially available antibodies, rendering same size bands than those observed for rat ER-alpha and PR isoforms.

ER-alpha and ER-beta isoforms have been identified in different tissues and their expression is tissue and species-specific. In rat uteri, ER-alpha expression is down-regulated by E2[21]. P4 also down-regulates ER-alpha expression in rat uteri, but increases ER-beta expression in the decidual tissue[10,21,22].

Both PR-A and PR-B are present in rodents, primates and birds[10,23,24], except for rabbits, where only PR-B isoform has been detected[25]. In humans and rats, both PR isoforms are encoded by the same gene, but transcribed from two different promoter sequences[26,27]. ER and PR isoforms in Mongolian gerbils have not been sequenced to date.

In the gerbil uterus, we observed a similar content of both PR isoforms, but they were not regulated by E2 and P4. This lack of regulation contrasts with the up- and down-regulation of PR expression by E2 and P4, respectively, reported in rat uterus[17].

There was a tendency of E2 treatment to increase ER-alpha content, but it was not significantly different. Since sexual hormone levels in gerbils have not been characterized, it is not known yet whether other hormone doses in gerbils are necessary to regulate ER and PR content.

To date, there are no reports on the regulation of the ER and PR isoforms in either the human or murine stomach; however, it is well known that this regulation is tissue-specific. In our previous study, we demonstrated an important role for E2 and P4 in the Mongolian gerbil gastric mucosa in response to early *H pylori* infection influencing the inflammatory response, proliferation, apoptosis and gastrin producing cell number in the gastric antrum[11]. Although the mechanisms involved in those effects are still unclear, the presence of ER-alpha and both PR isoforms in gastric tissue suggest that their effects are mediated by these receptors.

We have detected ER-alpha and both PR isoforms in gerbil gastric antrum and corpus. In the antrum, none of the receptors was regulated by E2 and P4, but in gastric corpus we observed a down-regulation by E2. Thus, our results show that ER-alpha regulation also depends on the type of cells present in the mucosa. The mechanisms involved in the lack of regulation of PR in gerbil stomach, as well as the differential regulation of ER-alpha by E2, deserve further research.

Parietal cells, the acid producing cells characteristic of gastric corpus, have steroidogenic activity. These cells synthesize E2, P4, testosterone and their metabolites and, therefore, an important role of sexual hormones in hepatic-gastric axis regulation has been suggested[28]. It has been shown that E2 participates in gastric mucous production, epithelial cell exchange, and induces G-cells to release gastrin. E2 produced in stomach is probably processed in the liver[28-30]. Parietal cells are localized only in gastric corpus, thus, it is possible for cells in the corpus to be more sensitive or prone to E2 regulation on ER as compared with antral cells. It is necessary to explore the specific cell types expressing these receptors in both corpus and antrum.

PR and ER expression has been reported in normal human stomach, in human gastric cancer samples[31,32] and in gastric cell lines[33-35]. Also, the differential expression of ER isoforms alpha and beta has been reported in intact rat antrum and corpus, with a higher expression of ER-beta both in human[31,32] and rat[36,37] stomach. In our study, we were unable to detect ER-beta probably due to a lack of cross reactivity of the tested antibodies with this gerbil receptor.

In conclusion, PR isoforms and ER-alpha are expressed in gastric tissue of female gerbils and these receptors are probably involved in E2 and P4 actions in the stomach.

COMMENTS

Background

Sex steroid hormones, estradiol and progesterone in particular, have multiple effects that have been widely described. These hormones act either *via* their nuclear receptors or activating second-messengers pathways. We, and others, have previously reported effects of estradiol and progesterone in the stomach. In this work, we studied the presence of estrogens receptor alpha isoform and progesterone receptors isoforms A and B, and their regulation by their respective ligands in Mongolian gerbils.

Research frontiers

Estradiol and progesterone have shown to have effects on the stomach response to damage and *Helicobacter pylori (H pylori)* infection; Mongolian gerbils are a very useful model for studying the latter. However, the general information available on Mongolian gerbils genome and proteome is scarce. This study will provide basis for further studies on the role of sex steroids on *H pylori* infection and gastric cancer.

Innovations and breakthroughs

In this study, we reported the presence of estrogen receptor alpha isoform, and progesterone receptor A and B isoforms in Mongolian gerbil antrum and corpus, as well as their regulation. The results showed that these receptors are detectable in whole tissue protein with commercially available antibodies, and that the regulation of sex steroid receptors in the stomach is tissue specific.

Applications

This study provides support to the possible genomic mechanism of action of sex steroid hormones in the stomach. Also, it describes an accessible method to detect the presence of these receptors in gastric tissue.

Peer review

In this manuscript, the authors examined the expression of receptors for estrogen (ERs) and progesterone (PRs) in ovarectomized female gerbils. PR isoforms and ER-alpha are expressed in gastric tissue of female gerbils. In humans, male are more prone than female to *H pylori*-related diseases such as peptic ulcer diseases and gastric cancers. These gender-related differences appear diminished after menopausal age. This paper is of special value in considering some interesting and unknown aspects of gastric physiology.

REFERENCES

1 **Barfield MA**, Beeman EA. The oestrous cycle in the Mongolian gerbil, Meriones unguiculatus. *J Reprod Fertil* 1968; **17**: 247-251
2 **Nishino N**, Totsukawa K. Study on the estrous cycle in the Mongolian gerbil (Meriones unguiculatus). *Exp Anim* 1996; **45**: 283-288
3 **Cunha GR**, Cooke PS, Kurita T. Role of stromal-epithelial interactions in hormonal responses. *Arch Histol Cytol* 2004; **67**: 417-434
4 **Peluso JJ**. Multiplicity of progesterone's actions and receptors in the mammalian ovary. *Biol Reprod* 2006; **75**: 2-8
5 **Yamashita S**. Localization and functions of steroid hormone receptors. *Histol Histopathol* 1998; **13**: 255-270
6 **Gustafsson JA**. Estrogen receptor beta--getting in on the action? *Nat Med* 1997; **3**: 493-494
7 **Green S**, Walter P, Kumar V, Krust A, Bornert JM, Argos P, Chambon P. Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature* 1986; **320**: 134-139
8 **Kuiper GG**, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* 1996; **93**: 5925-5930
9 **Anzaldua SR**, Camacho-Arroyo I, Reyna-Neyra A, Perez-Martinez M, Cerbon M. Regional differences in expression of progesterone receptor in oviduct and uterus of rabbit during early pregnancy. *Comp Biochem Physiol A Mol Integr Physiol* 2007; **147**: 685-690
10 **Camacho-Arroyo I**, Gonzalez-Arenas A, Gonzalez-Moran G. Ontogenic variations in the content and distribution of progesterone receptor isoforms in the reproductive tract and brain of chicks. *Comp Biochem Physiol A Mol Integr Physiol* 2007; **146**: 644-652
11 **Saqui-Salces M**, Rocha-Gutierrez BL, Barrios-Payan JA, Ruiz-Palacios G, Camacho-Arroyo I, Gamboa-Dominguez A. Effects of estradiol and progesterone on gastric mucosal response to early Helicobacter pylori infection in female gerbils. *Helicobacter* 2006; **11**: 123-130
12 **Ohtani M**, Garcia A, Rogers AB, Ge Z, Taylor NS, Xu S, Watanabe K, Marini RP, Whary MT, Wang TC, Fox JG. Protective role of 17 beta -estradiol against the development of Helicobacter pylori-induced gastric cancer in INS-GAS mice. *Carcinogenesis* 2007; **28**: 2597-2604
13 **Campbell-Thompson M**, Lauwers GY, Reyher KK, Cromwell J, Shiverick KT. 17Beta-estradiol modulates gastroduodenal preneoplastic alterations in rats exposed to the carcinogen N-methyl-N'-nitro-nitrosoguanidine. *Endocrinology* 1999; **140**: 4886-4894
14 **Aguwa CN**. Effects of exogenous administration of female sex hormones on gastric secretion and ulcer formation in the rat. *Eur J Pharmacol* 1984; **104**: 79-84
15 **Machowska A**, Szlachcic A, Pawlik M, Brzozowski T, Konturek SJ, Pawlik WW. The role of female and male sex hormones in the healing process of preexisting lingual and gastric ulcerations. *J Physiol Pharmacol* 2004; **55** Suppl 2: 91-104
16 **Montoneri C**, Drago F. Effects of pregnancy in rats on cysteamine-induced peptic ulcers: role of progesterone. *Dig Dis Sci* 1997; **42**: 2572-2575
17 **Gonzalez-Arenas A**, Villamar-Cruz O, Guerra-Araiza C, Camacho-Arroyo I. Regulation of progesterone receptor isoforms expression by sex steroids in the rat lung. *J Steroid Biochem Mol Biol* 2003; **85**: 25-31
18 **Bradford MM**. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248-254
19 **Estes PA**, Suba EJ, Lawler-Heavner J, Elashry-Stowers D, Wei LL, Toft DO, Sullivan WP, Horwitz KB, Edwards DP. Immunologic analysis of human breast cancer progesterone receptors. 1. Immunoaffinity purification of transformed receptors and production of monoclonal antibodies. *Biochemistry* 1987; **26**: 6250-6262
20 **Ikeno T**, Ota H, Sugiyama A, Ishida K, Katsuyama T, Genta RM, Kawasaki S. Helicobacter pylori-induced chronic active gastritis, intestinal metaplasia, and gastric ulcer in Mongolian gerbils. *Am J Pathol* 1999; **154**: 951-960
21 **Varayoud J**, Ramos JG, Monje L, Bosquiazzo V, Munoz-de-Toro M, Luque EH. The estrogen receptor alpha sigma3 mRNA splicing variant is differentially regulated by estrogen and progesterone in the rat uterus. *J Endocrinol* 2005; **186**: 51-60
22 **Tessier C**, Deb S, Prigent-Tessier A, Ferguson-Gottschall S, Gibori GB, Shiu RP, Gibori G. Estrogen receptors alpha and beta in rat decidua cells: cell-specific expression and differential regulation by steroid hormones and prolactin. *Endocrinology* 2000; **141**: 3842-3851
23 **Guerra-Araiza C**, Villamar-Cruz O, Gonzalez-Arenas A, Chavira R, Camacho-Arroyo I. Changes in progesterone receptor isoforms content in the rat brain during the oestrous cycle and after oestradiol and progesterone treatments. *J Neuroendocrinol* 2003; **15**: 984-990
24 **Guerra-Araiza C**, Cerbon MA, Morimoto S, Camacho-Arroyo I. Progesterone receptor isoforms expression pattern in the rat brain during the estrous cycle. *Life Sci* 2000; **66**: 1743-1752
25 **Loosfelt H**, Atger M, Misrahi M, Guiochon-Mantel A, Meriel C, Logeat F, Benarous R, Milgrom E. Cloning and sequence analysis of rabbit progesterone-receptor complementary DNA. *Proc Natl Acad Sci USA* 1986; **83**: 9045-9049
26 **Kastner P**, Krust A, Turcotte B, Stropp U, Tora L, Gronemeyer H, Chambon P. Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. *EMBO J* 1990; **9**: 1603-1614
27 **Kraus WL**, Katzenellenbogen BS. Regulation of progesterone receptor gene expression and growth in the rat uterus: modulation of estrogen actions by progesterone and sex steroid hormone antagonists. *Endocrinology* 1993; **132**: 2371-2379
28 **Ueyama T**, Shirasawa N, Numazawa M, Yamada K, Shelangouski M, Ito T, Tsuruo Y. Gastric parietal cells: potent endocrine role in secreting estrogen as a possible regulator of gastro-hepatic axis. *Endocrinology* 2002; **143**: 3162-3170
29 **Le Goascogne C**, Sananes N, Eychenne B, Gouezou M, Baulieu EE, Robel P. Androgen biosynthesis in the stomach: expression of cytochrome P450 17 alpha-hydroxylase/17,20-lyase messenger ribonucleic acid and protein, and metabolism of pregnenolone and progesterone by parietal cells of the rat gastric mucosa. *Endocrinology* 1995; **136**: 1744-1752
30 **Ueyama T**, Shirasawa N, Ito T, Tsuruo Y. Estrogen-producing steroidogenic pathways in parietal cells of the rat gastric mucosa. *Life Sci* 2004; **74**: 2327-2337
31 **Singh S**, Poulsom R, Wright NA, Sheppard MC, Langman MJ. Differential expression of oestrogen receptor and oestrogen inducible genes in gastric mucosa and cancer. *Gut* 1997; **40**: 516-520
32 **Matsuyama S**, Ohkura Y, Eguchi H, Kobayashi Y, Akagi K, Uchida K, Nakachi K, Gustafsson JA, Hayashi S.

Estrogen receptor beta is expressed in human stomach adenocarcinoma. *J Cancer Res Clin Oncol* 2002; **128**: 319-324

33 **Karat D**, Brotherick I, Shenton BK, Scott D, Raimes SA, Griffin SM. Expression of oestrogen and progesterone receptors in gastric cancer: a flow cytometric study. *Br J Cancer* 1999; **80**: 1271-1274

34 **Wu CW**, Chang YF, Yeh TH, Chang TJ, Lui WY, P'Eng FK, Chi CW. Steroid hormone receptors in three human gastric cancer cell lines. *Dig Dis Sci* 1994; **39**: 2689-2694

35 **Wu CW**, Chi CW, Chang TJ, Lui WY, P'eng FK. Sex hormone receptors in gastric cancer. *Cancer* 1990; **65**: 1396-1400

36 **Campbell-Thompson ML**. Estrogen receptor alpha and beta expression in upper gastrointestinal tract with regulation of trefoil factor family 2 mRNA levels in ovariectomized rats. *Biochem Biophys Res Commun* 1997; **240**: 478-483

37 **Gejima K**, Kawaguchi H, Souda M, Kawashima H, Komokata T, Hamada N, Umekita Y, Sakata R, Yoshida H. Expression of estrogen receptor-alpha protein in the rat digestive tract. *In Vivo* 2007; **21**: 487-492

S- Editor Zhong XY **L- Editor** Rippe RA **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5707

World J Gastroenterol 2008 October 7; 14(37): 5707-5711
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Effects of anatomical position on esophageal transit time: A biomagnetic diagnostic technique

Teodoro Cordova-Fraga, Modesto Sosa, Carlos Wiechers, Jose Maria De la Roca-Chiapas, Alejandro Maldonado Moreles, Jesus Bernal-Alvarado, Raquel Huerta-Franco

Teodoro Cordova-Fraga, Modesto Sosa, Carlos Wiechers, Jose Maria De la Roca-Chiapas, Alejandro Maldonado Moreles, Jesus Bernal-Alvarado, Instituto de Física, Universidad de Guanajuato, León 37150, Gto., Mexico
Raquel Huerta-Franco, Instituto de Investigaciones sobre el Trabajo, Universidad de Guanajuato, León 37150, Gto., Mexico
Author contributions: Cordova-Fraga T and Sosa M designed the study; De la Roca-Chiapas JM and Huerta-Franco R wrote the manuscript; Wiechers C, Maldonado Moreles A and Bernal-Alvarado J contributed in data collection and conducted research; Huerta-Franco R and De la Roca-Chiapas JM contributed in analytic input and data analysis.
Supported by CONACYT grant, No. J51082
Correspondence to: Jose Maria De la Roca-Chiapas, Instituto de Física, Universidad de Guanajuato, Loma del Bosque 103, Lomas del Campestre, León 37150, Gto., Mexico. joseroca@fisica.ugto.mx
Telephone: +52-477-7885100 Fax: +52-477-7885100-8410
Received: May 10, 2008 Revised: August 11, 2008
Accepted: August 18, 2008
Published online: October 7, 2008

Abstract

AIM: To study the esophageal transit time (ETT) and compare its mean value among three anatomical inclinations of the body; and to analyze the correlation of ETT to body mass index (BMI).

METHODS: A biomagnetic technique was implemented to perform this study: (1) The transit time of a magnetic marker (MM) through the esophagus was measured using two fluxgate sensors placed over the chest of 14 healthy subjects; (2) the ETT was assessed in three anatomical positions (at upright, fowler, and supine positions; 90º, 45º and 0º, respectively).

RESULTS: ANOVA and Tuckey post-hoc tests demonstrated significant differences between ETT mean of the different positions. The ETT means were 5.2 ± 1.1 s, 6.1 ± 1.5 s, and 23.6 ± 9.2 s for 90º, 45º and 0º, respectively. Pearson correlation results were r = -0.716 and $P < 0.001$ by subjects' anatomical position, and r = -0.024 and $P > 0.05$ according the subject's BMI.

CONCLUSION: We demonstrated that using this biomagnetic technique, it is possible to measure the ETT and the effects of the anatomical position on the ETT.

Key words: Transit time; Magnetic marker; Esophagus; Anatomical position; Non-invasive

Peer reviewer: Michael F Vaezi, Department of Gastroenterology and Hepatology, Vanderbilt University Medical Center, 1501 TVC, Nashville, TN 37232-5280, United States

Cordova-Fraga T, Sosa M, Wiechers C, De la Roca-Chiapas JM, Maldonado Moreles A, Bernal-Alvarado J, Huerta-Franco R. Effects of anatomical position on esophageal transit time: A biomagnetic diagnostic technique. *World J Gastroenterol* 2008; 14(37): 5707-5711 Available from: URL: http://www.wjgnet.com/1007-9327/14/5707.asp DOI: http://dx.doi.org/10.3748/wjg.14.5707

INTRODUCTION

The esophageal phase is the last phase in the swallow process; it includes the propulsion of the meal through the esophagus toward the stomach. The esophageal transit time (ETT) reported for solid and semisolid meals is between 4 and 8 s, whereas liquid ETT lasts approximately 1 to 2 s in healthy people[1]. A diagnosis of gastroesophageal reflux disease should include the presence of a pathological reflux in patients lacking another motility disorder or damage in the esophagus[2,3]. If this condition can not be met, then the evaluation should include the assessment of disintegration time of oral tablets before they enter the stomach[4]. Currently, diagnosis of gastroesophageal reflux diseases is made with endoscopy[5,6], manometry[3], imaging methods[7], impedance[8], scintigraphy[9] and other techniques[2,10]. These assessments are useful to quantify the liquid and solid volumes retained in the esophagus. Currently, the scintigraphic technique is the gold standard test accepted to assess ETT and it is indicated in cases where the manometric and barometric studies do not give a differential diagnosis[11].

The ETT assessment is used to complete the diagnosis of diseases, such as gastroesophageal reflux[12-14], dysphagia[15,16], esophagitis[17,18], and achalasia[6,19,20]. The latter studies are commonly performed with scintigraphic and manometric techniques[21], in healthy[22], geriatric[23], and pediatric patients[24], despite the use of ionizing radiation and catheters in each test, respectively. Recently, several assessments were performed using the biomagnetic technique, including gastric emptying time[25] and colon

transit time[26]. These studies have the advantage of being non-invasive, comfortable for the patient, and do not use ionizing radiation. Daghastanli *et al*[27] in 1998 reported an ETT study carried out with a biosusceptometer and a magnetic tracer, where they used 5 g of manganese (Mn) and ferrite power. In their study, they also measured the ETT using the scintigraphy technique and found that the ETT was 4.6 ± 0.9 s when biomagnetic technique was used, in comparison to a time of 3.8 ± 0.8 s as measured by the scintigraphy technique. The results of these studies demonstrated the efficacy of the magnetic techniques to carry out the ETT.

In this study, we implemented a novel modality of the biomagnetic technique using modern instruments, which included the monitoring of a magnetic marker (MM) traveling though the length of the esophagus. This was done using a pair of fluxgate magnetometers. We hypothesized that the esophagus motility and the mean ETT are significantly different when they are tested as a function of different anatomical positions of a subject (supine = 0°; fowler = 45°, and upright = 90°, respectively) (Figure 1).

MATERIALS AND METHODS

In Figure 1, we present a schematic set up of the biomagnetic probe used, which consisted of two digital tri-axis fluxgate magnetometers which were placed in an electronic device. They were separated 19 cm and were positioned in a line along the esophagus, just above the subjects' thorax (Figure 2). A 3-mm long and 4-mm high magnet was used as the biomagnetic source or MM. This magnet was enclosed in a polycarbonate sphere 6 mm in diameter (Figure 3) in order to avoid chemical reactions with the gastric acids.

Subjects

Fourteen normal and healthy adult subjects (10 men and 4 women) participated in the study; they did not have clinical antecedents of esophageal or gastrointestinal disease. The subject's mean age and body mass index (BMI) were 21.8 ± 1.5 years and 23.9 ± 2.7 kg/m^2, respectively. All volunteers received instructions before starting the experiment and signed an informed consent approved by the Institutional Review Board of our institution. The experiment was carried out according to the Declaration of Helsinki.

Procedure

Subjects were studied after fasting for 12 h. All of them were assessed in three anatomical positions: (1) upright position, 90°; (2) fowler position, this is a semi-laying position bent at 45°; and (3) supine position, this is a laying position with a bend of 0° (Figure 1). The MM or magnetic particle was introduced inside the mouth of the subjects and swallowed with 20 mL of yogurt (50 kcal), this substance was used as MM vehicle.

Data collection and signal processing

The magnetic signal was registered for 1 min, with a sampling rate of 30 samples/s. Data acquisition was carried out using a routine informatic implemented with software of LabVIEW 7 platform. Then, collected data were exported and graphically analyzed in Matlab 6.5 in order to measure the ETT.

Statistical analysis

We calculated the mean age and BMI of the subjects using descriptive statistics. Using one way ANOVA and Tuckey post-hoc test, we compared the differences among the mean ETT obtained when subjects adopted each of the three anatomical positions. A Pearson correlation was used to determine the correlation coefficient between the ETT and the subject's age, BMI and the angle of inclination of the anatomical position. $P < 0.05$ was considered statistically significant.

RESULTS

Figure 4 shows the raw signals recorded from one subject in three anatomical positions. The time signal shown as a continuous line is the recording with the fluxgate magnetometer added in the upper part of the esophagus, while the dashed line corresponds to the fluxgate magnetometer in the bottom part of the esophagus. The different time between the dominant peaks of each raw signal gives the ETT in each case. In Figure 4, it shows the raw recordings carried out in each anatomical position of one subject. We estimate that the differences in the time seen here was typical of all subjects.

Figure 5 demonstrates the mean and standard deviation values of the ETTs, which were significantly longer at 0° (23.6 ± 9.2 s) than at 45° (6.1 ± 1.5 s), and 90° (5.18 ± 1.8 s). The results of the ANOVA and Tuckey post-hoc test demonstrated the significant differences between the groups (Figure 5). Pearson correlation coefficient test demonstrated an indirect relationship between anatomical position and ETT. This means that when subjects adopt a greater angle of inclination, they will have shorter ETT values. This relationship had a coefficient of $r = -0.716$, $P < 0.001$. However, we did not find any statistically significant difference between the EET and weight, age or BMI.

DISCUSSION

The esophageal phase is the last phase in the swallow process; it includes the propulsion of the meal through the esophagus toward the stomach. The ETT reported for solid and semisolid meals is between 4 and 8 s, whereas liquid ETT lasts approximately 1 to 2 s in healthy people[1]. Using the biomagnetic technique, we demonstrated that ETT is affected by anatomical position, with a significantly larger transit time when subjects adopted an upright position. These results concur with previous reports[1], including studies in which a biosusceptometer magnetometer was used[27].

Previously, researchers reported that ETT in healthy individuals was approximately 4-8 s for solid and semisolid meals, and 1-2 s for liquid meals[11,28,29]. In

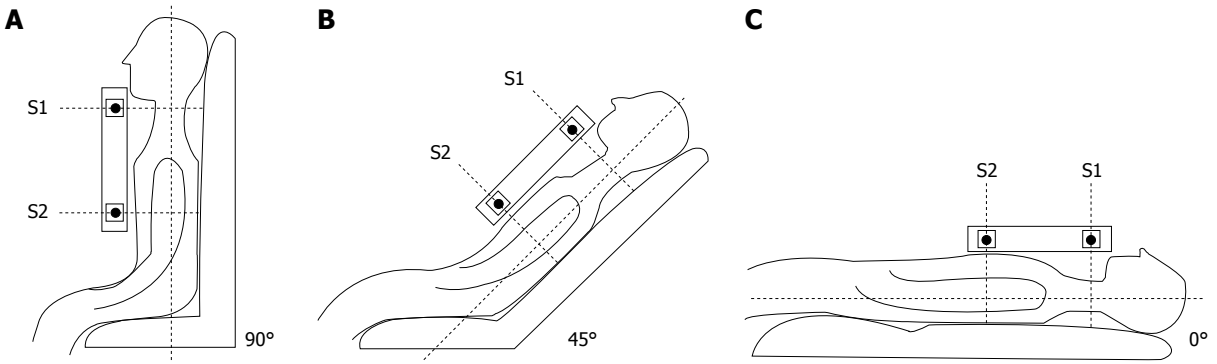


Figure 1 Measurement of ETT at different anatomical positions. **A**: 90° (upright); **B**: 45° (fowler); **C**: 0° (supine). The positions of the magnetic sensors are presented (S1 = sensor one, and S2 = sensor 2).

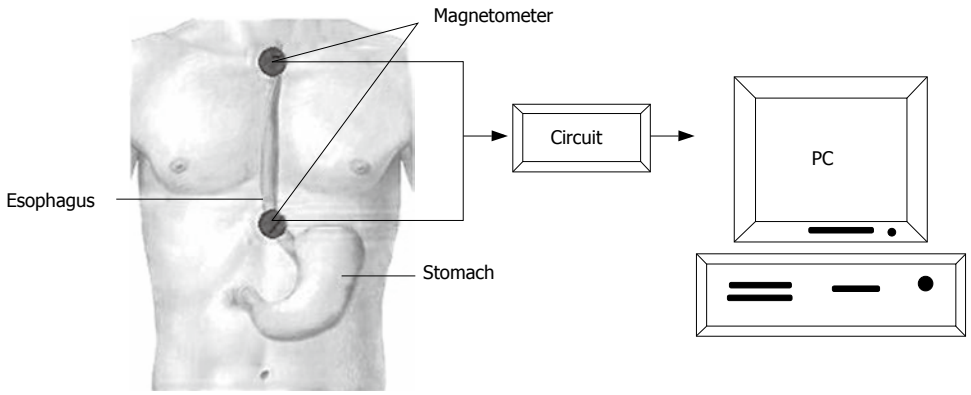


Figure 2 The schematic of the setup, anatomical location of the esophagus, and the circuits for data collection.

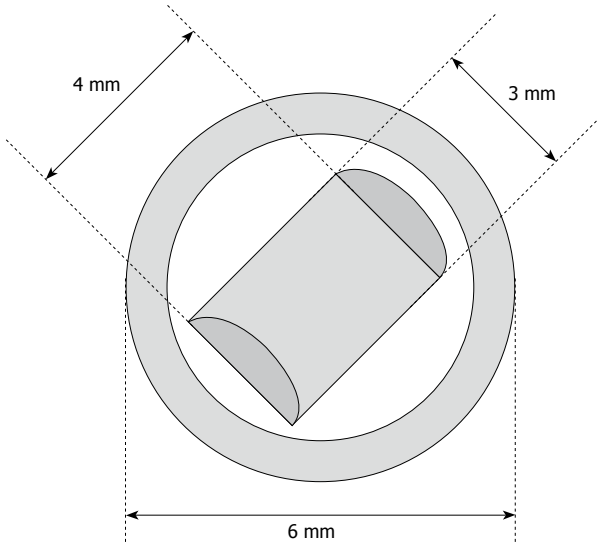


Figure 3 Cross-sectional profile of MM.

agreement with the aforementioned findings, our study demonstrated that the mean ETT was 6.1 ± 1.5 s in the upright position and 5.18 ± 1.8 s in the fowler position. However, the only value which was inconsistent with previous reports was that of the supine position, a transit time of 23.6 ± 9.2 s. This can be explained by the effects of gravity on the test meal and magnets.

Our study demonstrated that ETT is affected by gravity, and therefore, the subjects' anatomical position changes ETT. This phenomenon is explained by the physiology of the esophagus, which combines resistance and contraction to cause movement of the bolus or liquid. When gravity also contributes to the propulsion of the bolus or liquid through the esophagus, transit time is decreased and esophageal transit rates are increased.

Using this biomagnetic modality, we demonstrated that ETT varies depending on the subjects' anatomical position. In this study, we found no relationship between ETT and the subjects' age, which can be explained by the fact that we generally evaluated only young and healthy subjects (mean age: 21.8 ± 1.5 years). However, more studies assessing the ETT in older subjects and patients with different pathologies, such as gastroesophageal reflux[12-14], dysphagia[15,16], esophagitis[17,18], and achalasia[6,19,20], are necessary to determine the differences in ETT of healthy subjects *versus* patients. It is likely that the ETT will differ in patients with a clinical diagnosis of esophageal disease.

In this study, we found no significant correlation between ETT and the subject's BMI, which also may be explained by the samples from a largely homogeneous group of healthy and non-obese subjects (BMI: 23.9 ± 2.7 kg/m^2). Therefore, in order to demonstrate the relationship of BMI and ETT, additional studies are needed using males and females with BMI within normal and obese ranges. Other clinical applications of biomagnetic technique exist in gastro-pharmacology

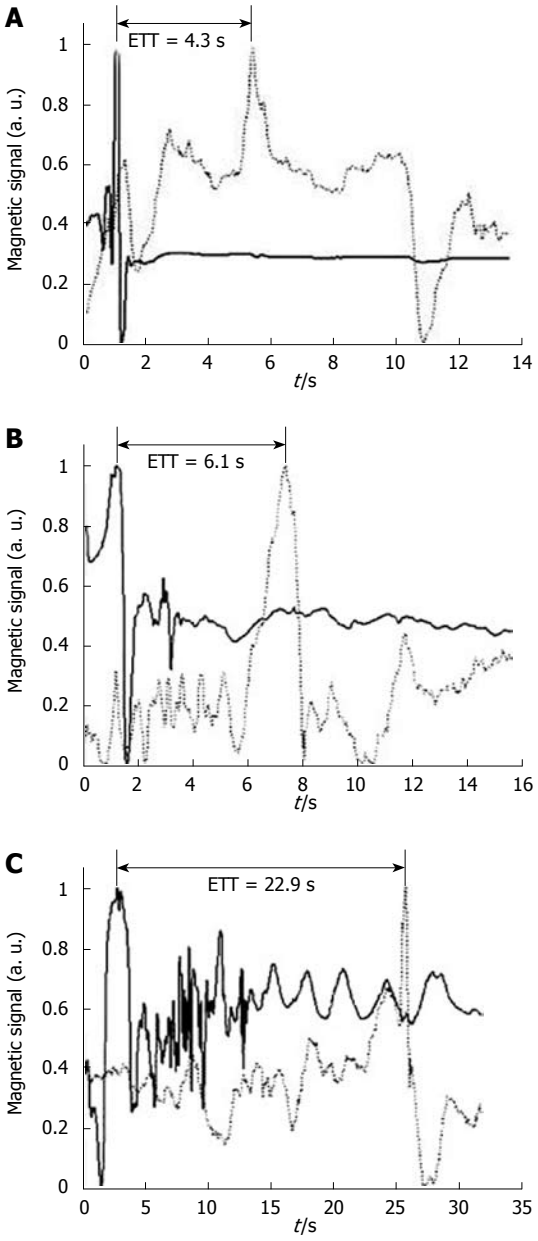


Figure 4 This series of graph showing the raw signal recorded from a single representative subject lying in the three angles of inclination: 90° (**A**), 45° (**B**) and 0° (**C**).

studies, where researchers are testing the efficacy of a drug to increase or decrease ETT.

An advantage of this new application of the biomagnetic technique, implemented for the measurement of ETT, is that it demands little space and hardware, since all that is needed is a low-cost magnetometer. Therefore, this technique could be implemented for clinical assessment of esophageal disorders in general practice medicine, for gastroenterologists studying drug transit time[4,11] and in other specialties. Additionally, because of its low cost and non-invasiveness, this technique could be implemented in small clinical areas and hospitals. Although this technique has already been validated[27], further studies are needed to compare biomagnetism with the most innovative and sophisticated techniques commonly used for esophageal evaluation in order to identify its sensitivity and reproducibility.

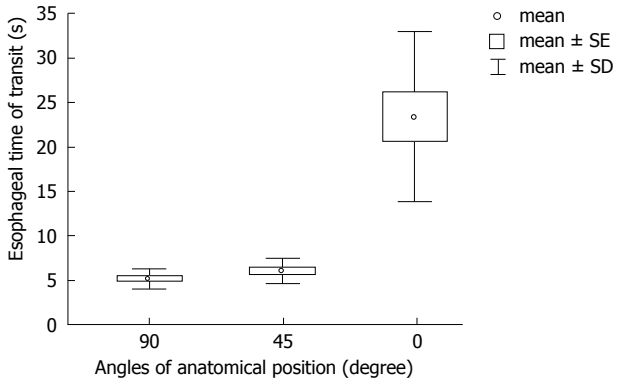


Figure 5 The results of one way ANOVA demonstrate statistically significant differences in the subjects' ETT between the supine position (0°) and both the fowler (45°) and the upright (90°) positions.

ACKNOWLEDGMENT

The authors would like to thank Kathleen M. Capaccione for review and revision of the manuscript.

COMMENTS

Background

Esophageal transit time (ETT) assessment is used to diagnose of gastroesophageal disease, such as esophagitis and achalasia. These studies are carried out using either scintigraphic or manometric techniques, but each has its own disadvantage: scintigraphy uses ionizing radiation, while manometry uses an invasive probe. Recently, several assessments of other body systems were performed using the biomagnetic technique (BMT), including ETT, gastric emptying time, and colon transit time. These studies have the advantage of being non-invasive, comfortable for the patient, and are conducted without ionizing radiation. Recently, researchers have tested the validity of BMT by comparing it with the scintigraphic technique (the gold standard test accepted to assess ETT) in the evaluation of ETT.

Research frontiers

In this study, authors implemented a novel use of the BMT using modern instruments, which included the monitoring of a magnetic marker (MM) traveling though the length of the esophagus. Using the BMT technique, they demonstrated that ETT varies depending on the subjects' anatomical position.

Innovations and breakthroughs

The Fluxgate magnetometer is a small device which functions at room temperature without a magnetic un-shielding room. Its range to precisely identify locations of a small MM is approximately a distance of less than 50 cm. It is possible to record magnetic signals through solid objects including the human body, and therefore, the technique is still valid if the MM is held in one's hand or is passing through the esophagus. Therefore, this is a portable evaluation system that can be implemented for the clinical study and diagnosis of esophegeal disorders in hospitals around the world.

Applications

A major application of this technique is the simultaneous collection of peristaltic activity data from different points along the esophagus. By a subject ingesting an MM similar in size and shape to a pill and following it as it passes through the esophageous by monitoring its progress at several sites along the esophagus, researchers can deduce the peristaltic behavior of the esophagus. Accordingly, ETT and propulsion velocity can accuratly be estimated using this technique.

Terminology

Body anatomical inclinations is an angle of the human body, such as standing upright (90º), fowler (45º) and supine positions (0º). Biomagnetic technique is a technique which uses either external or internal magnetic fields applied to biological materials. Biomagnetism is the phenomenon that involves magnetic fields produced by the human body and other living entities. It is to be distinguished from magnetic fields applied to the body, properly called magnetobiology. Biosusceptometer magnetometer is a probe instrument using room-temperature sensor(s) that can measure variations in magnetic

susceptibilities. MM means external magnetic substance or object used in this case as a vehicle to monitor the passage of an orally taken substance (food, drug, tablet, capsule, *etc*.) through the intestinal tract. Fluxgate sensor is a scientific instrument used to measure the strength and/or direction of the magnetic field in the vicinity of the instrument. Magnetic susceptibility means the magnetization of a material per unit applied field. It describes the magnetic response of a substance to an applied magnetic field.

Peer review

This is an interesting study. Authors used a BMT to monitor ETT to test the hypothesis that esophageal motility and the mean ETT are significantly different when subjects adopt different anatomical inclinations. With this technique, they demonstrated that the mean values of ETT vary depending on the subjects' anatomical inclination.

REFERENCES

1 **Ojeaburu J**, Benjamin SB. Normal Esophageal Fisiology. In: Antony JD, Stanley BB. Gastrointestinal Disease an Endoscopic Approach. New Jersey: Slack Incorporated, 2002: 173-190

2 **Mariani G**, Boni G, Barreca M, Bellini M, Fattori B, AlSharif A, Grosso M, Stasi C, Costa F, Anselmino M, Marchi S, Rubello D, Strauss HW. Radionuclide gastroesophageal motor studies. *J Nucl Med* 2004; **45**: 1004-1028

3 **Can MF**, Yagci G, Cetiner S, Gulsen M, Yigit T, Ozturk E, Gorgulu S, Tufan T. Accurate positioning of the 24-hour pH monitoring catheter: agreement between manometry and pH step-up method in two patient positions. *World J Gastroenterol* 2007; **13**: 6197-6202

4 **Perkins AC**, Blackshaw PE, Hay PD, Lawes SC, Atherton CT, Dansereau RJ, Wagner LK, Schnell DJ, Spiller RC. Esophageal transit and in vivo disintegration of branded risedronate sodium tablets and two generic formulations of alendronic acid tablets: a single-center, single-blind, six-period crossover study in healthy female subjects. *Clin Ther* 2008; **30**: 834-844

5 **Ahmadi A**, Draganov P. Endoscopic mucosal resection in the upper gastrointestinal tract. *World J Gastroenterol* 2008; **14**: 1984-1989

6 **Chung JJ**, Park HJ, Yu JS, Hong YJ, Kim JH, Kim MJ, Lee SI. A comparison of esophagography and esophageal transit scintigraphy in the evaluation of usefulness of endoscopic pneumatic dilatation in achalasia. *Acta Radiol* 2008; **49**: 498-505

7 **Gilja OH**, Hatlebakk JG, Odegaard S, Berstad A, Viola I, Giertsen C, Hausken T, Gregersen H. Advanced imaging and visualization in gastrointestinal disorders. *World J Gastroenterol* 2007; **13**: 1408-1421

8 **Blonski W**, Hila A, Jain V, Freeman J, Vela M, Castell DO. Impedance manometry with viscous test solution increases detection of esophageal function defects compared to liquid swallows. *Scand J Gastroenterol* 2007; **42**: 917-922

9 **Hike K**, Urita Y, Watanabe T, Sugimoto M, Miki K. Saliva transit from the oral cavity to the esophagus in GERD. *Hepatogastroenterology* 2008; **55**: 4-7

10 **Vergara MT**. Labotarorio en los trastornos motores del esófago. *Gastr Latinoam* 2005; **16**: 109-114

11 **Osmanoglou E**, Van Der Voort IR, Fach K, Kosch O, Bach D, Hartmann V, Strenzke A, Weitschies W, Wiedenmann B, Trahms L, Monnikes H. Oesophageal transport of solid dosage forms depends on body position, swallowing volume and pharyngeal propulsion velocity. *Neurogastroenterol Motil* 2004; **16**: 547-556

12 **Sidhu SS**, Bal C, Karak P, Garg PK, Bhargava DK. Effect of endoscopic variceal sclerotherapy on esophageal motor functions and gastroesophageal reflux. *J Nucl Med* 1995; **36**: 1363-1367

13 **Tolin RD**, Malmud LS, Reilley J, Fisher RS. Esophageal scintigraphy to quantitate esophageal transit (quantitation of esophageal transit). *Gastroenterology* 1979; **76**: 1402-1408

14 **Stanghellini V**, Tosetti C, Corinaldesi R. Standards for non-invasive methods for gastrointestinal motility: scintigraphy. A position statement from the Gruppo Italiano di Studio Motilita Apparato Digerente (GISMAD). *Dig Liver Dis* 2000; **32**: 447-452

15 **Tatum RP**, Shi G, Manka MA, Brasseur JG, Joehl RJ, Kahrilas PJ. Bolus transit assessed by an esophageal stress test in postfundoplication dysphagia. *J Surg Res* 2000; **91**: 56-60

16 **Zhu ZJ**, Chen LQ, Duranceau A. Long-term result of total versus partial fundoplication after esophagomyotomy for primary esophageal motor disorders. *World J Surg* 2008; **32**: 401-407

17 **Dantas RO**, Oliveira RB, Aprile LR, Hara SH, Sifrim DA. Saliva transport to the distal esophagus. *Scand J Gastroenterol* 2005; 40: 1010-1016

18 **De Jonge PJ**, Van Eijck BC, Geldof H, Bekkering FC, Essink-Bot ML, Polinder S, Kuipers EJ, Siersema PD. Capsule endoscopy for the detection of oesophageal mucosal disorders: a comparison of two different ingestion protocols. *Scand J Gastroenterol* 2008; **43**: 870-877

19 **Bonavina L**, Nosadini A, Bardini R, Baessato M, Peracchia A. Primary treatment of esophageal achalasia. Long-term results of myotomy and Dor fundoplication. *Arch Surg* 1992; **127**: 222-227

20 **Dogan I**, Mittal RK. Esophageal motor disorders: recent advances. *Curr Opin Gastroenterol* 2006; **22**: 417-422

21 **Mughal MM**, Marples M, Bancewicz J. Scintigraphic assessment of oesophageal motility: what does it show and how reliable is it? *Gut* 1986; **27**: 946-953

22 **Caleiro MT**, Lage LV, Navarro-Rodriguez T, Bresser A, da Costa PA, Yoshinari NH. Radionuclide imaging for the assessment of esophageal motility disorders in mixed connective tissue disease patients: relation to pulmonary impairment. *Dis Esophagus* 2006; **19**: 394-400

23 **Issa Okubo Pde C**, Dantas RO, Troncon LE, Moriguti JC, Ferriolli E. Clinical and scintigraphic assessment of swallowing of older patients admitted to a tertiary care geriatric ward. *Dysphagia* 2008; **23**: 1-6

24 **Warrington JC**, Charron M. Pediatric gastrointestinal nuclear medicine. *Semin Nucl Med* 2007; **37**: 269-285

25 **de la Roca-Chiapas JM**, Cordova T, Hernandez E, Solorio S, Solis S, Sosa M. Magnetogastrography (MGG) reproducibility assessments of gastric emptying on healthy subjects. *Physiol Meas* 2007; **28**: 175-183

26 **Cordova-Fraga T**, Carneiro AA, de Araujo DB, Oliveira RB, Sosa M, Baffa O. Spatiotemporal evaluation of human colon motility using three-axis fluxgates and magnetic markers. *Med Biol Eng Comput* 2005; **43**: 712-715

27 **Daghastanli NA**, Braga FJ, Oliveira RB, Baffa O. Oesophageal transit time evaluated by a biomagnetic method. *Physiol Meas* 1998; **19**: 413-420

28 **Wise JL**, Murray JA, Conklin JL. Regional differences in oesophageal motor function. *Neurogastroenterol Motil* 2004; **16**: 31-37

29 **Tutuian R**, Elton JP, Castell DO, Gideon RM, Castell JA, Katz PO. Effects of position on oesophageal function: studies using combined manometry and multichannel intraluminal impedance. *Neurogastroenterol Motil* 2003; **15**: 63-67

S- Editor Li DL **L- Editor** Kumar M **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5712

World J Gastroenterol 2008 October 7; 14(37): 5712-5716
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Esophageal epithelial surface in patients with gastroesophageal reflux disease: An electron microscopic study

Takane Azumi, Kyoichi Adachi, Kenji Furuta, Shuji Nakata, Shunji Ohara, Kenji Koshino, Masaharu Miki, Terumi Morita, Takashi Tanimura, Nobuo Ashizawa, Yoshikazu Kinoshita

Takane Azumi, Kyoichi Adachi, Kenji Furuta, Shuji Nakata, Shunji Ohara, Kenji Koshino, Masaharu Miki, Terumi Morita, Takashi Tanimura, Nobuo Ashizawa, Yoshikazu Kinoshita, Second Department of Internal Medicine, Shimane University School of Medicine, Shimane 693-8501, Japan
Author contributions: Azumi T and Adachi K contributed equally to this work; Adachi K, Ashizawa N and Kinoshita Y designed research; Azumi T, Adachi K, Furuta K, Nakata S, Ohara S, Koshino K, Miki M, Morita T, Tanimura T performed research; Azumi T and Adachi K contributed new reagents/analytic tools; Azumi T and Adachi K analyzed data; and Azumi T, Adachi K and Kinoshita Y wrote the paper.
Supported by The Grants-in-Aid from Science Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, No. 19590724
Correspondence to: Kyoichi Adachi, MD, PhD, Second Department of Internal Medicine, Shimane University School of Medicine, 89-1, Enya-cho, Izumo-shi, Shimane 693-8501, Japan. kadachi@med.shimane-u.ac.jp
Telephone: +81-853-202190 Fax: +81-853-202187
Received: August 3, 2008 Revised: September 16, 2008
Accepted: September 23, 2008
Published online: October 7, 2008

Abstract

AIM: To investigate the intercellular spaces between the most superficially located esophageal epithelial cells in patients with gastroesophageal reflux disease (GERD).

METHODS: Eighteen patients with erosive esophagitis, 10 patients with non-erosive reflux disease (NERD), and 18 normal asymptomatic volunteers were enrolled. Biopsy specimens were obtained from the lower esophageal mucosa without ulcer or erosion. Scanning electron microscopy was employed to investigate the tightness of the superficial cellular attachment.

RESULTS: The intercellular space between the most superficially located epithelial cells in patients with erosive esophagitis or NERD was not different from that in asymptomatic healthy individuals.

CONCLUSION: Widened luminal intercellular spaces of esophageal superficial epithelium are not responsible for the induction of reflux symptoms in patients with GERD.

Key words: Reflux esophagitis; Non-erosive gastroesophageal reflux diseases; Electron microscopy; Questionnaire

Peer reviewer: Gert De Hertogh, PhD, Department of Morphology and Molecular Pathology, University Hospitals KULeuven, Minderbroedersstraat 12, Leuven 3000, Belgium

Azumi T, Adachi K, Furuta K, Nakata S, Ohara S, Koshino K, Miki M, Morita T, Tanimura T, Ashizawa N, Kinoshita Y. Esophageal epithelial surface in patients with gastroesophageal reflux disease: An electron microscopic study. *World J Gastroenterol* 2008; 14(37): 5712-5716 Available from: URL: http://www.wjgnet.com/1007-9327/14/5712.asp DOI: http://dx.doi.org/10.3748/wjg.14.5712

INTRODUCTION

Gastroesophageal reflux disease (GERD) is a symptom complex caused by the reflux of gastric contents into the esophagus with and without complications[1,2]. Patients with esophageal complications, including esophageal erosions and ulcers, are reported to have a higher incidence of gastroesophageal acid reflux than those without complications[3,4]. The esophageal mucosa, composed of stratified squamous epithelium, has limited resistance against gastric juice because its secretion of mucus and bicarbonate is negligible in comparison with the gastric mucosa. Mucus is important for protecting the mucosal surface from pepsin, a protease present in gastric juice. Bicarbonate secretion into the mucus gel layer is necessary to neutralize hydrogen ions diffusing into it[5]. Therefore, when the esophageal mucosa is exposed to acidic gastric juice for a longer period, erosions and ulcers on the esophageal mucosa can easily be formed. GERD with these mucosal complications is known as reflux esophagitis (RE) and that without such complications is referred to as non-erosive reflux disease (NERD)[2]. Patients with NERD suffer significant reflux symptoms including heartburn and acid regurgitation, even in the absence of esophageal complications[2,6]. Afferent neurons with chemoreceptors, including transient receptor potential vanilloid type 1 (TRPV-1), are reported to terminate in the esophageal epithelial

intercellular spaces[7,8]. In healthy individuals, these spaces are narrow and hydrogen ions and pepsin have difficulty to penetrate into them to stimulate noxious receptors on nerve terminals[5,9]. In order for acid and pepsin to penetrate into the intercellular spaces, the intercellular adhesion of the uppermost squamous epithelial cells needs to be damaged or widened[5,9,10]. Several reports have described the widening of intercellular spaces between squamous epithelial cells in mid-stratified epithelial layers by using transmission electron microscopy (TEM)[9-12]. However, there has been no description of intercellular adhesion between cells located in the uppermost part of the stratified squamous epithelium, since intercellular spaces of esophageal superficial epithelium could not be observed by TEM. Thus, this study was designed to investigate the luminal surface of the esophageal squamous epithelium in patients with GERD by using scanning electron microscopy and to examine the relationship between the tightness of esophageal superficial epithelial adhesion and the presence or absence of reflux symptoms.

MATERIALS AND METHODS

Patients

Eighteen symptomatic patients with RE (male/female = 14/4, mean age 58), 10 patients with NERD (male/female = 3/4, mean age 61), and 18 normal volunteers without any gastrointestinal and reflux symptoms (male/female = 13/5, mean age 65) were enrolled in this study. None of the subjects had a history of gastrointestinal surgery or had been taking drugs that would influence gastric acid secretion or esophageal function. Thirteen of the patients with RE were diagnosed as grade A according to the Los Angeles classification[3], 3 as grade B, and 2 as grade C. All the patients with RE or NERD had typical reflux symptoms and their scores for the Japanese version of the Carlsson-Dent self-administered questionnaire (QUEST)[13] were over 4.

This study was performed in accordance with the declaration of Helsinki. Written informed consent was obtained from all the patients and volunteers. The protocol of this study was approved by the ethics committee of Shimane University School of Medicine.

Methods

All the enrolled subjects were investigated by upper gastrointestinal endoscopy for the possible presence of esophageal mucosal breaks and to grade any mucosal breaks according to the Los Angeles classification. One biopsy specimen was taken from the esophageal mucosa without mucosal breaks at the frontal wall 2 cm above the squamous-columnar junction (SCJ), since the evident mucosal injuries are frequently observed at the level of SCJ in patients with RE. Hiatus hernia over a length of 2 cm was not found in any of the subjects.

The biopsy samples were immediately immersed in fixative liquid and routinely processed for scanning electron microscopic observation as described previously[14-16]. The epithelial surface of the processed biopsy specimens was observed at a magnification of × 2000 using a Hitachi S-800 scanning electron microscope (Hitachi Co., Ltd., Tokyo, Japan). Three areas on the epithelial surface of each biopsy sample were randomly selected and electron micrographs were taken for analysis. The photographs were reviewed and graded by three gastroenterologists (KF, KK, and MM) who were blinded to the clinical diagnosis of each case. The reviewers graded the intercellular spaces between the surface epithelial cells on each of three photographs taken from a biopsy sample into 3 grades according to the sample photographs shown in Figure 1. Grade 1 represented a close and tight attachment of the surface epithelial cells. Grade 2 represented a loose attachment evident in some areas of the photo and the presence of widened intercellular spaces. Grade 3 represented looser intercellular adhesion and identifiable large intercellular spaces in over 50% of the area of intercellular attachment. If there was an inconsistency in the reading of photographs between 3 reviewers, the final diagnosis was decided by simultaneous review by the 3 authors. The mean value of these grades determined on each photo was calculated as the representative grade for each individual case. The intercellular space between the surface epithelial cells was also quantified with NIH image software (version 1.61, U.S. National Institute of Health) based on the threshold technique.

The intercellular adhesion, cellular surface structure of the uppermost epithelial cells was also observed and graded (Figure 2). When the cellular surface had regularly shaped multiple folds, the cellular surface structure was graded as 1. When no such regularly shaped folds were observed, it was graded as 3. A cellular surface intermediate between grades 1 and 3 was graded as 2.

Statistical analysis

Statistical comparison between normal controls, patients with NERD, and patients with RE was performed by Kruskal-Wallis test followed by Mann-Whitney *U*-test. Differences at $P < 0.05$ were considered to be statistically significant.

RESULTS

Cellular attachments between surface squamous epithelial cells identified by scanning electron microscopy were remarkably diverse, as shown in Figure 3A. Even in normal asymptomatic individuals, grades of attachment between cells ranged from grade 1.0 to grade 2.4. Attachment grades in patients with symptomatic RE and NERD showed almost the same range of distribution as those in normal individuals. When the median and mean of each group were calculated, there were no statistically significant differences among the three groups. The percentage intercellular space quantified by NIH image in the three groups is shown in Figure 3B. Even quantitative measurement by NIH image revealed no significant difference between patients with GERD and normal individuals.

Grades of epithelial cellular surface structure in individual cases are shown in Figure 3C. The grades of

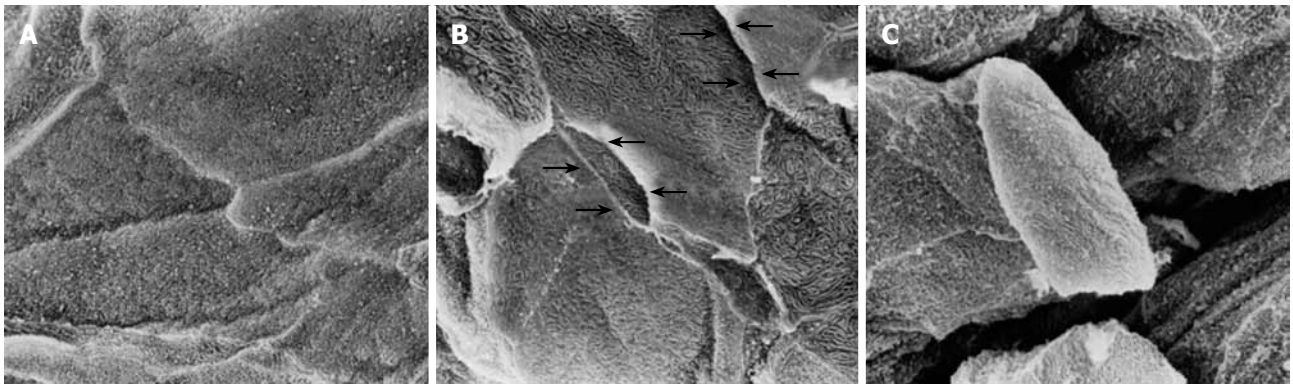


Figure 1 Intercellular spaces between the most superficially located squamous epithelial cells were graded from 1 to 3. Grade 1 (**A**) represents close and tight attachment. Grade 2 (**B**) represents loose attachment in some areas, with widened intercellular spaces (arrows). Grade 3 (**C**) represents further loosening of intercellular adhesion (x 2000).

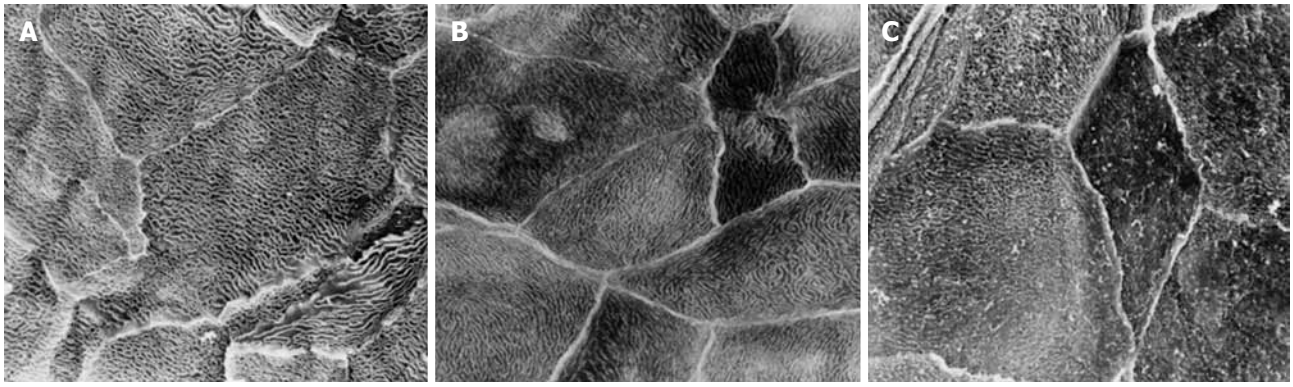


Figure 2 The cellular surface structure of the uppermost epithelial cells was graded from 1 to 3. Grade 1 (**A**) represented regularly shaped multiple folds. Grade 3 (**C**) represented an absence of such regularly shaped folds. A cellular surface intermediate between grades 1 and 3 was classified as grade 2 (**B**).

intercellular attachment and irregularity of the superficial cellular surface structure were distributed over a wide range. The median and mean grades among the three groups showed no statistically significant differences.

DISCUSSION

The presence of esophageal lesions and the presence of reflux symptoms are not well correlated[2]. Many patients with endoscopically evident esophageal erosions have no reflux symptoms[2,17-19]. In fact, our recent study confirmed that 74% of patients with esophageal erosions/ulcers do not have reflux symptoms, including heartburn or regurgitation[20]. Therefore, other pathological changes in the esophageal mucosa may have some role in the induction of reflux symptoms. Tobey and colleagues found widening of squamous epithelial intercellular spaces, not only in patients with RE, but also in patients with NERD[10]. In the epithelial intercellular space, many terminals of afferent neurons are present and convey various noxious stimuli to the central nervous system. When the intercellular spaces become widened, acid and pepsin refluxed from the stomach can easily penetrate the epithelial layer and stimulate noxious receptors on the afferent neurons and induce reflux symptoms[5]. An association between widened intercellular spaces and reflux symptoms has been reported[11,12]. In order to enter the mucosal intercellular spaces, acid and pepsin have to penetrate the intercellular adhesion sites of the uppermost superficial epithelial cells. In this study, the intercellular attachment of the most superficial squamous epithelial cells in the esophagus was investigated by scanning electron microscopy in asymptomatic normal individuals and symptomatic patients with RE or NERD. It was found that the gap between superficial epithelial cells varied markedly in individuals, even in healthy volunteers. Some individuals had tight attachment between superficial cells whereas others had loose intercellular attachment. The tightness of superficial cellular attachment did not differ significantly among normal volunteers, patients with NERD, and patients with RE.

Patients with NERD complain of significant reflux symptoms, even if their gastroesophageal acid reflux is limited[2,21,22]. Some investigators have reported that patients with NERD show hypersensitivity of the esophagus to esophageal extension and acid perfusion[23,24]. Loose attachment between esophageal squamous cells is the most easily suspected cause of esophageal acid hypersensitivity. In the present study, however, it was clarified that loose attachment between the most superficially located squamous cells was not a cause of acid hypersensitivity in patients with NERD.

There were several limitations to this study. The number of subjects investigated in this study was relatively

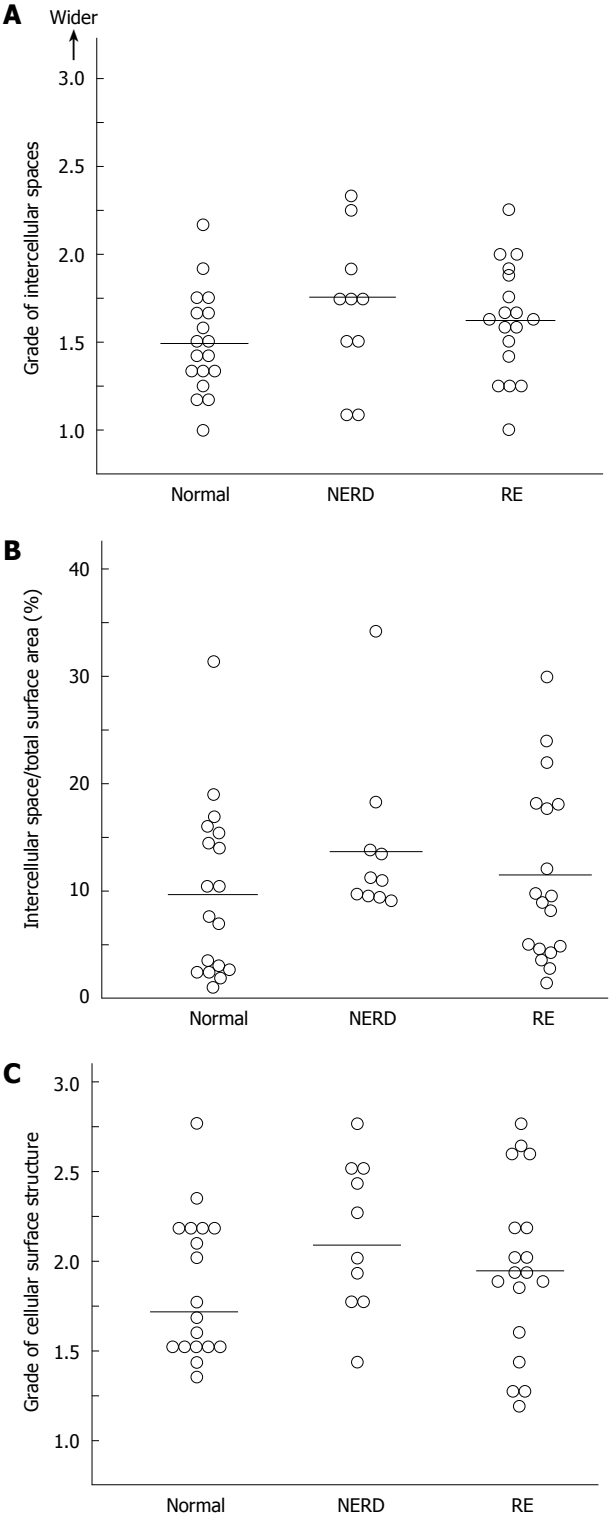


Figure 3 **A**: The intercellular spaces between the most superficially located squamous epithelial cells are shown. Each dot represents an individual case and horizontal lines show the median values. There was no statistically significant difference among the groups; **B**: The intercellular spaces between the most superficially located squamous epithelial cells were quantified using the NIH Image program. The proportion of intercellular space relative to total superficial area was determined in individuals with NERD and RE, and in asymptomatic healthy individuals. Each dot represents an individual case. Horizontal lines show the median values. There was no statistically significant difference among the groups; **C**: The regularity of the luminal cellular surface structure of the squamous cells was compared between normal individuals and patients with RE or NERD. There was no difference in the cellular surface structure between normal individuals and patients with RE or NERD. Each dot represents an individual case and horizontal lines show the median values.

small. In addition, we could not demonstrate the influence of esophageal acid reflux on the attachment of superficial squamous epithelium, since 24-h esophageal pH monitoring study was not performed in this study and the diagnosis of NERD was made by reflux symptoms and endoscopic findings. The location, from which the biopsy samples were obtained, also might influence the results of this study, since we took only one biopsy from the esophageal mucosa without mucosal breaks at the frontal wall 2 cm above the SCJ. Further study will be necessary to clarify the relationship between the attachment of superficial squamous cells and the attachment of more deeply located squamous cells. For this purpose, the combination of transmission and scanning electron microscopy might be useful.

In summary, using scanning electron microscopy, we have clarified that the intercellular adhesion of esophageal superficial squamous cells is diverse among individuals, and does not differ among normal individuals and patients with NERD and RE.

ACKNOWLEDGMENTS

We wish to thank Ms. Rika Tohma and Ms. Keiko Masuzaki for their technical support.

COMMENTS

Background

To investigate the intercellular spaces between the most superficially located esophageal epithelial cells in patients with gastroesophageal reflux disease (GERD).

Research frontiers

The widening of squamous epithelial intercellular spaces was demonstrated in patients with reflux esophagitis (RE), but also patients with non-erosive reflux disease (NERD), by using transmission electron microscopy (TEM), and an association between widened intercellular spaces and reflux symptoms has been reported. However, the intercellular attachment of the most superficial squamous epithelial cells in the esophagus was not investigated by using scanning electron microscopy.

Innovations and breakthroughs

The intercellular space between the most superficially located epithelial cells in patients with erosive esophagitis or NERD was not different from that in asymptomatic healthy individuals. Widened luminal intercellular spaces of esophageal superficial epithelium are not responsible for the induction of reflux symptoms in patients with GERD.

Applications

The relationship between the attachment of superficial squamous cells and the attachment of more deeply located squamous cells should be investigated in future by using the combination of transmission and scanning electron microscopy.

Terminology

The majority of patients with reflux symptoms do not have esophageal mucosal injury; these patients are referred to as NERD. Scanning electron microscopy used in this study can reveal the minimal changes of the luminal surface of the esophageal squamous epithelium.

Peer review

This is an interesting study. Authors investigated the intercellular spaces between the most superficially located esophageal epithelial cells in patients with GERD.

REFERENCES

1 **Dodds WJ**, Hogan WJ, Helm JF, Dent J. Pathogenesis of

reflux esophagitis. *Gastroenterology* 1981; **81**: 376-394
2 **Fass R**, Fennerty MB, Vakil N. Nonerosive reflux disease--current concepts and dilemmas. *Am J Gastroenterol* 2001; **96**: 303-314
3 **Lundell LR**, Dent J, Bennett JR, Blum AL, Armstrong D, Galmiche JP, Johnson F, Hongo M, Richter JE, Spechler SJ, Tytgat GN, Wallin L. Endoscopic assessment of oesophagitis: clinical and functional correlates and further validation of the Los Angeles classification. *Gut* 1999; **45**: 172-180
4 **Adachi K**, Fujishiro H, Katsube T, Yuki M, Ono M, Kawamura A, Rumi MA, Watanabe M, Kinoshita Y. Predominant nocturnal acid reflux in patients with Los Angeles grade C and D reflux esophagitis. *J Gastroenterol Hepatol* 2001; **16**: 1191-1196
5 **Orlando RC**. Review article: oesophageal mucosal resistance. *Aliment Pharmacol Ther* 1998; **12**: 191-197
6 **Wiklund I**, Bardhan KD, Muller-Lissner S, Bigard MA, Bianchi Porro G, Ponce J, Hosie J, Scott M, Weir D, Fulton C, Gillon K, Peacock R. Quality of life during acute and intermittent treatment of gastro-oesophageal reflux disease with omeprazole compared with ranitidine. Results from a multicentre clinical trial. The European Study Group. *Ital J Gastroenterol Hepatol* 1998; **30**: 19-27
7 **Matthews PJ**, Aziz Q, Facer P, Davis JB, Thompson DG, Anand P. Increased capsaicin receptor TRPV1 nerve fibres in the inflamed human oesophagus. *Eur J Gastroenterol Hepatol* 2004; **16**: 897-902
8 **Bhat YM**, Bielefeldt K. Capsaicin receptor (TRPV1) and non-erosive reflux disease. *Eur J Gastroenterol Hepatol* 2006; **18**: 263-270
9 **Tobey NA**, Carson JL, Alkiek RA, Orlando RC. Dilated intercellular spaces: a morphological feature of acid reflux--damaged human esophageal epithelium. *Gastroenterology* 1996; **111**: 1200-1205
10 **Tobey NA**, Hosseini SS, Argote CM, Dobrucali AM, Awayda MS, Orlando RC. Dilated intercellular spaces and shunt permeability in nonerosive acid-damaged esophageal epithelium. *Am J Gastroenterol* 2004; **99**: 13-22
11 **Calabrese C**, Fabbri A, Bortolotti M, Cenacchi G, Areni A, Scialpi C, Miglioli M, Di Febo G. Dilated intercellular spaces as a marker of oesophageal damage: comparative results in gastro-oesophageal reflux disease with or without bile reflux. *Aliment Pharmacol Ther* 2003; **18**: 525-532
12 **Calabrese C**, Bortolotti M, Fabbri A, Areni A, Cenacchi G, Scialpi C, Miglioli M, Di Febo G. Reversibility of GERD ultrastructural alterations and relief of symptoms after omeprazole treatment. *Am J Gastroenterol* 2005; **100**: 537-542
13 **Carlsson R**, Dent J, Bolling-Sternevald E, Johnsson F, Junghard O, Lauritsen K, Riley S, Lundell L. The usefulness of a structured questionnaire in the assessment of symptomatic gastroesophageal reflux disease. *Scand J Gastroenterol* 1998; **33**: 1023-1029
14 **Ashizawa N**, Niigaki M, Hamamoto N, Niigaki M, Kaji T, Katsube T, Sato S, Endoh H, Hidaka K, Watanabe M, Kinoshita Y. The morphological changes of exocrine pancreas in chronic pancreatitis. *Histol Histopathol* 1999; **14**: 539-552
15 **Kaji T**, Ishihara S, Ashizawa N, Hamamoto N, Endo H, Fukuda R, Adachi K, Watanabe M, Nakao M, Kinoshita Y. Adherence of Helicobacter pylori to gastric epithelial cells and mucosal inflammation. *J Lab Clin Med* 2002; **139**: 244-250
16 **Ashizawa N**, Sakai T, Yoneyama T, Naora H, Kinoshita Y. Three-dimensional structure of peripheral exocrine gland in rat pancreas: reconstruction using transmission electron microscopic examination of serial sections. *Pancreas* 2005; **31**: 401-404
17 **Locke GR 3rd**, Talley NJ, Fett SL, Zinsmeister AR, Melton LJ 3rd. Prevalence and clinical spectrum of gastroesophageal reflux: a population-based study in Olmsted County, Minnesota. *Gastroenterology* 1997; **112**: 1448-1456
18 **Isolauri J**, Laippala P. Prevalence of symptoms suggestive of gastro-oesophageal reflux disease in an adult population. *Ann Med* 1995; **27**: 67-70
19 **Kennedy T**, Jones R. The prevalence of gastro-oesophageal reflux symptoms in a UK population and the consultation behaviour of patients with these symptoms. *Aliment Pharmacol Ther* 2000; **14**: 1589-1594
20 **Mishima I**, Adachi K, Arima N, Amano K, Takashima T, Moritani M, Furuta K, Kinoshita Y. Prevalence of endoscopically negative and positive gastroesophageal reflux disease in the Japanese. *Scand J Gastroenterol* 2005; **40**: 1005-1009
21 **Martinez SD**, Malagon IB, Garewal HS, Cui H, Fass R. Non-erosive reflux disease (NERD)--acid reflux and symptom patterns. *Aliment Pharmacol Ther* 2003; **17**: 537-545
22 **Shi G**, Bruley des Varannes S, Scarpignato C, Le Rhun M, Galmiche JP. Reflux related symptoms in patients with normal oesophageal exposure to acid. *Gut* 1995; **37**: 457-464
23 **Miwa H**, Minoo T, Hojo M, Yaginuma R, Nagahara A, Kawabe M, Ohkawa A, Asaoka D, Kurosawa A, Ohkusa T, Sato N. Oesophageal hypersensitivity in Japanese patients with non-erosive gastro-oesophageal reflux diseases. *Aliment Pharmacol Ther* 2004; **20** Suppl 1: 112-117
24 **Trimble KC**, Pryde A, Heading RC. Lowered oesophageal sensory thresholds in patients with symptomatic but not excess gastro-oesophageal reflux: evidence for a spectrum of visceral sensitivity in GORD. *Gut* 1995; **37**: 7-12

S- Editor Li DL **L- Editor** Rippe RA **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5717

World J Gastroenterol 2008 October 7; 14(37): 5717-5722
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Clinical outcome after pulmonary metastasectomy from primary hepatocellular carcinoma: Analysis of prognostic factors

Jong-Bum Kwon, Khun Park, Young-Du Kim, Jong-Hee Seo, Seok-Whan Moon, Deog-Gon Cho, Yong-Whan Kim, Dong-Goo Kim, Seung-Kew Yoon, Hyeon-Woo Lim

Jong-Bum Kwon, Khun Park, Young-Du Kim, Jong-Hee Seo, Seok-Whan Moon, Deog-Gon Cho, Yong-Whan Kim, Department of Thoracic and Cardiovascular Surgery, the Catholic University of Korea, Seoul 137-701, Korea
Dong-Goo Kim, Department of Surgery, the Catholic University of Korea, Seoul 137-701, Korea
Seung-Kew Yoon, Department of Internal Medicine, the Catholic University of Korea, Seoul 137-701, Korea
Hyeon-Woo Lim, Department of Preventive Medicine, the Catholic University of Korea, Seoul 137-701, Korea
Author contributions: Kwon JB and Park K contributed equally to this work; Kwon JB, Park K, Moon SW, Kim YD, Seo JH, Cho DG, and Kim YW performed research; Moon SW, Kim DG, and Yoon SK designed research; Lim HW analyzed data.
Supported by 21C Frontier Functional Human Genome Project from the Ministry of Science & Technology in Korea, No. FG06-11-11
Correspondence to: Seok-Whan Moon, MD, Department of Thoracic and Cardiovascular Surgery, the Catholic University of Korea, St. Paul Hospital, 620-56 Jeonnong-Dong, Dongdaemun-Ku, Seoul 130-709, Korea. swmoon@catholic.ac.kr
Telephone: +82-2-5902768 Fax: +82-2-5948644
Received: April 29, 2008 Revised: June 23, 2008
Accepted: June 30, 2008
Published online: October 7, 2008

Abstract

AIM: To review the surgical outcomes in terms of the surgical indications and relevant prognostic factors.

METHODS: Sixteen patients underwent therapeutic lung surgery between March 1999 and May 2006. The observation period was terminated on May 31, 2007. The surgical outcomes and the clinicopathological factors were compared.

RESULTS: There was no mortality or major morbidity encountered in this study. The mean follow-up period after metastasectomy was 26.7 ± 28.2 (range: 1-99 mo), and the median survival time was 20 mo. The 1- and 5-year survival rates were 56% and 26%, respectively. At the end of the follow-up, 1 patient died from hepatic failure without recurrence, 6 died from hepatic failure with a recurrent hepatocellular carcinoma (HCC), and 4 died from recurrent HCC with cachexia. Among several clinical factors, Kaplan-Meier analysis revealed that liver transplantation as a treatment for the primary lesion, grade of cell differentiation, and negative evidence HBV infection were independent predictive factors. On Cox's proportional hazard model, there were no significant factors affecting survival after pulmonary metastasectomy in patients with HCC.

CONCLUSION: A metastasectomy should be performed before other treatments in selected patients. Although not significant, patients with liver transplantation of a primary HCC survived longer. Liver transplantation might be the most beneficial modality that can offer patients better survival. A multi-institutional and collaborative study would be needed for identifying clinical prognostic factors predicting survival in patients with HCC and lung metastasis.

Key words: Hepatocellular carcinoma; Pulmonary metastasis; Metastasectomy; Liver transplantation; Thoracoscopy

Peer reviewer: Akihito Tsubota, Assistant Professor, Institute of Clinical Medicine and Research, Jikei University School of Medicine, 163-1 Kashiwa-shita, Kashiwa, Chiba 277-8567, Japan

Kwon JB, Park K, Kim YD, Seo JH, Moon SW, Cho DG, Kim YW, Kim DG, Yoon SK, Lim HW. Clinical outcome after pulmonary metastasectomy from primary hepatocellular carcinoma: Analysis of prognostic factors. *World J Gastroenterol* 2008; 14(37): 5717-5722 Available from: URL: http://www.wjgnet.com/1007-9327/14/5717.asp DOI: http://dx.doi.org/10.3748/wjg.14.5717

INTRODUCTION

Pulmonary metastasis from a hepatocellular carcinoma (PM-HCC) is quite frequent occurring in approximately 10% of patients treated with curative surgery for HCC[1-3]. However, its incidence varies according to the diagnostic modality, stage of the HCC, treatment plan, and observation time[1,4,5]. Unlike a PM from other solid

tumors, it is difficult to manage PM-HCC, partly because it is commonly associated with advanced liver disease, or the patients cannot tolerate systemic chemotherapy for an intrahepatic recurrence or extrahepatic metastasis (EHM)[6,7]. In terms of tumor staging, patients who develop an EHM often have a tumor that is too advanced for surgical treatment and are offered systemic chemotherapy with or without combined locoregional therapy for the concurrent intrahepatic tumor[7,8]. Although few experiences have been reported, an active treatment strategy for better outcomes was proposed for an EHM with or without an intrahepatic recurrence[3,9,10]. Regarding the surgical indications for PM, the primary solid tumors should be controlled before or soon after performing a metastasectomy. However, transcatheter or percutaneous local therapy for a primary HCC has excellent therapeutic results. Therefore, a pulmonary metastasectomy with diagnostic or therapeutic intent may provide adequate management plans, such as liver transplantation or local therapy[10,11]. The treatment plans for a PM-HCC can be individualized by taking these factors into consideration. A large series of surgical treatments for PM from solid tumors has increasingly shown that a pulmonary metastasectomy is effective in prolonging the survival rate. Similarly, surgical experience for PM-HCC has increased, and favorable results on a metastasectomy have been reported even though they were from a select group of cases or a small series[2,3,10,12]. Therefore, a more aggressive approach to PM or extended surgical indication has been adopted to extend the survival rates of oncology patients. The aim of this study was to identify the appropriate candidates for aggressive intervention as well as to assess the clinical prognostic factors in patients with PM-HCC.

MATERIALS AND METHODS

From March 1999 to May 2006, 23 patients with a primary HCC were referred to our department for an evaluation of indeterminate pulmonary lesion(s) that had been resected by an open thoracotomy, or thoracoscopy. Patients with benign pulmonary lesions were excluded from the analysis of the survival rate and prognostic factors. The primary HCC was treated or planned to be treated by a hepatic resection, percutaneous or transcatheter local therapy before or after the pulmonary resection[13]. The surgical indications, which were similar to the previously described guidelines at our institution, included those patients who had resectable pulmonary metastases from the HCC before or after the treatment for the primary HCC, with the potential of complete control of the HCC after the metastasectomy or with a lower surgical risk (normal or mild liver dysfunction, acceptable lung function for partial resection or extended resection, normal cardiac function shown by Doppler echocardiography) for a metastasectomy (Table 1). Sixteen of 23 (60%) patients showed a true PM-HCC on the pathologic diagnosis. None of the patients were lost to follow-up and all patients were observed until May, 2007 after the pulmonary metastasectomy.

Table 1 Guidelines for pulmonary metastasectomy[1]

Primary lesion	Metastatic lesion	Patient factor
Completely cured	Therapeutic intents	Tolerable margin of safety in lung function
Controllable surgically or with other modalities after metastasectomy	Diagnostic intents (true malignancy, evaluate chemotherapy)	Good risk for other organ functions (heart and cerebral status)

[1]Modified from others and us[18,19].

The clinical outcomes were evaluated at regular 3-mo intervals with laboratory tests, which included serum α-feto protein and radiological imaging studies, such as a conventional or spiral chest computed tomography (CT) scan. In particular, the PET-CT scan was performed to evaluate the other EHM before thoracic surgery in selective cases. Although general adjuvant chemotherapy after a metastasectomy was not applied to the patients with the exception of one, locoregional therapy, such as percutaneous ethanol injection therapy (PEIT), radiofrequency ablation (RFA) or transcatherter arterial chemotherapy for the primary HCC, or radiation therapy, including tomotherapy for recurrent metastatic tumors in the lung, was used where needed. The overall survival time after the pulmonary metastasectomy was evaluated with respect to the disease-free interval (DFI), which is defined as the interval between the completion of the HCC treatment and the time the PM was diagnosed, the number of lung metastases, the therapeutic modality used for the primary lesion, such as surgical resection or liver transplantation, the number of pulmonary metastasectomies performed, and other clinical factors.

Surgical treatment for PM-HCC

The surgical plan for PM was considered based on the anatomical characteristics of the lung lesions, patients' medical status including the liver function values or potential risk factors for general anesthesia and operation. The tumor was resected conservatively by a pulmonary wedge resection using either a thoracoscopy or an open thoracotomy in order to preserve the lung function and minimize the surgical risk to the patients with chronic liver diseases. There was only one case of an anatomical lobectomy. For such reasons, a limited resection through a thoracoscopy was applied widely to the patients in this study. However, the thoracoscopic technique was modified in that for difficult lesions, such as a small nodule or deep seated pulmonary lesion, barium sulfate or microcoils were injected into or around the nodule under CT guidance[14]. Moreover, metastatic nodules localized using radiopaque materials, in which the radiopacity was visualized on the portable fluoroscopic monitor and was helpful to guide thoracoscopic resection, could be detected and resected easily. This technique enabled us to avoid an open thoracotomy and allowed for an additional repeat thoracotomy with minimal morbidities. In addition, an open thoracotomy was adopted for some specific lesions

Table 2 Clinical findings of 16 patients with HCC who underwent pulmonary metastasectomy

Clinical findings	Number of patients (n = 16)	Percents of patients (%)
Gender: male/female	14/2	87.5/12.5
Age at pulmonary resection (yr)	45 ± 9.8 (31-67)[1]	
Etiology of liver diseases		
Cirrhosis + HCC + HBV	5	31.3
Cirrhosis + HCC	2	12.5
Alcoholic liver disease + HCC	2	12.5
HCC + HBV	7	43.8
Maximal diameter of lung lesions	13 ± 10 (3-50 mm)	
Treatment of primary lesion[2]		
Hepatic resection	5	31.3
Liver transplantation	7	43.8
Local therapy	5	31.3
Treatment of lung lesions[2]		
Thoracoscopic resection	15	93.8
Open wedge resection	9	56.3
Lobectomy	1	6.3
Tomotherapy after recurrence	2	12.5
Maximal diameter of lung lesions	13 ± 10 (3-50 mm)	
Number of lung metastasectomy		
1	9	56.2
⩾ 2	7	43.8
HBV hepatitis		
Yes	12	75.0
No	4	25.0
HCC pathologic finding		
Well differentiated	7	43.8
Poorly differentiated	9	56.2
Alpha fetoprotein		
> 20 ng/mL	8	50.0
< 20 ng/mL	8	50.0
Liver cirrhosis		
Yes	7	43.8
No	9	56.3

[1]mean ± SD (range); [2]Multiple therapies were performed.

that were centrally located or recurrent lesions with possible dense pleural adhesion caused by the previous thoracotomy.

Treatment of primary HCC

The treatment for the primary HCC or intrahepatic recurrent tumor was performed before (n = 21) or after (n = 2) the pulmonary metastasectomy: hepatic resection, liver transplantation and local therapy for 5, 7 and 5 patients with an intrahepatic recurrence or concurrent PM-HCC, respectively.

Statistical analysis

The survival curves of 16 patients who were pathologically proven to have a metastatic lesion were calculated using the Kaplan-Meier method, and the survival according to the prognostic factors was compared using a log-rank test. Using Cox's proportional hazard model, clinical prognostic indicators predicting increased survival of patients with HCC and pulmonary metastasectomy were tested. The statistical study was performed with SAS statistical software package (version 6.12, SAS Institute, Cary, NC). $P < 0.05$ was considered statistically significant.

Table 3 Follow-up results of 16 patients with HCC after pulmonary metastasectomy

	Number of patients (n = 16)	Percentages of patients (%)
Status at the last observation period		
Survival without recurrence	4	25.0
Survival with recurrence	1	6.25
Died of recurrence, cachexia	4	25.0
Died of hepatic failure + recurrence	6	37.5
Died of hepatic failure	1	6.25

RESULTS

Sixteen of 23 (60%) patients, who had undergone a pulmonary resection, were diagnosed with a PM-HCC on pathology examination. Of the 16 PM-HCC, 12 patients had a large primary tumor or multicentricity, and 9 patients showed poor differentiation according to the Edmonson-Steiner grading system, which determines the grade of HCC differentiation. Table 2 summarizes the clinical features of the 16 PM-HCC before or after the pulmonary metastasectomy. Of the 16 patients, 2 had a concurrent PM-HCC at the time of the initial presentation, and the PM-HCC was developed in sequence in 14 patients after treatment for the primary lesion. There was a mean 14 ± 4 mo (range: 0-38 mo) DFI between the completion of the HCC treatment and the time the PM was diagnosed. Twenty-five thoracic procedures were performed for 30 pulmonary lesions of PM-HCC. Among them, 4 concurrent nodules showed benign characteristics. There were no surgery-related deaths or major complications but one patient had a prolonged hospital stay in consequence of air leakage after a repeated resection by an open thoracotomy.

Figure 1 shows the overall survival rates after the initial diagnosis of the primary HCC and pulmonary metastasectomy. The median survival time after the pulmonary metastasectomy was 20 mo. The 1- and 5 year survival rates after the pulmonary metastasectomy were 56% and 26%, respectively. When the prognosis after the pulmonary metastasectomy was compared according to the therapeutic modality for primary HCC, the survival was found to be significantly higher in patients who had undergone liver transplantation than in those who had received other treatments, such as a surgical resection, locoregional therapy (Figure 2). In addition, among clinical factors, liver transplantation, negative evidence of an HBV infection (Figure 3) and well differentiation of HCC (Figure 4) showed longer survival by log rank test.

A total of 11 patients died during the follow-up period (Table 3). Six patients developed an intrahepatic recurrence during the follow-up after the metastasectomy: one patient was treated successfully by local therapy but another two developed an extrahepatic metastasis during treatment. This suggests that control of the primary lesion can result in a favorable prognosis even in the advanced stage. Among the 9 patients with recurrent PM-HCC after the pulmonary metastasectomy,

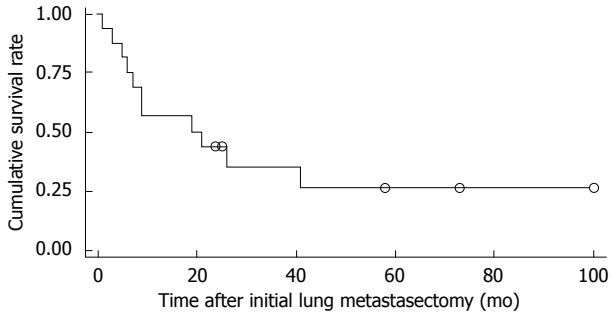


Figure 1 Overall cumulative survival curves of 16 patients with metastasectomy (Open circles indicate censored cases).

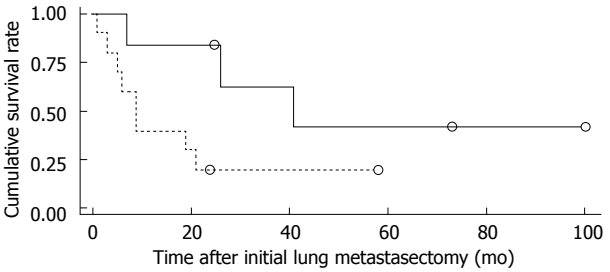


Figure 2 Comparison of survival in patients with HCC after pulmonary metastasectomy by liver transplantation. Patients who had undergone liver transplantation survived longer significantly (log-rank test, $P = 0.014$).

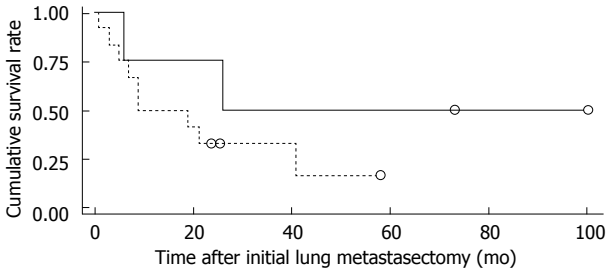


Figure 3 Comparison of survival in patients with HBV hepatitis-related HCC after pulmonary metastasectomy. A significant difference was found between the two groups (log-rank test, $P = 0.0386$).

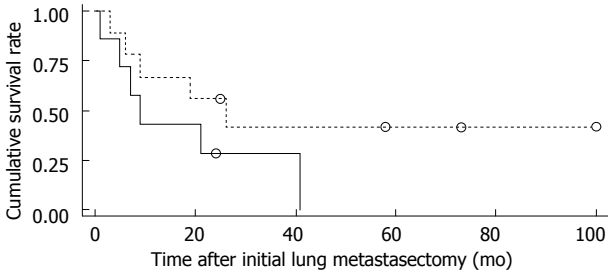


Figure 4 Comparison of survival in patients with HCC after pulmonary metastasectomy by pathologic findings for histologic differentiation. A significant difference was found between the two groups (log-rank test, $P = 0.0392$).

they all underwent repeated pulmonary metastasectomy more than twice, of whom two received tomotherapy for recurrence. Five of these patients are still alive, four of whom show no evidence of recurrent disease (98, 73, 25, 58 mo, respectively).

DISCUSSION

HCC is a major public health problem in Asian countries, including Korea, where HBV infections are endemic[2,6,14]. A longer survival of HCC has been achieved with the recent advances in diagnostic and therapeutic modalities. However, the presence of an extrahepatic metastasis (EHM) of an HCC, which frequently occurs in the lung, regional nodes, bone and brain[4,6], is an indicator of a poor prognosis with a very low 1-year survival rate[6,10]. Despite the advances in therapeutic strategies, an EHM is still a major obstacle for further improving the overall survival and prognosis of the patients. Unfortunately, patients with an EHM do not tolerate systemic chemotherapy well, which is first considered as the therapeutic modality for EHM that finally leads to a shorter survival time[6,8,15,16]. For these reasons, the surgical management of PM-HCC might be a good option. With increasing experience[14], a pulmonary metastasectomy has become more acceptable. Hence, we have developed new guidelines by modifying previous results[17,18] (Table 1).

The incidence or prevalence of PM-HCC depends on the T category, imaging technique, observation time, and therapeutic modality[1,4,5,9]. Because most patients with PM-HCC do not show pulmonary symptoms until they progress to full-blown disease, spiral CT scan is essential for making an early and accurate diagnosis of a PM-HCC. Although PET is effective in detecting distant metastasis in other solid tumors, the diagnostic accuracy of primary HCC showed limited clinical significance[19]. In the present series, 5 true metastatic lesions in 3 patients were confirmed by pathological examination but PET-CT did not show any diagnostic accuracy for these lesions, which might be due to the small lesions or the low standardized uptake values (SUV). By contrast, serial monitoring of the α-feto protein level might help to confirm PM-HCC in the absence of a primary HCC or detect an early recurrence or EHM. However, in this study, the α-feto protein level was elevated by more than 20 ng/mL in only 8 (50%) of the 16 patients with a PM-HCC. The reason why only 50% of patients with an EHM had an elevated α-feto protein level might be due to a small lesion (< 1 cm) or severe liver dysfunction.

The pathological characteristics of the concurrent or sequential pulmonary lesions in patients with a primary HCC can affect the treatment plan and prognosis[15]. Of the 23 patients with a preoperative suspicious metastasis in this series, only 16 (70%) had a true PM-HCC. In addition, the therapeutic plan was consequently changed in 9 of the 23 (39%) patients according to the pathology results. A less invasive or conservative pulmonary resection is recommended for treating a PM from a solid tumor in order to preserve the lung function in the case of a possible PM in future. As shown in this series, the thoracoscopic resection was performed in 10 of 25 (40%) procedures. Although thoracoscopy did not affect the surgical outcome or survival compared with

Table 4 Cox's proportional hazards model of all factors predicting increased survival after pulmonary metastasectomy in patients with HCC

Prognostic factors	Parameter estimate (β)	*P*	HR	95% confidence interval
Disease-free interval (≤ 12 *vs* > 12 mo)	1.97545	0.4005	7.21	0.072-721.009
HBV viral hepatitis (*vs* no)	-33.8718	0.9906	0	0-.
Well differentiation grade (*vs* poorly)	-18.73237	0.9896	0	0-.
α-feto protein > 20 ng/mL (yes *vs* no)	-4.49444	0.0748	0.011	.-1.569
Liver transplantation (*vs* no)	32.71851	0.9818	1.62E + 14	0-.
Single metastasectomy (*vs* multiple)	-15.0999	0.0572	0	0-1.83
Thoracoscopy (*vs* others)	-13.02123	0.9928	0	0-.
Age	0.55293	0.0419	1.738	1.021-2.961

Table 5 Cox's proportional hazards model of liver transplantation, HBV viral hepatitis, well differentiation grade predicting increased survival after pulmonary metastasectomy in patients with HCC

Prognostic factors	Parameter estimate (β)	*P*	HR	95% confidence interval
HBV viral hepatitis (*vs* no)	-0.33272	0.7674	0.717	0.079-6.504
Well differentiation grade (*vs* poorly)	-0.7663	0.3007	0.465	0.109-1.984
Liver transplantation (*vs* no)	1.26672	0.1568	3.549	0.615-20.495

an open thoracotomy in this and other series, it may allow for better satisfaction and an additional pulmonary metastasectomy[14,20].

The prognosis of patients with a PM-HCC has been reported to be as low as 20%-30% at 1 yr despite receiving systemic chemotherapy[6,16]. A pulmonary metastasectomy of an HCC increases the median survival or long-term survival[2,10]. Although there were no consistent and complete prognostic indicators, several clinical or pathological factors have been reported[2,3,5,16,20]. Generally, patient selection according to the prognostic factors, such as single metastasis and small primary HCC, is essential for a survival benefit by surgery. In this series, liver transplantation, negative evidence of an HBV infection, and well differentiation of HCC were significant prognostic indicators among the other clinical factors. Accordingly, this finding suggests that hepatic factors indicating a complete resection and good liver function might be essential for better survival[11,16]. However, Cox's proportional hazard model (Tables 4 and 5) showed no significant clinical indicators, which might be attributed to small numbers of patients and poor survival due to high virulence of HCC. More importantly, a brain metastasis of HCC is not common but life threatening, and should be detected early, especially in patients with PM-HCC[5,20,21].

During the follow-up after the metastasectomy, EHM or intrahepatic recurrence may be controlled by irradiation therapy[20,22], local therapy[10,13] as well as by a repeated metastasectomy[10,12,20,23]. In this series, a repeated pulmonary resection for a recurrent PM-HCC after a metastasectomy was performed in 6 patients. Although patients with a single metastasis or single pulmonary surgery might show better survival[2,20], multiple metastases or repeated metastasectomies are not risk factors for long-term survival, as shown in this and previous studies[12,24].

When considering the cause of death in this and other reports, hepatic dysfunction complicating liver cirrhosis was one of the most important factors for the long-term survival[2,16,25]. Considerable attention should be paid to patient selection, which may be essential for achieving long-term survival in patients with a PM-HCC according to prognostic analysis. As shown in this study and suggested by others, the prognosis may be affected by many clinical or pathologic parameters, which may be major limitations in clinical reports including these results.

In summary, selected patients without an intrahepatic HCC and with a good hepatic reserve showed that a pulmonary metastasectomy is effective in controlling a PM-HCC and can offer the only chance for long-term survival.

COMMENTS

Background

Treatment of patients with primary hepatocellular carcinoma (HCC) and concurrent or subsequent pulmonary metastasis (PM) has no definite or proper guidelines, but it has been reported that pulmonary metastasectomy has been proven to prolong the survival in a selected group or a small series. Local therapy and surgical resection have been effective in treating the primary HCC. However, systemic chemotherapy for distant metastasis had limitations, such as poor toleration and aggravation of liver function. Although a few clinical prognostic factors affected the survival rate in patients after pulmonary metastasectomy, there were limitations in terms of small number and heterogenosity of patients.

Research frontiers

Diagnostic and therapeutic surgical interventions for PM in patients with HCC affected the treatment plans depending on the result of pathology. In fact, surgical metastasectomy of PM may provide perioperative and anaesthetic risk, particularly in patients with hepatic dysfunction. Moreover, repeat metastasectomy for patients with recurrent PM who had no other distant metastasis may have technical difficulties despite of good results. Adequate guidelines for management of PM-HCC have been proposed based on significant prognostic factors increasing survival. Pulmonary metastasectomy may be a good option for PM-HCC.

Innovations and breakthroughs

A better result on surgical management of PM would be achieved with collaborative investigation and adequate guidelines. This strategy provided better survival and none to minimal surgical risk. Moreover, significant prognostic factors, such as liver transplantation as a treatment modality for

primary disease, absence of HBV infection, and well differentiation in pathology, were identified despite of small numbers of patients.

Applications

Pulmonary metastasectomy or more extended resection should be considered for diagnostic or therapeutic surgical interventions in managing patients with collaborative approaches, otherwise most of them might survive for less than one year. When considering surgery, the adequate guidelines, as proposed in this series should be adopted for better results. Firstly, adequate control over primary liver cancer is prerequisite. Aggressive management has the possibility of better results in patients who had undergone liver transplantation. Secondly, conservative resection or minimally invasive surgery may help to treat recurrent lung lesions or to provide less risk. Lastly, PM usually indicates advanced stage in patients with HCC, therefore other distant metastasis, particularly brain metastasis, should be excluded in surgical indications and be carefully detected by MRI on follow-up.

Peer review

This paper reported the clinical significance of pulmonary metastasectomy to improve the prognosis of patients with HCC. This manuscript arouses interest for readers and provides an important clue to treat such patients and alter the survival rate.

REFERENCES

1 **Inagaki Y**, Unoura M, Urabe T, Ogino H, Terasaki S, Matsushita E, Kaneko S, Morioka T, Furusawa A, Wakabayashi T. Distant metastasis of hepatocellular carcinoma after successful treatment of the primary lesion. *Hepatogastroenterology* 1993; **40**: 316-319

2 **Lam CM**, Lo CM, Yuen WK, Liu CL, Fan ST. Prolonged survival in selected patients following surgical resection for pulmonary metastasis from hepatocellular carcinoma. *Br J Surg* 1998; **85**: 1198-1200

3 **Tomimaru Y**, Sasaki Y, Yamada T, Eguchi H, Takami K, Ohigashi H, Higashiyama M, Ishikawa O, Kodama K, Imaoka S. The significance of surgical resection for pulmonary metastasis from hepatocellular carcinoma. *Am J Surg* 2006; **192**: 46-51

4 **Katyal S**, Oliver JH 3rd, Peterson MS, Ferris JV, Carr BS, Baron RL. Extrahepatic metastases of hepatocellular carcinoma. *Radiology* 2000; **216**: 698-703

5 **Natsuizaka M**, Omura T, Akaike T, Kuwata Y, Yamazaki K, Sato T, Karino Y, Toyota J, Suga T, Asaka M. Clinical features of hepatocellular carcinoma with extrahepatic metastases. *J Gastroenterol Hepatol* 2005; **20**: 1781-1787

6 **Okusaka T**, Okada S, Ishii H, Nose H, Nagahama H, Nakasuka H, Ikeda K, Yoshimori M. Prognosis of hepatocellular carcinoma patients with extrahepatic metastases. *Hepatogastroenterology* 1997; **44**: 251-257

7 **Choi TK**, Lee NW, Wong J. Chemotherapy for advanced hepatocellular carcinoma. Adriamycin versus quadruple chemotherapy. *Cancer* 1984; **53**: 401-405

8 **Jang JW**, Park YM, Bae SH, Choi JY, Yoon SK, Chang UI, Nam SW, Kim BS. Therapeutic efficacy of multimodal combination therapy using transcatheter arterial infusion of epirubicin and cisplatin, systemic infusion of 5-fluorouracil, and additional percutaneous ethanol injection for unresectable hepatocellular carcinoma. *Cancer Chemother Pharmacol* 2004; **54**: 415-420

9 **Shimada M**, Takenaka K, Gion T, Fujiwara Y, Kajiyama K, Maeda T, Shirabe K, Nishizaki T, Yanaga K, Sugimachi K. Prognosis of recurrent hepatocellular carcinoma: a 10-year surgical experience in Japan. *Gastroenterology* 1996; **111**: 720-726

10 **Poon RT**, Fan ST, O'Suilleabhain CB, Wong J. Aggressive management of patients with extrahepatic and intrahepatic recurrences of hepatocellular carcinoma by combined resection and locoregional therapy. *J Am Coll Surg* 2002; **195**: 311-318

11 **Schwartz M**, Roayaie S, Llovet J. How should patients with hepatocellular carcinoma recurrence after liver transplantation be treated? *J Hepatol* 2005; **43**: 584-589

12 **O'Suilleabhain CB**, Poon RT, Lau CW, Fan ST. Repeated resections of extrahepatic metastases after hepatic resection: an aggressive approach to hepatocellular carcinoma. *Hepatogastroenterology* 2004; **51**: 825-829

13 **Jang JW**, Choi JY, Bae SH, Kim CW, Cho SH, Yoon SK, Yang JM, Han JY, Lee YS, Kim DG. The best candidates for transarterial chemotherapy in patients with hepatocellular carcinoma awaiting liver transplantation: a cohort-based characterization of dropout times. *Aliment Pharmacol Ther* 2007; **26**: 87-94

14 **Moon SW**, Wang YP, Jo KH, Kwack MS, Kim SW, Kwon OK, Jang HS. Fluoroscopy-aided thoracoscopic resection of pulmonary nodule localized with contrast media. *Ann Thorac Surg* 1999; **68**: 1815-1820

15 **Utsunomiya T**, Shimada M, Shirabe K, Kajiyama K, Gion T, Takenaka K, Sugimachi K. Clinicopathological characteristics of patients with extrahepatic recurrence following a hepatectomy for hepatocellular carcinoma. *Hepatogastroenterology* 2001; **48**: 1088-1093

16 **Arii S**, Monden K, Niwano M, Furutani M, Mori A, Mizumoto M, Imamura M. Results of surgical treatment for recurrent hepatocellular carcinoma; comparison of outcome among patients with multicentric carcinogenesis, intrahepatic metastasis, and extrahepatic recurrence. *J Hepatobiliary Pancreat Surg* 1998; **5**: 86-92

17 **Thomford NR**, Woolner LB, Clagett OT. The surgical treatment of metastatic tumors in the lungs. *J Thorac Cardiovasc Surg* 1965; **49**: 357-363

18 **McCormack P**. Surgical resection of pulmonary metastases. *Semin Surg Oncol* 1990; **6**: 297-302

19 **Hustinx R**. PET imaging in assessing gastrointestinal tumors. *Radiol Clin North Am* 2004; **42**: 1123-39, ix

20 **Nakajima J**, Tanaka M, Matsumoto J, Takeuchi E, Fukami T, Takamoto S. Appraisal of surgical treatment for pulmonary metastasis from hepatocellular carcinoma. *World J Surg* 2005; **29**: 715-718

21 **Tunc B**, Filik L, Tezer-Filik I, Sahin B. Brain metastasis of hepatocellular carcinoma: A case report and review of the literature. *World J Gastroenterol* 2004; **10**: 1688-1689

22 **Aramaki M**, Kawano K, Sasaki A, Matsumoto T, Kai S, Iwashita Y, Himeno Y, Kitano S. Prolonged survival after repeat resection of pulmonary metastasis from hepatocellular carcinoma. *J Hepatobiliary Pancreat Surg* 2002; **9**: 386-388

23 **Gwak GY**, Jung JO, Sung SW, Lee HS. Long-term survival after pulmonary metastatectomy of hepatocellular carcinoma; treatment outcome or natural history? *Hepatogastroenterology* 2004; **51**: 1428-1433

24 **Koide N**, Kondo H, Suzuki K, Asamura H, Shimada K, Tsuchiya R. Surgical treatment of pulmonary metastasis from hepatocellular carcinoma. *Hepatogastroenterology* 2007; **54**: 152-156

25 **Chen YJ**, Hsu HS, Hsieh CC, Wu YC, Wang LS, Hsu WH, Huang MH, Huang BS. Pulmonary metastasectomy for hepatocellular carcinoma. *J Chin Med Assoc* 2004; **67**: 621-624

S- Editor Li DL **L- Editor** Kumar M **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5723

World J Gastroenterol 2008 October 7; 14(37): 5723-5729
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Severe thrombocytopenia before liver transplantation is associated with delayed recovery of thrombocytopenia regardless of donor type

Jae Hyuck Chang, Jong Young Choi, Hyun Young Woo, Jung Hyun Kwon, Chan Ran You, Si Hyun Bae, Seung Kew Yoon, Myung-Gyu Choi, In-Sik Chung, Dong Goo Kim

Jae Hyuck Chang, Jong Young Choi, Hyun Young Woo, Jung Hyun Kwon, Chan Ran You, Si Hyun Bae, Seung Kew Yoon, Myung Gyu Choi, In Sik Chung, Department of Internal Medicine, College of Medicine, the Catholic University of Korea, 505 Banpo-Dong, Seocho-Gu, Seoul 137-040, Korea
Dong Goo Kim, Department of Surgery, College of Medicine, the Catholic University of Korea, 505 Banpo-Dong, Seocho-Gu, Seoul 137-040, Korea
Author contributions: Choi JY designed the study; Chang JH, Woo HY, Kwon JH, You CR, Bae SH, Yoon SK, Choi MG, Chung IS performed the research; Kim DG performed the operations; Chang JH and Choi JY wrote the paper.
Supported by The Grant (Clinical Research Center of Liver Cirrhosis) of the Korea Health 21 Research and Development Project from Ministry of Health and Welfare, Republic of Korea, No. A050021
Correspondence to: Jong Young Choi, Division of Hepatology and Gastroenterology, Department of Internal Medicine, College of Medicine, the Catholic University of Korea, 505 Banpo-Dong, Seocho-Gu, Seoul 137-040, Korea. jychoi@catholic.ac.kr
Telephone: +82-2-5902529 Fax: +82-2-34814025
Received: May 4, 2008 Revised: August 18, 2008
Accepted: August 25, 2008
Published online: October 7, 2008

Abstract

AIM: To compare the recovery of thrombocytopenia and splenomegaly during long-term follow-up after liver transplantation in patients receiving a living donor transplant or a cadaveric donor transplant.

METHODS: This was a retrospective cohort study of 216 consecutive liver transplant patients who survived for > 6 mo after transplantation; 169 received a liver transplant from a living donor and 47 from a cadaveric donor. The platelet counts or spleen volumes were examined before transplant, 1, 6, and 12 mo after transplant, and then annually until 5 years after transplant.

RESULTS: The mean follow-up period was 49 mo (range, 21-66). Platelet counts increased continuously for 5 years after orthotopic liver transplant. The restoration of platelet counts after transplant was significantly slower in patients with severe pretransplant thrombocytopenia (< 50 000/μL) until 4 years after transplant (P = 0.005). Donor type did not significantly affect the recovery of platelet count and spleen volume in either patient group. In multivariate analysis, pretransplant severe thrombocytopenia (< 50 000/μL) was an independent factor associated with sustained thrombocytopenia (P < 0.001, odds ratio 6.314; confidence interval, 2.828-14.095). Thrombocytopenia reappeared after transplant in seven patients with portal flow disturbance near the anastomosis site.

CONCLUSION: Our study suggests that severe thrombocytopenia before transplant is closely associated with delayed recovery of platelet count after transplant and donor type did not affect the recovery of thrombocytopenia. The reappearance of thrombocytopenia after transplant should be considered a possible indicator of flow disturbance in the portal vein.

Key words: Liver transplantation; Thrombocytopenia; Splenomegaly; Hypersplenism

Peer reviewer: Adam G Testro, PhD, Department of Gastroenterology and Liver Transplantation, Austin Health Institution, Heidelberg 3032, Australia

Chang JH, Choi JY, Woo HY, Kwon JH, You CR, Bae SH, Yoon SK, Choi MG, Chung IS, Kim DG. Severe thrombocytopenia before liver transplantation is associated with delayed recovery of thrombocytopenia regardless of donor type. *World J Gastroenterol* 2008; 14(37): 5723-5729 Available from: URL: http://www.wjgnet.com/1007-9327/14/5723.asp DOI: http://dx.doi.org/10.3748/wjg.14.5723

INTRODUCTION

Thrombocytopenia develops commonly in the early postoperative period after liver transplantation[1-3], and most instances of early thrombocytopenia recover with restoration of hepatic function[4,5]. By contrast, the recovery of pretransplant long-lasting thrombocytopenia differs among patients, and some patients experience persistent thrombocytopenia even several years after the operation[6]. Persistent thrombocytopenia increases the risk of bleeding-related complications, which worsen the

prognosis for the transplanted patients[1,7,8].

The possible causes of thrombocytopenia soon after the operation include consumption of platelets in the graft liver, impairment of platelet synthesis, small graft, and sepsis[1,3,7-10]. However, the causes or contributing factors of persistent thrombocytopenia (< 100 000/μL) beyond one year after the operation are not well known. A previous report suggested that thrombocytopenia at 3 and 6 mo after transplantation is an independent contributing factor to persistent thrombocytopenia[11].

Patients receiving a living donor transplant restore the liver volume up to 80% of the whole liver, in contrast with cadaveric donor transplant. Because of this deficient restoration in liver volume, the posttransplant recovery of thrombocytopenia or splenomegaly in living donor transplant is assumed to be different than that in cadaveric donor transplant[10].

The aims of this study were to compare the recovery of thrombocytopenia and splenomegaly in patients receiving a living donor liver transplantation (LDLT) and cadaveric donor liver transplantation (CDLT), and to identify the factors contributing to persistent thrombocytopenia during long-term follow-up after orthotopic liver transplantation (OLT).

MATERIALS AND METHODS

Patients

We performed 289 OLTs in our transplantation center between July 1996 and June 2006. Among them, 49 patients had liver transplant from cadaveric donor and 240 patients from living donor who donated right lobe. In addition, 19 patients prepared their transplants with us and then had CDLT performed in China during the same period. They were sent to us soon after transplants and followed up. Ninety-two patients were excluded from this study because of death within 6 mo after OLT (n = 52), post-OLT chemotherapy (n = 21), acute liver failure (n = 7), post-OLT interferon treatment (n = 7), age less than 20 years old (n = 4), and post-OLT portal vein obstruction (n = 1). The remaining 216 patients (169 LDLT, 47 CDLT) were recruited consecutively (Figure 1). Patient's anonymity was preserved and the study protocol conforms to the ethical standards of the responsible committee on human experimentation and with the Helsinki declaration of 1975, as revised in 1983.

The patients comprised 158 men and 58 women with a mean age at transplant of 49.1 ± 7.7 years (range, 21-66 years) (Table 1). The indications for OLT included complications of advanced cirrhosis in 133 patients, hepatocellular carcinoma in 75 patients, and other causes in seven patients. The causes of liver diseases were hepatitis B virus in 188 patients, hepatitis C virus in 9 patients, alcoholic liver diseases in 13 patients, autoimmune diseases in 5 patients, and other causes in 2 patients. The follow-up duration after the operation ranged from 6 to 178 mo, with a mean of 49 mo.

Methods

Serial platelet counts were obtained from the medical

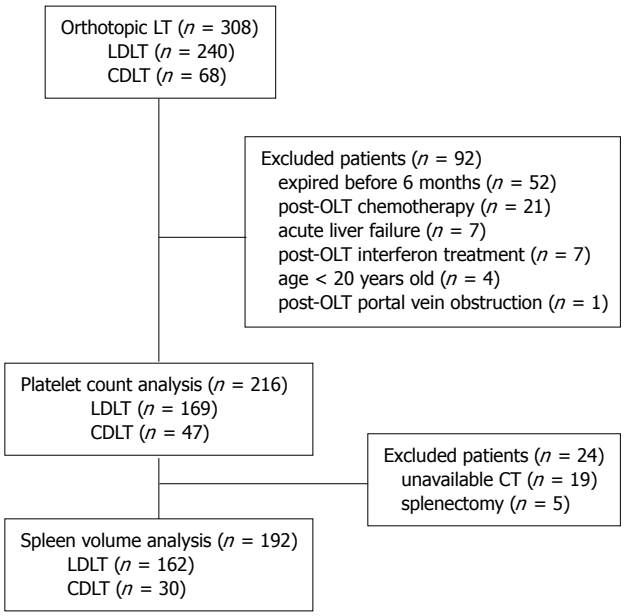


Figure 1 Study design.

Table 1 Baseline characteristics of patients

Characteristics (n = 216)	Data
Age at transplant (range), yr	49.1 ± 7.7 (21-66)
Duration posttransplant (range), mo	49.1 ± 29.5 (6-178)
Male/Female (%)	158/58 (73/27)
Donor type (%)	
LDLT	169 (78)
CDLT	47 (22)
Indication for transplant (%)	
Hepatitis B cirrhosis	113 (52.3)
Hepatitis C cirrhosis	3 (1.4)
Alcoholic cirrhosis	8 (3.7)
Mixed type cirrhosis[1]	6 (2.8)
Cryptogenic cirrhosis	3 (1.4)
Hepatocellular carcinoma[2]	75 (34.7)
Autoimmune hepatitis	5 (2.3)
Wilson's disease	1 (0.5)
Primary biliary cirrhosis	1 (0.5)
Child-Pugh score	9.5 ± 2.2 (5-14)
Cirrhosis complication[3] (%)	
Ascites	130 (60)
Variceal bleeding	53 (25)
Encephalopathy	81 (38)
SBP	24 (11)
Hepato-renal syndrome	4 (2)
Platelet count, pretransplant (× 10^3/μL)	58 ± 36 (9-340)
Platelet group (%)	
Group 1 (< 30 000/μL)	29 (13)
Group 2 (30 000-50 000/μL)	80 (37)
Gourp 3 (50 000-100 000/μL)	88 (41)
Group 4 (≥ 100 000/μL)	19 (9)
Spleen volume, pretransplant (mm^3, n = 193)	1105 ± 636 (128-4858)

[1]Alcohol cirrhosis + hepatitis B cirrhosis (n = 5); hepatitis B cirrhosis + hepatitis C cirrhosis (n = 1); [2]With hepatitis B virus (n = 70), hepatitis C virus (n = 5); [3]Included overlap; SBP: Spontaneous bacterial peritonitis; LDLT: Living donor liver transplantation; CDLT: Cadaveric donor liver transplantation.

record before the OLT and 1, 6, 12, 24, 36, 48, and 60 mo after the OLT. Pretransplant thrombocytopenia was classified arbitrarily according to platelet count as follows: group 1 (< 30 000/μL), group 2 (30 000-50 000/μL), group 3 (50 000-100 000/μL), group 4 (≥ 100 000/μL), which was modified according to the WHO adverse event criteria for hematologic toxicity. To evaluate spleen volume, the greatest length, transverse diameter, and thickness at the hilum of the spleen were measured using abdominal computed scanning tomography before the OLT and 1, 6, 12, 24 and 36 mo after the OLT. These values were multiplied together and then by the factor of 0.6 to obtain an estimated spleen volume as described previously[12]. Sustained thrombocytopenia was defined as platelets count < 100 000/μL 12 mo after the OLT. Factors such as demographic and clinical features associated with sustained thrombocytopenia were also assessed.

The immunosuppressive regimen used comprised cyclosporine or tacrolimus combined with or without mycophenolate mofetil, which was withdrawn in patients with severe leucopenia (< 2 000/μL). In addition, corticosteroid was used for 3-6 mo after operation, and then it was tapered. Among 216 patients, 132 (61%) patients had taken cyclosporine, 130 (60%) patients had tacrolimus, and 127 (59%) patients had mycophenolate mofetil. Mycophenolate mofetil was used for short duration, less than 6 mo, in most patients.

Rejection was diagnosed by liver biopsy, which showed Banff rejection activity index more than 3. Patients who had endoscopic retrograde biliary procedures or percutaneous transhepatic biliary procedures performed were diagnosed as those with biliary complication. We defined patients as those with portal flow disturbance if there was clear evidence in abdominal computerized tomography or magnetic resonance imaging.

Statistical analysis

Continuous variables are expressed as mean ± SD unless stated otherwise. Factors associated with sustained thrombocytopenia were analyzed by univariate analysis. Significant factors by univariate analysis ($P < 0.05$) were subjected to multivariate analysis using logistic regression (forward selection). Continuous variables were compared by paired *t* test and repeated-measures analysis of variance (ANOVA), and categorical variables were compared by either the chi-square test or Fisher exact test, as appropriate. The statistical analyses were performed using SPSS for Windows version 14 (Chicago: SPSS Inc., USA). $P < 0.05$ was considered significant.

RESULTS

Ninety-one percent (197/216) of our studied population demonstrated evidence of moderate to severe thrombocytopenia, as indicated by a platelet count < 100 000/μL before the OLT. The percentages of patients according to platelet count before the operation were 13% in group 1 (< 30 000 μL), 37% in group 2 (30 000-50 000/μL), 41% in group 3 (50 000-100 000/μL), and 19% in group 4 (≥ 100 000/μL). The mean platelet counts increased continuously for 5 years after orthotopic liver transplant: 58 000/μL before OLT, 121 000/μL at 1 mo, 128 000/μL at 6 mo, 132 000/μL at 12 mo, 145 000/μL at 24 mo, 148 000/μL at 36 mo, 149 000/μL at 48 mo, and 158 000/μL at 60 mo after OLT. The platelet counts increased significantly during the first month after OLT compared with later periods ($P < 0.001$), and then increased gradually until 5 years after the operation (Figure 2A). The donor type did not affect the recovery of platelet count after the operation and the recovery of platelet count in patients with severe thrombocytopenia before the operation (groups 1 and 2) was also not different between LDLT and CDLT (Figure 2B).

Recovery of platelet count after OLT differed significantly between the four groups classified according to pretransplant thrombocytopenia until 5 years after the OLT ($P = 0.015$, repeated-measures ANOVA) (Figure 3A). In those who survived more than 1 year, 58% (15/26) of patients in group 1, 30% (30/75) in group 2, 12% (10/82) in group 3, and 6% (1/17) in group 4 had moderate to severe thrombocytopenia (< 100 000/μL) one year after the OLT. Five years after the OLT, the mean platelet count in patients with pretransplant severe thrombocytopenia (groups 1 and 2) was < 150 000/μL. In contrast, patients with pretransplant moderate thrombocytopenia (group 3) showed a faster recovery of platelet count to a mean value near 150 000/μL (148 000/μL) 6 mo after the OLT. The restoration of platelet counts after the OLT was significantly slower in patients with severe pretransplant thrombocytopenia (groups 1 and 2) than in the groups with mild or moderate thrombocytopenia (groups 3 and 4) until 4 years after the OLT ($P = 0.005$, Figure 3A).

In univariate analysis, clinical factors associated with sustained thrombocytopenia (< 100 000/μL at 12 mo after OLT) were pretransplant severe thrombocytopenia (< 50 000/μL) and pretransplant large spleen volume (> 2000 mm^3) ($P < 0.001$, respectively; Table 2). In the multivariate analysis, pretransplant severe thrombocytopenia and splenomegaly were independent factors associated with sustained thrombocytopenia ($P < 0.001$ and $P = 0.004$, respectively).

Seven patients with portal vein thrombosis or stenosis showed a decrease in platelet count in proportion to the progression of portal flow disturbance. The disturbance of portal flow appeared between 6 mo and 1 year after the operation. In these patients, the platelet count increased initially as in the other patients but decreased gradually with disturbed portal flow (Figure 3B). The causes of portal flow disturbance were attributed to main portal vein thrombosis (three patients) and stenosis of the portal vein anastomosis site (four patients). The donor types for these patients were three cadaveric donors and four living donors.

All patients with splenomegaly showed decreased spleen volume 6 mo after the operation. The mean volumes of spleens were 1105 ± 636 cm^3 before transplant, 810 ± 469 mm^3 6 mo after OLT, 761 ± 385 mm^3 12 mo after OLT, 711 ± 399 mm^3 24 mo after

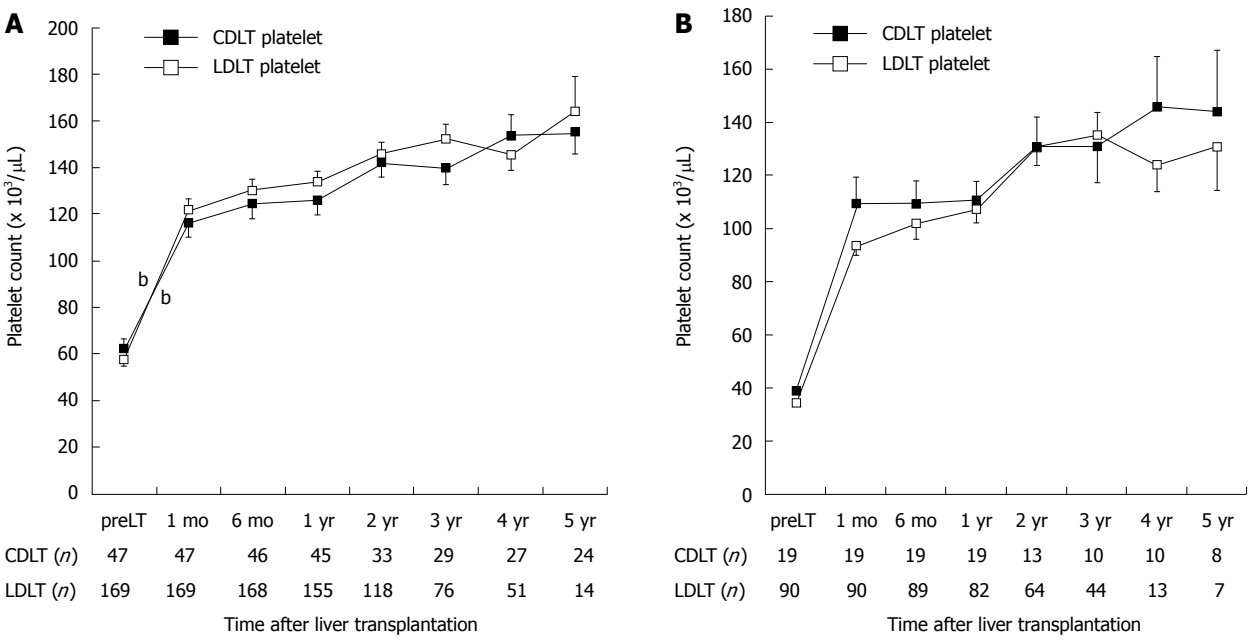


Figure 2 Change in platelet counts during the 5 years after OLT (mean ± SE). **A**: In both LDLT and CDLT, the platelet counts within 1 mo after OLT increased significantly ($^bP < 0.001$). The increase of platelet counts within first 1 mo after OLT was significantly larger than the later periods ($P < 0.001$). Platelet counts in LDLT and CDLT did not differ significantly during the 5 years after OLT ($P = 0.437$, repeated-measures ANOVA); **B**: In patients with severe preoperative thrombocytopenia (< 50 000/μL), donor type also did not affect the recovery of platelet count after the OLT ($P = 0.541$).

OLT, and 648 ± 415 mm^3 36 mo after OLT, and it did not return to normal until 3 years. The reduction rate of spleen volume relative to the pretransplant volume was 26.7% and 31.1% 6 and 12 mo after the operation, respectively. The speed of volume reduction was fastest within the first 6 mo after the operation compared with later periods up to 2 years ($P \leqslant 0.02$; Figure 3C). The rate of reduction in spleen volume did not differ significantly between the LDLT and CDLT groups. The restoration of spleen volume after the OLT was significantly slower in patients who had severe pre-OLT thrombocytopenia (groups 1 and 2) than in those with mild to moderate thrombocytopenia (groups 3 and 4) until 2 years after the operation ($P = 0.006$, repeated-measures ANOVA; Figure 3D).

DISCUSSION

This long-term cohort study clearly demonstrated that liver transplantation affects the rate of recovery of thrombocytopenia. The recovery was fastest within the first month and then decreased gradually for 5 years. Most previous reports have shown the impact of whole liver transplantation from cadaveric donor on hypersplenism during the early postoperative period[13-16]. Recent reports showed that partial liver transplantation from living donor effectively reduces spleen size and resolves thrombocytopenia in adults and children, but the follow-up duration was shorter than in the present study[17-19]. Compared with CDLT, LDLT could not restore the whole liver volume. This suggests that the recovery rate of thrombocytopenia after the operation may differ between patients receiving LDLT and CDLT. However, our study included a large number of LDLT patients, showed that LDLT effectively restored platelet count at a rate similar to that observed in CDLT patients. This indicates that LDLT rapidly resolved the portal hypertension despite the partial restoration of the liver up to 80% of the whole liver volume, and regeneration of a living donor liver is as effective as regeneration after CDLT.

The causes of thrombocytopenia during the early postoperative period include sequestration of platelets in the liver graft, reduced platelet production, immunological reaction, and graft dysfunction[1,3,7-9]. However, the causes of sustained long-term thrombocytopenia for more than 1 year are not fully understood. A previous study suggested that pretransplant variceal bleeding, pretransplant splenomegaly, and thrombocytopenia at 3 and 6 mo after LT are predicting factors of persistent thrombocytopenia[11]. In our study, pretransplant severe thrombocytopenia (< 50 000/μL) and splenomegaly (> 2000 mm^3) were significantly associated with sustained thrombocytopenia. In addition, delayed graft failure, biliary complication, and infections are considered other possible causes of sustained thrombocytopenia lasting more than 1 year. In our study, most biliary complication induced transient thrombocytopenia rather than sustained thrombocytopenia. Almost these patients with biliary complication recovered from thrombocytopenia after biliary intervention.

Splenomegaly is sustained for several years after the operation even though the graft liver restores normal portal hemodynamics relatively soon after the operation[16]. In a prospective hemodynamic study, hemodynamic parameters including cardiac index, mean arterial pressure, portal flow velocity, and hepatic artery resistance index improved within 6 mo after liver transplantation[15]. However, the spleen size decreased gradually beyond 2 years. Our study

Table 2 Univariate and multivariate analysis of clinical factors associated with sustained thrombocytopenia (< 100 000/μL) at 12 mo after the operation

	Sustained thrombocytopenia[1]		Univariate analysis	Multivariate analysis	
	Absent (*n* = 144)	Present (*n* = 56)	*P*	*P*	OR (95% CI)
Age at transplant (yr)	48.8 ± 7.9	48.6 ± 7.3	ns		
Gender			ns		
Male	108	40			
Female	36	16			
Cirrhosis complication, pretransplant			ns		
Present	110	44			
Absent	34	12			
MELD, pretransplant (LDLT)			ns		
> 25	20	6			
≤ 25	91	38			
Child classification, preptranplant			ns		
C	75	34			
A and B	69	22			
Platelet count, pretransplant[a]			< 0.001	< 0.001	6.314 (2.828-14.095)
Group 1 and 2	56	45			
Group 3 and 4	88	11			
Spleen volume, pretransplant[a]			< 0.001	0.004	8.464 (2.001-35.810)
> 2000 mm	3	10			
≤ 2000 mm^3	124	41			
Donor type			ns		
LDLT	111	44			
CDLT	33	12			
Acute rejection			ns		
Present	21	5			
Absent	123	51			
Mycophenolate mofetil use			ns		
Yes	44	13			
No	100	43			
Biliary complication, posttransplant			ns		
Present	36	16			
Absent	108	40			
Portal flow disturbance, posttransplant			ns		
Present	3	4			
Absent	141	52			

[a]$P < 0.05$. [1]Included the patients who survived more than 12 mo. OR: Odds ratio; CI: Confidence interval; HCC: Hepatocellular carcinoma; MELD: Model for end-stage liver disease; ns: Non-specific ($P > 0.05$).

is consistent with the results of previous studies in showing that the long-lasting severe splenomegaly takes several years to resolve although liver synthetic function recovers rapidly.

In seven of our patients, thrombocytopenia reappeared after the initial recovery of platelet counts because of disturbed portal flow. The possibility of portal vein complications is considered greater after LDLT than after CDLT[20,21]. Vascular complications in pediatric patients with whole liver grafts and segmental grafts in a large series of 600 transplants was reported[20]. The incidence of portal vein complications was higher in LDLT patients (27%) than in patients receiving reduced-size or split-liver transplantation (1%) or whole liver transplantation (1%) from a cadaveric donor. Portal vein thrombosis or stenosis appearing in immediate postoperative period should be treated with surgical treatment or radiological intervention[22-25]. If the stenosis or thrombosis progresses slowly beyond 1 year, as in our patients, the patients may not have any symptoms because of reopening of the previously established collateral circulation. Thus, the clinician should monitor the recovery of platelet count after the operation carefully. If thrombocytopenia reappears after the initial recovery of platelet count, the degree of portal vein stenosis or collateral circulation should be evaluated by dynamic computed tomography. In addition, biliary tract infection or interferon therapy for recurrent hepatitis C may cause the reappearance of thrombocytopenia[26,27].

Our study has some limitations. First, it is a retrospective cohort study. However, a large number of patients (216 patients) were included consecutively in the cohort and the follow-up duration was longer than that of other studies. Second, we did not perform hemodynamic studies to evaluate the resolution of portal hypertension after the operation and did not measure serum thrombopoietin level to evaluate the synthetic function of the platelet[28-30]. Further study including hemodynamic analysis is needed to identify why the patients with severe pretransplant thrombocytopenia improve slowly after the operation.

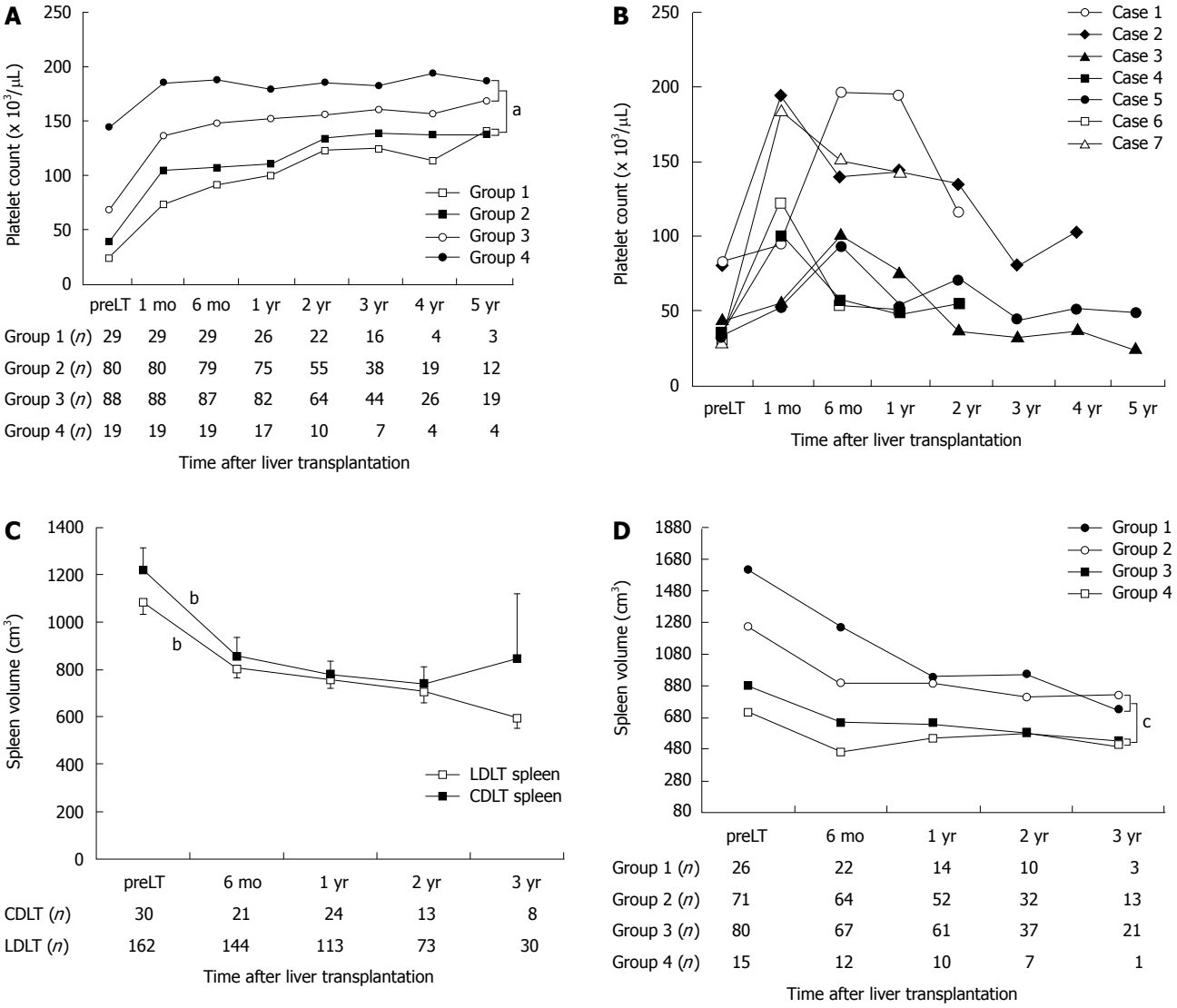


Figure 3 **A**: Change in platelet counts during the 5 years after OLT grouped according to pre-OLT platelet count. Patients with severe pretransplant thrombocytopenia (groups 1 and 2) showed a slower recovery of thrombocytopenia compared with those with mild to moderate thrombocytopenia (groups 3 and 4) until 4 years after the OLT (^{a}P = 0.005, repeated-measures ANOVA); **B**: Change in platelet counts after the OLT in seven patients with portal vein thrombosis or stenosis. The increase of initial platelet count was similar to that observed in the other patients, but the count decreased gradually in proportion to the disturbance of portal flow; **C**: Change in spleen volumes during the first 3 years after the OLT (mean with standard error). In both LDLT and CDLT, the spleen volumes within 6 mo after OLT decreased significantly ($^{b}P < 0.001$). The decrease of spleen volumes within first 6 mo after OLT was significantly larger than the later periods ($P \leqslant 0.02$). The reduction in spleen volume after OLT did not differ significantly between LDLT and CDLT patients until 2 years after the OLT (P = 0.850, repeated-measures ANOVA); **D**: Change in spleen volumes for the first 3 years after the OLT grouped according to the pre-OLT platelet count. Patients with severe pretransplant thrombocytopenia (groups 1 and 2) showed a slower reduction of spleen volume compared with those with mild to moderate thrombocytopenia (groups 3 and 4) until 2 years after the OLT (^{c}P = 0.006, repeated-measures ANOVA).

In conclusion, this study demonstrated that severe thrombocytopenia before transplantation is closely associated with delayed recovery of platelet count after transplantation. Our data also showed that hypersplenism improve at the same rate after LDLT as after CDLT. If thrombocytopenia reappears beyond 6-12 mo without any other cause, disturbance of portal flow, especially in the anastomosis site of the portal vein, should be evaluated.

COMMENTS

Background

The recovery of pretransplant long-lasting thrombocytopenia differs among patients. Because of the deficient restoration in liver volume, the posttransplant recovery of thrombocytopenia or splenomegaly in living donor transplant is assumed to be different from that in cadaveric donor transplant.

Research frontiers

The study has not been done to compare the recovery of thrombocytopenia and splenomegaly in patients receiving a living donor liver transplantation and cadaveric donor liver transplantation during long-term follow-up after orthotopic liver transplantation.

Innovations and breakthroughs

This study suggests at the first that severe thrombocytopenia before transplant is closely associated with delayed recovery of platelet count and spleen volume after transplant, and donor type does not affect the recovery of thrombocytopenia.

Applications

The degree of thrombocytopenia before transplant can be used to predict delayed recovery of platelet count and spleen volume after transplant.

Peer review

This is a retrospective study comparing recovery of thrombocytopenia and sple-

nomegaly in patients receiving a LDLT and CDLT. The manuscript is generally well written and has a concise, simple methodology with good long-term follow-up.

REFERENCES

1 **McCaughan GW**, Herkes R, Powers B, Rickard K, Gallagher ND, Thompson JF, Sheil AG. Thrombocytopenia post liver transplantation. Correlations with pre-operative platelet count, blood transfusion requirements, allograft function and outcome. *J Hepatol* 1992; **16**: 16-22

2 **Randoux O**, Gambiez L, Navarro F, Declerck N, Labalette M, Dessaint JP, Pruvot FR. Post-liver transplantation thrombocytopenia: a persistent immunologic sequestration? *Transplant Proc* 1995; **27**: 1710-1711

3 **Richards EM**, Alexander GJ, Calne RY, Baglin TP. Thrombocytopenia following liver transplantation is associated with platelet consumption and thrombin generation. *Br J Haematol* 1997; **98**: 315-321

4 **Chatzipetrou MA**, Tsaroucha AK, Weppler D, Pappas PA, Kenyon NS, Nery JR, Khan MF, Kato T, Pinna AD, O'Brien C, Viciana A, Ricordi C, Tzakis AG. Thrombocytopenia after liver transplantation. *Transplantation* 1999; **67**: 702-706

5 **Pereboom IT**, Lisman T, Porte RJ. Platelets in liver transplantation: friend or foe? *Liver Transpl* 2008; **14**: 923-931

6 **Iglesias-Berengue J**, Lopez-Espinosa JA, Ortega-Lopez J, Sanchez-Sanchez L, Asensio-Llorente M, Margarit-Creixell C, Diaz-Heredia C. Hematologic abnormalities in liver-transplanted children during medium- to long-term follow-up. *Transplant Proc* 2003; **35**: 1904-1906

7 **Plevak DJ**, Halma GA, Forstrom LA, Dewanjee MK, O'Connor MK, Moore SB, Krom RA, Rettke SR. Thrombocytopenia after liver transplantation. *Transplant Proc* 1988; **20**: 630-633

8 **Munoz SJ**, Carabasi AR, Moritz MJ, Jarrell BE, Maddrey WC. Postoperative thrombocytopenia in liver transplant recipients: prognostic implications and treatment with high dose of gamma-globulin. *Transplant Proc* 1989; **21**: 3545-3546

9 **Miyata T**, Yokoyama I, Todo S, Tzakis A, Selby R, Starzl TE. Endotoxaemia, pulmonary complications, and thrombocytopenia in liver transplantation. *Lancet* 1989; **2**: 189-191

10 **Marubashi S**, Dono K, Miyamoto A, Takeda Y, Nagano H, Umeshita K, Monden M. Impact of graft size on postoperative thrombocytopenia in living donor liver transplant. *Arch Surg* 2007; **142**: 1054-1058

11 **Sutedja DS**, Wai CT, Teoh KF, Lee HL, DaCosta M, Kaur M, Lee YM, Lee KH, Mak K, Quak SH, Isaac J, Lim SG, Prabhakaran K. Persistent thrombocytopenia in liver transplant patients. *Transplant Proc* 2004; **36**: 2331-2333

12 **Shah SH**, Hayes PC, Allan PL, Nicoll J, Finlayson ND. Measurement of spleen size and its relation to hypersplenism and portal hemodynamics in portal hypertension due to hepatic cirrhosis. *Am J Gastroenterol* 1996; **91**: 2580-2583

13 **Yanaga K**, Tzakis AG, Shimada M, Campbell WE, Marsh JW, Stieber AC, Makowka L, Todo S, Gordon RD, Iwatsuki S. Reversal of hypersplenism following orthotopic liver transplantation. *Ann Surg* 1989; **210**: 180-183

14 **Soresi M**, Bascone F, Magliarisi C, Campagna P, Di Giovanni G, Riili A, Carroccio A, Montalto G. Hemodynamic changes in splanchnic circulation after orthotopic liver transplantation in patients with liver cirrhosis. *Abdom Imaging* 2002; **27**: 541-545

15 **Piscaglia F**, Zironi G, Gaiani S, Mazziotti A, Cavallari A, Gramantieri L, Valgimigli M, Bolondi L. Systemic and splanchnic hemodynamic changes after liver transplantation for cirrhosis: a long-term prospective study. *Hepatology* 1999; **30**: 58-64

16 **Witte M**, Langnas AN, Hirst K, Stratta RJ, Shaw BW Jr. Impact of liver transplantation on the reversal of hypersplenism. *Transplant Proc* 1993; **25**: 1987

17 **Kaneko J**, Sugawara Y, Akamatsu N, Kokudo N, Makuuchi M. Spleen volume and platelet number changes after living donor liver transplantation in adults. *Hepatogastroenterology* 2004; **51**: 262-263

18 **Egami S**, Sugawara Y, Mizuta K, Kaneko J, Kawarasaki H, Makuuchi M. Effect of pediatric living-donor liver transplantation on splenomegaly. *Transplantation* 2002; **74**: 1639-1642

19 **Kim SH**, Lee JM, Choi JY, Suh KS, Yi NJ, Han JK, Choi BI. Changes of portosystemic collaterals and splenic volume on CT after liver transplantation and factors influencing those changes. *AJR Am J Roentgenol* 2008; **191**: W8-W16

20 **Buell JF**, Funaki B, Cronin DC, Yoshida A, Perlman MK, Lorenz J, Kelly S, Brady L, Leef JA, Millis JM. Long-term venous complications after full-size and segmental pediatric liver transplantation. *Ann Surg* 2002; **236**: 658-666

21 **Broniszczak D**, Szymczak M, Kaminski A, Chyzynska A, Ismail H, Drewniak T, Nachulewicz P, Markiewicz M, Teisseyre J, Dzik E, Lembas A, Kalicinski P. Vascular complications after pediatric liver transplantation from the living donors. *Transplant Proc* 2006; **38**: 1456-1458

22 **Koo BY**, Yu HC, So MC, Jin GY, Kwak HS, Cho BH. Endovascular management of early portal vein thrombosis caused by coronary vein steal after liver transplantation and its outcome. *Clin Transplant* 2008; **22:** 668-671

23 **Lendoire J**, Raffin G, Cejas N, Duek F, Barros Schelotto P, Trigo P, Quarin C, Garay V, Imventarza O. Liver transplantation in adult patients with portal vein thrombosis: risk factors, management and outcome. *HPB* (Oxford) 2007; **9**: 352-356

24 **Yerdel MA**, Gunson B, Mirza D, Karayalcin K, Olliff S, Buckels J, Mayer D, McMaster P, Pirenne J. Portal vein thrombosis in adults undergoing liver transplantation: risk factors, screening, management, and outcome. *Transplantation* 2000; **69**: 1873-1881

25 **Ko GY**, Sung KB, Lee S, Yoon HK, Kim KR, Kim KM, Lee YJ. Stent placement for the treatment of portal vein stenosis or occlusion in pediatric liver transplant recipients. *J Vasc Interv Radiol* 2007; **18**: 1215-1221

26 **Iacob S**, Gheorghe L, Hrehoret D, Becheanu G, Herlea V, Popescu I. Pegylated interferon alpha-2a and ribavirin combination therapy in HCV liver transplant recipients. Experience of 7 cases. *J Gastrointestin Liver Dis* 2008; **17**: 165-172

27 **Ross AS**, Bhan AK, Pascual M, Thiim M, Benedict Cosimi A, Chung RT. Pegylated interferon alpha-2b plus ribavirin in the treatment of post-liver transplant recurrent hepatitis C. *Clin Transplant* 2004; **18**: 166-173

28 **Peck-Radosavljevic M**, Wichlas M, Zacherl J, Stiegler G, Stohlawetz P, Fuchsjager M, Kreil A, Metz-Schimmerl S, Panzer S, Steininger R, Muhlbacher F, Ferenci P, Pidlich J, Gangl A. Thrombopoietin induces rapid resolution of thrombocytopenia after orthotopic liver transplantation through increased platelet production. *Blood* 2000; **95**: 795-801

29 **Peck-Radosavljevic M**, Zacherl J, Wichlas M, Sims P, Meng YG, Panzer S, Lipinski E, Steininger R, Muhlbacher F, Pidlich J, Gangl A. Thrombopoietic cytokines and reversal of thrombocytopenia after liver transplantation. *Eur J Gastroenterol Hepatol* 1999; **11**: 151-156

30 **Chang FY**, Singh N, Gayowski T, Wagener MM, Mietzner SM, Stout JE, Marino IR. Thrombocytopenia in liver transplant recipients: predictors, impact on fungal infections, and role of endogenous thrombopoietin. *Transplantation* 2000; **69**: 70-75

S- Editor Li DL **L- Editor** Li M **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5730

World J Gastroenterol 2008 October 7; 14(37): 5730-5737
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Characterization of hepatic progenitors from human fetal liver during second trimester

Mekala Subba Rao, Aleem Ahmed Khan, Nyamath Parveen, Mohammed Aejaz Habeeb, Chittoor Mohammed Habibullah, Gopal Pande

Mekala Subba Rao, Gopal Pande, Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500007, India
Aleem Ahmed Khan, Nyamath Parveen, Mohammed Aejaz Habeeb, Chittor Mohammed Habibullah, Centre for Liver Research and Diagnostics, Kanchanbagh, Hyderabad 500058, India
Supported by Council of Scientific and Industrial Research Network Grant CMM002 and ICMR Grant (GAP 0215)
Author contributions: Rao MS is a lead worker of this study; Khan AA, Parveen N and Habeeb MA actively carried out the study; Pande G and Habibullah CM supervised the study.
Correspondence to: Gopal Pande, Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500007, India. gpande@ccmb.res.in
Telephone: +91-40-27192605 Fax: +91-40-27160591
Received: May 23, 2008 Revised: July 19, 2008
Accepted: July 26, 2008
Published online: October 7, 2008

Abstract

AIM: To enrich hepatic progenitors using epithelial cell adhesion molecule (EpCAM) as a marker from human fetal liver and investigate the expression of human leukocyte antigen (HLA) and their markers associated with hepatic progenitor cells.

METHODS: EpCAM +ve cells were isolated using magnetic cell sorting (MACS) from human fetuses (n = 10) at 15-25 wk gestation. Expression of markers for hepatic progenitors such as albumin, alpha-fetoprotein (AFP), CD29 (integrin β1), CD49f (integrin α6) and CD90 (Thy 1) was studied by using flow cytometry, immunocytochemistry and RT-PCR; HLA class Ⅰ (A, B, C) and class Ⅱ (DR) expression was studied by flow cytometry only.

RESULTS: FACS analysis indicated that EpCAM +ve cells were positive for CD29, CD49f, CD90, CD34, HLA class Ⅰ, albumin and AFP but negative for HLA class Ⅱ (DR) and CD45. RT PCR showed that EpCAM +ve cells expressed liver epithelial markers (CK18), biliary specific marker (CK19) and hepatic markers (albumin, AFP). On immunocytochemical staining, EpCAM +ve cells were shown positive signals for CK18 and albumin.

CONCLUSION: Our study suggests that these EpCAM +ve cells can be used as hepatic progenitors for cell transplantation with a minimum risk of alloreactivity and these cells may serve as a potential source for enrichment of hepatic progenitor.

Key words: Epithelial cell adhesion molecule; Human fetal liver; Hepatic progenitors; Human leukocyte antigen; Hepatic cell therapy

Peer reviewer: Paul E Sijens, PhD, Associate Professor, Department of Radiology, UMCG, Groningen 9713GZ, the Netherlands

Rao MS, Khan AA, Parveen N, Habeeb MA, Habibullah CM, Pande G. Characterization of hepatic progenitors from human fetal liver during second trimester. *World J Gastroenterol* 2008; 14(37): 5730-5737 Available from: URL: http://www.wjgnet.com/1007-9327/14/5730.asp DOI: http://dx.doi.org/10.3748/wjg.14.5730

INTRODUCTION

Hepatocyte transplantation has been reported as a useful bridge/alternative therapy for the treatment of various types of liver diseases. The potential of hepatocyte transplantation to provide metabolic support for acutely injured liver tissues has been suggested by earlier workers[1,2]. However, due to the scarcity of HLA matched adult human liver donor cells and the inability of hepatocytes to proliferate *in vitro* the usage of hepatocyte-based cell therapy in liver diseases has been limited. The existence of bi-potential progenitor cells in the liver of rodents using different phenotypic markers was demonstrated many years ago[3]. More recently the same markers have been demonstrated in human fetal liver and it has been argued that these progenitor cells are derived from a common cell compartment and they can be referred to as the hepatoblasts[4]. Similar cells obtained from the rat fetus could reconstitute bile ducts and structures resembling hepatocytes after being transplanted in retrosine damaged liver of syngeneic rats[5].

Progenitors isolated from adult or fetal liver can generate hepatocytes *in vitro* and mature liver cells *in vivo*. Fetal liver CD117+/CD34+/Lin-progenitors and their progeny proliferated *in vitro* and also functionally

differentiated into mature hepatic cells in an acute liver injury model[6]. Epithelial progenitor cells (EPC) from human fetal liver possessed highly proliferative ability and sub-passaged for more than 25 passages. Two months after EPC transplantation, the grafted cells differentiate into hepatocyte like cells[7]. Epithelial cell adhesion molecule positive (EpCAM +) cells are 80% hepatoblasts and 0.1%-0.7% is hepatic stem cells in human fetal liver[8]. Transplantation of these EpCAM + cells or hepatic stem cells expanded in culture into NOD/SCID mice results in mature liver tissue expressing human specific proteins[9]. It has been reported that cells in embryonic day (ED) 13.5 fetal mouse liver cells, which co-express CD49f and CD29 (α6, β1 integrin subunits) but do not express c-kit, CD45 or TER 119 are the best candidate for hepatic stem /progenitor cells[10].

Human fetal liver cells also offer an important source for isolating hepatic and hematopoietic progenitors for clinical application. After 5 wk of gestation hematopoiesis starts to shift from the yolk sac to the liver[11], and during the first trimester fetal liver contains both hepatic and hematopoietic progenitors[12-14]. During this period, fetal liver cells express hepatoblasts and biliary cell specific markers, such as albumin, alpha-fetoprotein (AFP), β-1 microglobulin, glycogen, glucose-6-phosphatase (G-6-P), gamma glutamyl transpeptidase (GGT), dipeptidyl peptidase Ⅳ (DPPIV) and cytokeratin (CK) 19; they also express hematopoietic markers such as CD34[15,16]. During the second trimester markers for hepatoblasts continue to be expressed but the expression of hematopoietic precursors is reduced. This offers an opportunity to isolate and study large numbers of hepatic progenitor cells without hematopoietic potential during the second trimester of gestation. Several years ago we, for the first time, had shown the use of human fetal hepatocytes isolated from the second trimester, for the treatment of fulminant hepatic failure patients[17]. Subsequently other investigators have also isolated hepatic progenitor cells from human fetal liver in the second trimester and demonstrated the higher ability to proliferate without changing their karyotypes[4]. It is therefore important to characterize the cells that could be responsible for the regenerative potential of the fetal hepatocytes in the second trimester of gestation.

Recently we have reported the expression of CD34 antigen (a hematopoietic marker) in human fetal liver cells during the second trimester, and the co-expression of hepatic markers such as AFP and albumin in these cells[18]. In this study we have described the expression of hepatic progenitor and HLA markers in the EpCAM +ve cells isolated from the second trimester fetal liver. Our results show that EpCAM +ve fetal liver cells at this stage express nil levels of HLA-DR marker but they show expression of progenitor and liver specific markers suggesting that these EpCAM +ve cells are hepatic progenitors. Our results also indicate that the EpCAM +ve cells include most of the CD34 +ve cells that are seen in the fetal liver during the second trimester.

MATERIALS AND METHODS

Isolation of fetal liver cells

Human fetal liver tissues were obtained from aborted fetuses at 15-25 wk gestation in accordance with the Institute ethical guidelines. The fetuses were collected under sterile condition within 2 h of the termination of pregnancy. The liver tissue from the fetus was initially perfused twice with cold PBS for 5 min to eliminate circulating cell contaminants, followed by digestion with 0.025% collagenase prepared in PBS for 5 min. Then the liver was disintegrated into a single cell suspension by passing through 70 μm cell strainer (BD Biosciences). Viable cell count was determined by the trypan blue dye exclusion test. All donors of the fetus used in the study had been serologically screened for syphilis, toxoplasmosis, rubella, hepatitis B and C, human immunodeficiency virus 1, cytomegalovirus, parvovirus, and herpes simplex types 1 and 2. All cell isolation procedures were carried out under sterile condition in class 100-biosafety cabinet.

Enrichment and culture of EpCAM +ve cells

EpCAM positive cells were sorted by using the magnetic cell sorter Auto MACS according to the manufacturer's instructions (Miltenyi Biotec, Germany, http//www.miltenyibiotec.com). Labeling of EpCAM positive cells was done by incubating with 5×10^7 total fetal liver cells in 500 μL of buffer containing FCR blocking reagent and EpCAM micro beads at 4℃ for 30 min. Cells were filtered using 70 μm cell strainer to remove clumps and loaded on magnetic column. EpCAM +ve cells were collected in the second fraction form the column.

The enriched EpCAM +ve cells were collected and suspended in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS, Sigma). Cells were plated at 1.5×10^6 per well in a six well plates and maintained at 37℃ in a humidified environment containing 5% carbon dioxide. After 72 h, the nonadherent cells were removed and the medium replaced. Colonies that formed during the culture period were picked after 10 d and 20 d and kept on ice for further study of CK18 and albumin expression by immunofluorescence.

FACS analysis

EpCAM +ve cells were stained with Phycoerythrin (PE) labeled anti-CD29 (Integrin β1, 1:100, BD Biosciences, USA), CD90 (Thy 1) or fluorescein isothiocynate (FITC) labeled anti CD49f (integrin α6) antibodies (1:100, BD Biosciences, USA). Staining for HLA-A, HLA-B, HLA-C and HLA-DR was done incubating with the respective primary antibodies (Chemicon, USA) at 4℃ for overnight. Subsequently the cells were incubated with FITC labeled rabbit anti-mouse IgG FITC (1:100, Sigma) at room temperature for 40 min. Unstained

Table 1 List of primer sequences used in the study

Genes	Primer sequence	Product size (bp)
CK18	Sense-TGGTACTCTCCTCAATCTGCTG	148
	Anti-sense-CTCTGGATTGACTGTGGAAGT	
CK19	Sense-CCTGCGGGACAAGATTCTTG	326
	Anti-sense-ACGGGCGTTGTCGATCTG	
Albumin	Sense-GCTTTGCCGAGGAGGGTAA	161
	Anti-sense-GTAGGCTGAGATGCTTTATGT	
AFP	Sense-GCAAAGCTGAAAATGCAGTTGA	216
	Anti-sense-GGAAAGTTCGGGTCCCAAAA	

Table 2 Expression of hepatic and progenitor markers on EpCAM +ve cells

Antibody	Group 1 (n = 5), cells%	Group 2 (n = 5), cells%	P
CD29	3.9 ± 1.3	1.9 ± 0.7	0.005
CD49f	2.1 ± 0.6	1.5 ± 0.7	0.372
CD90	3.5 ± 1.9	1.3 ± 0.3	0.044
Albumin	19.2 ± 3.1	21.3 ± 4.2	0.817
AFP	7.3 ± 0.6	7.2 ± 1.0	0.865
HLA-A, B, C (HLA Class Ⅰ)	6.5 ± 2.4	8.2 ± 2.1	0.279
HLA-DR (HLA class Ⅱ)	0.6 ± 0.4	0.35 ± 0.1	0.612

EpCAM positive cells were used as negative control to subtract auto fluorescence.

For albumin and AFP staining in the cytoplasm, cells were fixed with 4% paraformaldehyde in PBS and then permeated with 0.5% Triton X-100 for 10 min. After two washes cells were blocked with 0.5% bovine serum albumin (BSA, Sigma), at room temperature for 1 h. Cells were incubated with primary antibodies at room temperature for 2 h and subsequently incubated with secondary antibodies, FITC labeled rabbit anti-goat IgG (1:200, Bangalore Genei, India) at room temperature for 40 min. Stained cells were analyzed on FACS Calibur flow cytometry (BD Biosciences, USA).

Immunocytochemistry

In addition to phenotyping by flow cytometry, EpCAM +ve cells were stained for albumin and CK18 by immunocytochemistry. For albumin and CK18 staining in the cytoplasm, Cultured EpCAM +ve cells were fixed with 4% paraformaldehyde in PBS and then permeated with 0.5% Triton X-100 for 10 min. After two washes cells were blocked with 0.5% BSA at room temperature for 1 h. Cells were incubated with primary antibodies include albumin (1:100, MP biomedical), CK18 (1:200, Sigma) at room temperature for 2 h and subsequently incubated with secondary antibodies, FITC labeled rabbit anti-goat IgG (1:200, Bangalore Genei, India) at room temperature for 40 min. The signal was detected using a fluorescence microscope (Axio Plan upright; Carl Zeiss Vision Co., Ltd., Hallbergmoos, Germany).

Reverse transcription-PCR analysis

RT PCR analysis of cultured EpCAM +ve cells was done for 3 samples (n = 3). Total RNA from cultured EpCAM +ve cells was isolated using RNAeasy Mini Kit (Qiagen). One μg of RNA was reversed transcribed using Moloney murine leukemia virus Reverse transcriptase and Oligo dT (Promega). PCR reaction was performed using cDNA amount representing 100 ng of total RNA and 1 unit of Taq polymerase (Invitrogen, reaction mixture containing 10 mmol/L Tris-Hcl, 200 mmol/L dNTP and 20 pmol of gene specific primer). PCR was performed in a thermal cycler (Programmable Thermal Controller) following an initial denaturation step of 5 min at 95℃. Thermal program was consisting of 35 cycles of 94℃ for 1 min, 56℃ for 1 min and 72℃ for 2 min. This was followed by a final extension of 10 min at 72℃. PCR products were separated by electrophoresis in 1.5% agarose gel. The primer sequences (Invitrogen) used in the study are listed in Table 1.

Statistical analysis

The data were categorized as group 1, cells obtained from abortuses of gestational age 15 to 20 wk, and group 2, gestational age 21 wk to 25 wk. Student t-test was used to determine the likelihood of a significant difference ($P < 0.05$) between these two groups in the percentage of EpCAM +ve cells that express phenotypes with hepatic and progenitor markers and in the percentage of cells that express HLA classes Ⅰ and Ⅱ.

RESULTS

EpCAM +ve cell recovery fetal liver cells

Fetal liver cells in the study have been divided into two age groups, 15-20 wk (group 1) and 21-25 wk (group 2). The total number of cells obtained from each group was (140.0 ± 30.8) × 10^6 and (193.4 ± 96.8) × 10^6, respectively, and the cell viability in both groups was > 80%. This indicated that the total number of cells in fetal liver during the late second trimester is higher in the early second trimester. Microscopic examination of the cells before EpCAM +ve cell enrichment showed a heterogeneous population in both age groups (Figure 1B). Purification of EpCAM +ve cells was done by using magnetically tagged EpCAM antibodies and MACS as described. The number of EpCAM +ve cells obtained in both fetal age groups were (22 ± 4.8) × 10^6 to (34.4 ± 11.4) × 10^6, thus indicating a decrease in the yield of EpCAM +ve cells during the last part of the second trimester from about 16% to 15% of the total fetal liver cells, during the later second trimester. Morphologically most EpCAM +ve cells were homogenous (Figure 1C). On the contrary EpCAM -ve cells were a mixed population of cells (Figure 1D).

FACS analysis

We have analyzed the expression of HLA class Ⅰ and Ⅱ and hepatic progenitor markers such as CD29, CD49f, CD90 and hepatic specific markers such as albumin and AFP in EpCAM +ve cells by flow cytometry (Figure 2, Table 2). The results are depicted in Figure 2, gives representative histograms of the FACS analysis for HLA and hepatic progenitor markers in

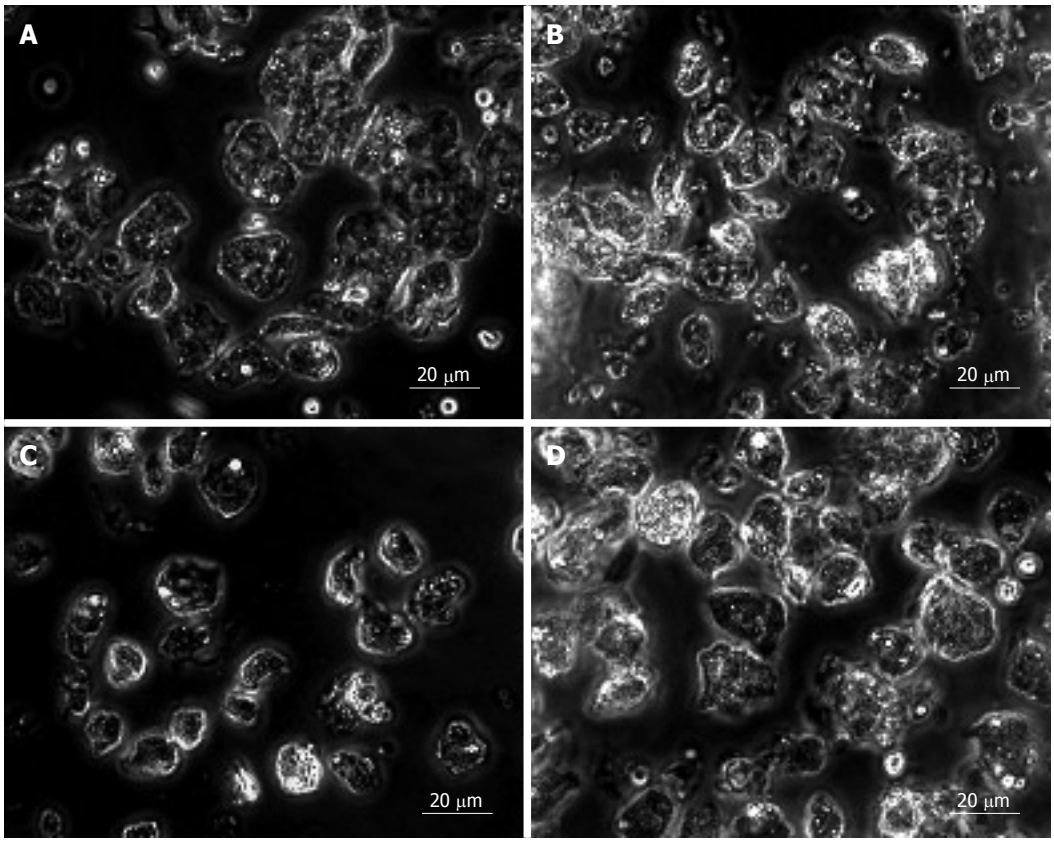


Figure 1 Purification of EpCAM +ve cell population. An unsorted fetal liver cell shows a heterogeneous population in group 1 (**A**) and group 2 (**B**). After double selection, the EpCAM +ve sorted fetal liver cells shows a homogeneous epithelial cell population (**C**). EpCAM -ve sorted fetal liver cells shows irregular shape of cells (**D**).

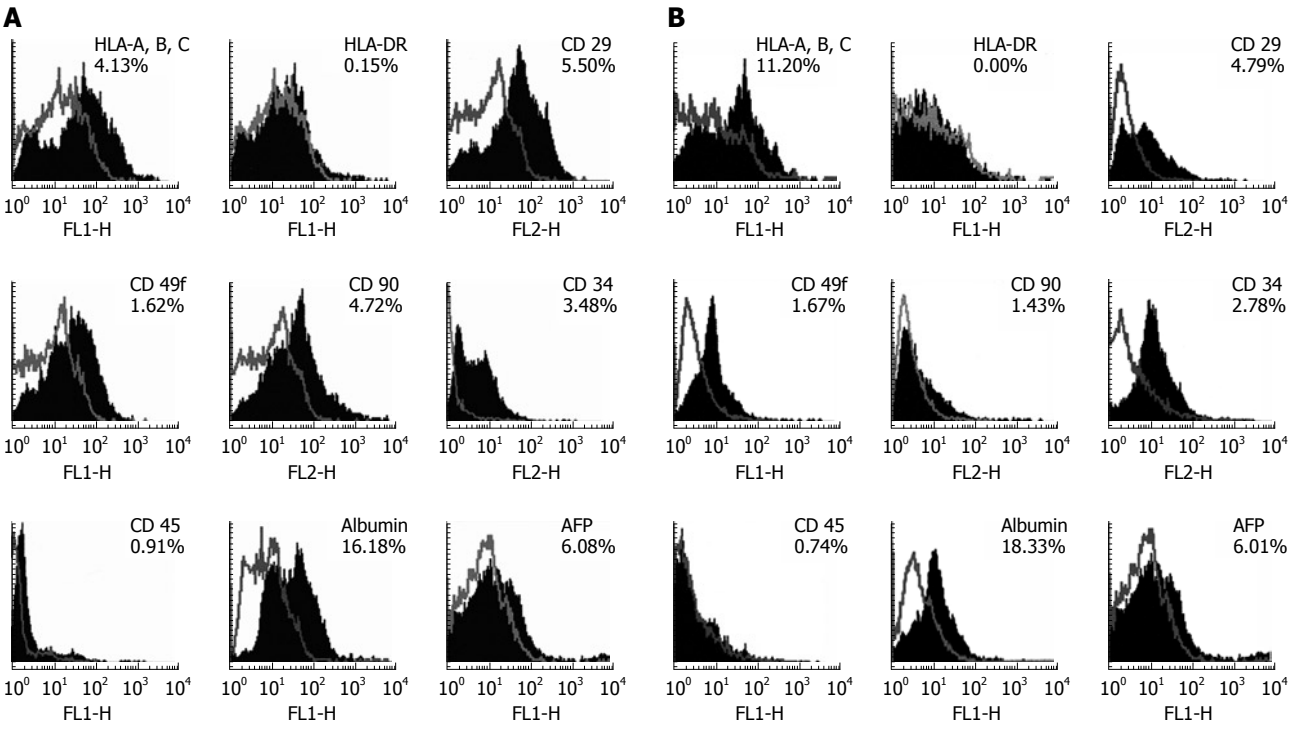


Figure 2 Identification of hepatic progenitor and HLA markers on EpCAM + sorted fetal liver cells by Flow cytometry. **A**: The overlaid histograms of analyzed markers with their unstained controls. Results indicated EpCAM +ve cells expressed surface markers such as, HLA-A, B, C, HLA-DR, CD29, CD49f, CD90, Albumin, AFP, CD34, CD45 By contrast HLA-DR did not express on EpCAM + cells in group 1. **B**: Expression of markers in group 2. In which HLA-A, HLA-B, HLA-C, and albumin was increased but CD90 expression was decreased on EpCAM +ve cells. Other markers such as CD29, CD49f, AFP, CD34 and CD45 expression levels were almost same in both age groups.

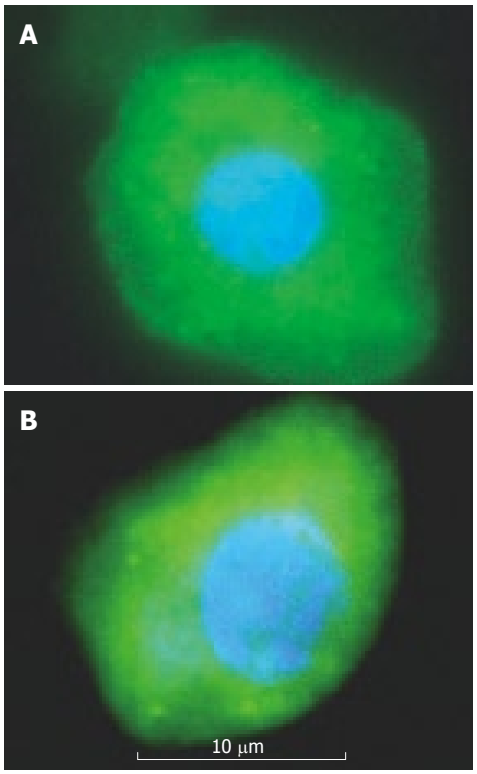


Figure 3 EpCAM + sorted fetal liver cells that are positive for CK18 (**A**) and albumin (**B**) in 18 wk gestation. These cells have high cytoplasm-to-nucleus ratio and observed mononucleated cells.

EpCAM +ve cells in group 1 (Figure 2A) and group 2 (Figure 2B). Numbers indicated in Table 2, gives mean percentage expression of HLA and hepatic progenitor markers in EpCAM +ve cells in group 1 and group 2.

Expression of HLA A, B and C and DR

HLA class Ⅰ expression was seen in approximately 6.52% cells in group 1. 8.2% in group 2. Expression of HLA class Ⅱ expression in the same period was similar 0.6% and 0.4% cells respectively (Table 2). HLA class Ⅰ and Ⅱ expression levels were not significantly different with respect to their gestational age groups.

Expression of integrins (CD29/49f), CD90, albumin and AFP

CD29 (β-1 integrin) and CD49f (α-6 integrin) are cell-adhesion molecules that are important for cell-matrix interactions, these markers are also used to study early hepatocyte differentiation. The expression of β1 integrin was observed in about 3.9% cells in-group 1 and in 2.0% cells in group 2. During the corresponding periods CD49f expression was seen in 2.2% and 1.6% cells. These data indicate the β-1 integrin cells in the EpCAM +ve population reduce by half in group 2 but there is no significant change in α-6 integrin +ve cells (Table 2).

CD90 is a cell surface protein is also one of the hepatic progenitor markers. The expression of CD90 was observed in 3.5% cells in group 1 and in 1.3% cells in group 2 and significant difference of expression of CD90 marker was observed with respect to gestational ages (Table 2).

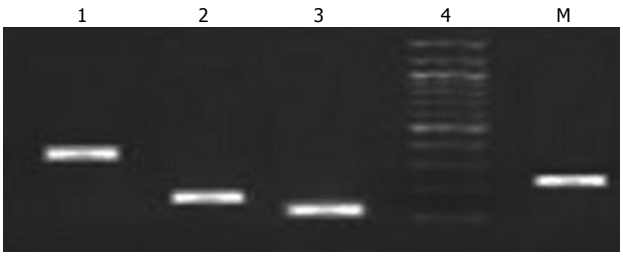


Figure 4 RT-PCR results indicate that the EpCAM + cells expressed hepatic stem cells markers such as CK18, CK19, albumin and AFP. 1: CK 19; 2: Albumin; 3: CK 18; 4: AFP; M: 100 bp Marker.

Albumin is used as a classic hepatic indicative markers differentiation for hepatic stem cells. We observed strong levels of albumin expression in EpCAM +ve but it did not differ significantly in gestational age groups (Table 2). FACS data revealed that albumin expression was 19.2% cells in group 1 and 21.3% cells in group 2.

AFP used as a marker for hepatic stem cells was observed strong levels in EpCAM +ve cells (Figure 2A and B), but it did not differ significantly in gestational age groups (Table 2). FACS data revealed that AFP expression was 7.3% in group 1 and 7.2% in group 2.

Analysis of markers expressed by Immunocytochemistry

On Immunocytochemical staining, EpCAM +ve cells were more strongly stained for CK18 and albumin. Albumin positive stained cells possessed large nuclei and more cytoplasm (Figure 3A). CK18 positive stained cells possessed large nuclei and highly granulated cytoplasm (Figure 3B).

RT-PCR analysis

To confirm that the sorted cells were of hepatic lineage, cultured Epcam +ve sorted cells were further analyzed for the expression of CK18, CK19, albumin, and AFP. These results suggest that EPCAM +ve cells strongly express genotypic CK18, CK19, Albumin, AFP (Figure 4) and these cells were capable of differentiating into hepatic and biliary lineages.

DISCUSSION

A progenitor or precursor for hepatocytes seen in fetal or adult tissues give rise to differentiated cells in a specialized way. Researchers often distinguish progenitor precursor cells from adult stem cells in the following way: when a stem cell divides, one of the two new cells is often a stem cell capable of replicating itself again where as the other cell is commited to differentiation. In contrast, when a progenitor/precursor cell divides, it can either form more progenitor/precursor cells or it can form two differentiated cells directly, neither of

which is capable of replicating itself. During normal homeostasis of tissues progenitor/precursor cells are used to replace cells that are damaged or dead, thus maintaining the integrity and functions of a tissue like liver.

This idea of progenitor cell dependent liver regeneration started with oval cells. Although neither cell replacement during normal tissue turnover nor after injury by partial hepatectomy requires stem cells for organ regeneration, this is not true for all types of liver injury. In some types of liver damage, for example primary biliary cirrhosis Alcoholic liver disease NASH, small cells (oval cells) with a high nuclear/cytoplasmic ratio emerge in the portal zone, proliferative extensively, and migrate into the lobule[19]. But the engraftment of oval cells may be less efficient than for hepatoctyes because of their smaller size and less efficient trapping in the liver[20].

Earlier studies have shown hepatic progenitors that express markers such as CD117/CD34[21], CD90[22], CD34/AFP[18]. Hepatoblasts are common progenitors for hepatocytes and biliary epithelial cells[23]. EpCAM has first been identified as a tumor-specific antigen on several carcinomas of different origin.

In our study we have observed EpCAM +ve cell enrichment of up to 15% after MACS sorting. These cells did not clump during purification and there was no change in the cell morphology or viability before and after sorting. In an earlier study have reported 12% EpCAM + enrichment from human fetal liver[8].

In our recent study we have shown an average of 5% CD34 +ve cells with hepatic progenitor properties on human total fetal liver cells[18]. Interestingly in the present study we observed CD34 expression (an average of 3%) on EpCAM +ve cells also. However these cells showed low or nil (0.91%) expression of CD45, the marker that distinguishes hematopoietic cells from non-hematopoietic cells. Therefore we can say that EpCAM +ve and CD34 +ve cells are not committed to hematopoietic lineage and they may differentiate into hepatic cells in the second trimester of fetal liver. Recently CD90 expressing cells were recognized as hepatic progenitors [22]. In our study progenitor markers such as CD90+, CD34+ expressions were observed on EpCAM +ve cells and these cells were negative for hematopoietic lineage (CD45). Where as CD90 and CD34 double positive expression ranged 0.44%-2.04% in total fetal liver cells and these cells were positive for both hepatic lineage (CK19) and hematopoietic lineage (CD45) markers in the second trimester[22].

In our study we found that positive cells of integrin CD29 (β-1 integrin) and CD49f (α-6) on EpCAM +ve cells. Earlier investigators have reported that CD29 as a specific hepatic stem cell marker, was expressed on an average of 0.08%-2% of the human total fetal liver cells[21]. It has been shown that CD49f +ve cells (an average of 48.51%) are primitive hepatic endodermal cells with the capacity to differentiate into hepatocytes in the mouse fetal liver[24]. Taken together presence of stem cell marker (β-1 integrin) and primitive hepatic endodermal (α-6 integrin) indicate that it is possible to designate our cells as possessing progenitor phenotype.

It has been speculated that progenitor cells are less immunogenic than mature cells; however, immunological rejection would still be an issue. We believe that transplantation of cells having negligible would certainly reduce GvHd development. Earlier investigators reported that high percentage of cells expressing the histocompatibility markers HLA class Ⅰ and HLA class Ⅱ were found in the 5th and 8th wk of gestation in total fetal liver cells after which the expression levels were decreased with advanced gestation age 21 wk This higher expression of HLA molecules in these cells may be because of hematopoietic cell contamination in the fetal liver in early gestation. Therefore in our study, we have enriched the EpCAM +ve cells without hematopoietic cell contamination and investigated the pattern of HLA molecules expressed by EpCAM +ve cells from human fetal liver by using flow cytometry. We found that EpCAM +ve cells expressed intermediate levels HLA class Ⅰ but no HLA class Ⅱ. Although controversial, it seems that, to varying degree, the human fetus can respond to allogenic cells from the beginning of the second trimester[25]. The allogenic response is usually HLA mediated and it is expected that transplantation with cells that do not express HLA class Ⅱ antigen may minimize the risk of alloreactivity. It has been stated that embryonic hepatocytes were still immature cells bearing incomplete MHC Ⅱ surface antigen, thus possessing lower immunogenicity[26].

Earlier investigators reported that EpCAM +ve cells were not immunogenic as they failed to stimulate a significant amount of T cell proliferation. Besides, these cells were not immunosuppressive as they failed to suppress the mixed lymphocyte reaction (MLR) significantly. Therefore EpCAM +ve cells might be an ideal candidate donor cells for hepatic cell therapy in liver disorders.

RT-PCR analysis on the EpCAM +ve cells showed that expression of albumin, AFP, CK18 and CK19 genes was detectable significantly with gestational ages. CK18 expression further confirms that the cells are epithelial lineage. It has been shown that EpCAM sorted hepatic progenitor cells have the high gene expression levels of AFP and albumin on hepatoblasts[8]. This suggests that EpCAM is a hepatic progenitor cell marker.

Fetal liver cells have been shown to be a rich source of progenitor/stem cells. However enrichment of hepatic progenitors from the developing human liver, with more viability and with minimum rejection in allogenic transplantation remains a significant challenge for the advancement of therapeutic approaches to liver disorders.

To summarize, the current study demonstrates that EpCAM is a novel and useful marker for enrichment of hepatic progenitors with moderate expression of HLA-class Ⅰ and negative for HLA- class Ⅱ. Thus this finding supports the use of EpCAM positive fetal liver cells for allogenic transplantation since these cells negative for class Ⅱ HLA antigen on their surface which otherwise can trigger a rejection reaction in allogenic transplantation. In our present study we determined the characteristic of cell type and purity. Further more, MLR and animal model studies are warranted to assess immunogenicity and in vivo engraftment respectively, of enriched EpCAM positive cells of fetal liver prior to pre-clinical transplantation.

ACKNOWLEDGEMENTS

The authors express their deep sense of gratitude to Mr Santhose Tiwari, Mr SM Vali, Mr KS Ahmed, Mr A Rajendraprasad of CLRD and Dr. O G Ram Prasad, Dr. Rajesh Gupta of CCMB for their technical help in the study.

COMMENTS

Background

Liver transplantation is the primary treatment for various end-stage hepatic diseases but is hindered by the lack of donor organs and by complications associated with rejection and immunosuppression. There is increasing evidence to suggest that the aborted human fetal liver is a transplantable source of hepatic progenitors. The aim of this study was to critically analyze the various phenotypic markers to enrich hepatic progenitor cells from human fetal liver. We have previously reported a marker (CD34) to enrich hepatic progenitor from human fetal liver.

Research frontiers

The hepatic progenitors cells offer a potential source for cell therapy and doing bridge transplantation for treatment of liver diseases.

Innovations and breakthroughs

This article helps to enrich hepatic progenitor cells using epithelial cell adhesion molecule (EpCAM) as a marker. We have found that EpCAM +ve cells expressed intermediate levels human leukocyte antigen (HLA) class Ⅰ but no HLA class Ⅱ. Our results provide evidence why enriched EpCAM + cells can be used for the treatment of liver diseases.

Applications

The sorting of stem cells using EpCAM can be used as hepatic as an alternative for hepatic cell therapy in liver cell disorders.

Peer review

This article tries to explore the enrichment of hepatic progenitor using EpCAM as a marker for the treatment of liver diseases. The result revealed that the EpCAM +ve cells can be used as hepatic progenitors for transplantation in patient with liver diseases.

REFERENCES

1 **Sutherland DE**, Numata M, Matas AJ, Simmons RL, Najarian JS. Hepatocellular transplantation in acute liver failure. *Surgery* 1977; **82**: 124-132
2 **Sommer BG**, Sutherland DE, Matas AJ, Simmons RL, Najarian JS. Hepatocellular transplantation for treatment of D-galactosamine-induced acute liver failure in rats. *Transplant Proc* 1979; **11**: 578-584
3 **Farber E**. Similarities in the sequence of early histological changes induced in the liver of the rat by ethionine, 2-acetylamino-fluorene, and 3'-methyl-4-dimethylaminoazobenzene. *Cancer Res* 1956; **16**: 142-148
4 **Malhi H**, Irani AN, Gagandeep S, Gupta S. Isolation of human progenitor liver epithelial cells with extensive replication capacity and differentiation into mature hepatocytes. *J Cell Sci* 2002; **115**: 2679-2688
5 **Sigal SH**, Brill S, Reid LM, Zvibel I, Gupta S, Hixson D, Faris R, Holst PA. Characterization and enrichment of fetal rat hepatoblasts by immunoadsorption ("panning") and fluorescence-activated cell sorting. *Hepatology* 1994; **19**: 999-1006
6 **Nowak G**, Ericzon BG, Nava S, Jaksch M, Westgren M, Sumitran-Holgersson S. Identification of expandable human hepatic progenitors which differentiate into mature hepatic cells in vivo. *Gut* 2005; **54**: 972-979
7 **Liu YN**, Zhang J, He QH, Dai X, Shen L. Isolation and characterization of epithelial progenitor cells from human fetal liver. *Hepatol Res* 2008; **38**: 103-113
8 **Schmelzer E**, Wauthier E, Reid LM. The phenotypes of pluripotent human hepatic progenitors. *Stem Cells* 2006; **24**: 1852-1858
9 **Schmelzer E**, Zhang L, Bruce A, Wauthier E, Ludlow J, Yao HL, Moss N, Melhem A, McClelland R, Turner W, Kulik M, Sherwood S, Tallheden T, Cheng N, Furth ME, Reid LM. Human hepatic stem cells from fetal and postnatal donors. *J Exp Med* 2007; **204**: 1973-1987
10 **Suzuki A**, Zheng YW, Kaneko S, Onodera M, Fukao K, Nakauchi H, Taniguchi H. Clonal identification and characterization of self-renewing pluripotent stem cells in the developing liver. *J Cell Biol* 2002; **156**: 173-184
11 **Migliaccio G**, Migliaccio AR, Petti S, Mavilio F, Russo G, Lazzaro D, Testa U, Marinucci M, Peschle C. Human embryonic hemopoiesis. Kinetics of progenitors and precursors underlying the yolk sac----liver transition. *J Clin Invest* 1986; **78**: 51-60
12 **Rowley PT**, Ohlsson-Wilhelm BM, Farley BA. Erythroid colony formation from human fetal liver. *Proc Natl Acad Sci USA* 1978; **75**: 984-988
13 **Hann IM**, Bodger MP, Hoffbrand AV. Development of pluripotent hematopoietic progenitor cells in the human fetus. *Blood* 1983; **62**: 118-123
14 **Roy V**, Miller JS, Verfaillie CM. Phenotypic and functional characterization of committed and primitive myeloid and lymphoid hematopoietic precursors in human fetal liver. *Exp Hematol* 1997; **25**: 387-394
15 **Haruna Y**, Saito K, Spaulding S, Nalesnik MA, Gerber MA. Identification of bipotential progenitor cells in human liver development. *Hepatology* 1996; **23**: 476-481
16 **Badve S**, Logdberg L, Sokhi R, Sigal SH, Botros N, Chae S, Das KM, Gupta S. An antigen reacting with das-1 monoclonal antibody is ontogenically regulated in diverse organs including liver and indicates sharing of developmental mechanisms among cell lineages. *Pathobiology* 2000; **68**: 76-86
17 **Habibullah CM**, Syed IH, Qamar A, Taher-Uz Z. Human fetal hepatocyte transplantation in patients with fulminant hepatic failure. *Transplantation* 1994; **58**: 951-952
18 **Nyamath P**, Alvi A, Habeeb A, Khosla S, Khan AA, Habibullah CM. Characterization of hepatic progenitors from human fetal liver using CD34 as a hepatic progenitor marker. *World J Gastroenterol* 2007; **13**: 2319-2323
19 **Shinozuka H**, Lombardi B, Sell S, Iammarino RM. Early histological and functional alterations of ethionine liver carcinogenesis in rats fed a choline-deficient diet. *Cancer Res* 1978; **38**: 1092-1098
20 **Wang X**, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Grompe M. The origin and liver repopulating capacity of murine oval cells. *Proc Natl Acad Sci USA* 2003; **100** Suppl 1: 11881-11888
21 **Nava S**, Westgren M, Jaksch M, Tibell A, Broome U, Ericzon BG, Sumitran-Holgersson S. Characterization of cells in the developing human liver. *Differentiation* 2005; **73**: 249-260

22 **Masson NM**, Currie IS, Terrace JD, Garden OJ, Parks RW, Ross JA. Hepatic progenitor cells in human fetal liver express the oval cell marker Thy-1. *Am J Physiol Gastrointest Liver Physiol* 2006; **291**: G45-G54
23 **Tanimizu N**, Nishikawa M, Saito H, Tsujimura T, Miyajima A. Isolation of hepatoblasts based on the expression of Dlk/Pref-1. *J Cell Sci* 2003; **116**: 1775-1786
24 **Hoppo T**, Fujii H, Hirose T, Yasuchika K, Azuma H, Baba S, Naito M, Machimoto T, Ikai I. Thy1-positive mesenchymal cells promote the maturation of CD49f-positive hepatic progenitor cells in the mouse fetal liver. *Hepatology* 2004; **39**: 1362-1370
25 **Lindton B**, Markling L, Ringden O, Kjaeldgaard A, Gustafson O, Westgren M. Mixed lymphocyte culture of human fetal liver cells. *Fetal Diagn Ther* 2000; **15**: 71-78
26 **Shi Z**, Liang XL, Lu BX, Pan SY, Chen X, Tang QQ, Wang Y, Huang F. Diminution of toxic copper accumulation in toxic milk mice modeling Wilson disease by embryonic hepatocyte intrasplenic transplantation. *World J Gastroenterol* 2005; **11**: 3691-3695

S- Editor Zhong XY **L- Editor** Alpini GD **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5738

World J Gastroenterol 2008 October 7; 14(37): 5738-5743
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Computed tomography perfusion in evaluating the therapeutic effect of transarterial chemoembolization for hepatocellular carcinoma

Guang Chen, Da-Qing Ma, Wen He, Bao-Feng Zhang, Li-Qin Zhao

Guang Chen, Da-Qing Ma, Wen He, Bao-Feng Zhang, Li-Qin Zhao, Department of Radiology, Beijing Friendship Hospital, Capital Medical University, 95 YongAn Road, Xuan Wu District, Beijing 100050, China
Author contributions: Ma DQ and He W designed the research; Chen G and Zhao LQ performed the data collection, data analysis and statistical analysis; Chen G and Zhang BF performed the clinical studies; Chen G, He W and Zhao LQ drafted and revised the manuscript.
Supported by The Science Technology Program of Beijing Education Committee: KM200810025002
Correspondence to: Wen He, MD, Department of Radiology, Beijing Friendship Hospital, Capital Medical University, 95 Yongan Road, Xuanwu District, Beijing 100050, China. hewen1724@hotmail.com
Telephone: +86-10-63138470 Fax: +86-10-63037216
Received: July 15, 2008 Revised: August 26, 2008
Accepted: September 2, 2008
Published online: October 7, 2008

Abstract

AIM: To prospectively assess the changes in parameters of computed tomography (CT) perfusion pre- and post-transarterial chemoembolization (TACE) of hepatocellular carcinoma (HCC) in different treatment response groups, and to correlate the changes with various responses of HCC to TACE.

METHODS: Thirty-nine HCC patients underwent CT perfusion examinations pre-(1 d before TACE) and post-treatment (4 wk after TACE). The response evaluation criteria for solid tumors (RECIST) were referred to when treatment responses were distributed. Wilcoxon-signed ranks test was used to compare the differences in CT perfusion parameters pre- and post-TACE for different response groups.

RESULTS: Only one case had treatment response to CR and the CT perfusion maps of post-treatment lesion displayed complete absence of signals. In the PR treatment response group, hepatic artery perfusion (HAP), hepatic arterial fracture (HAF) and hepatic blood volume (HBV) of viable tumors post-TACE were reduced compared with pre-TACE (P = 0.001, 0.030 and 0.001, respectively). In the SD group, all CT perfusion parameters were not significantly different pre- and post-TACE. In the PD group, HAP, HAF, portal vein perfusion (PVP) and hepatic blood flow (HBF) of viable tumors post-TACE were significantly increased compared with pre-TACE (P = 0.005, 0.012, 0.035 and 0.005, respectively).

CONCLUSION: Changes in CT perfusion parameters of viable tumors are correlated with different responses of HCC to TACE. Therefore, CT perfusion imaging is a feasible technique for monitoring response of HCC to TACE.

Key words: Hepatocellular carcinoma; Computed tomography; Transarterial chemoembolization; Digital subtraction arteriography; Region of interest

Peer reviewer: Dr. Serdar Karakose, Professor, Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey

Chen G, Ma DQ, He W, Zhang BF, Zhao LQ. Computed tomography perfusion in evaluating the therapeutic effect of transarterial chemoembolization for hepatocellular carcinoma. *World J Gastroenterol* 2008; 14(37): 5738-5743 Available from: URL: http://www.wjgnet.com/1007-9327/14/5738.asp DOI: http://dx.doi.org/10.3748/wjg.14.5738

INTRODUCTION

Transarterial chemoembolization (TACE) has been widely accepted as a choice of treatment for advanced hepatocellular carcinoma (HCC) and shows promising results[1-3]. The therapeutic efficacy of TACE has been evaluated by various imaging modalities. Digital subtraction angiography (DSA) is probably the most sensitive and effective imaging modality[4,5]. However, angiography is an invasive procedure and its routine use in evaluation of therapeutic effectiveness needs justification[5]. CT examination after TACE has been widely used to assess the efficacy of TACE[6], but its limitation is that a quantitative evaluation cannot be provided and an incomplete lipiodol accumulation may disturb the assessment of viable tumors on contrast-enhanced CT imaging[7].

Alternatively, CT perfusion imaging is a non-invasive technique for assessment of tissue perfusion in locally

advanced HCC[8-10]. The response to TACE may be evaluated by comparing the difference in perfusion parameters pre- and post-treatment[11,12].

To our knowledge, there are few reports on the application of this technique in evaluating the efficacy of TACE based on the quantitative analysis of perfusion parameters. In this study, we prospectively assessed the changes in perfusion parameters of viable tumors after TACE for different treatment response groups, and correlated the changes in CT perfusion parameters to different responses of tumors to TACE.

MATERIALS AND METHODS

Patients

Thirty-nine consecutive patients (28 men and 11 women; age range, 36-82 years; mean, 60.1 years) with histopathologically proven HCC (fine needle aspiration) referred to our center for TACE from September 2007 to June 2008 were included in the study. The average size of HCC was 6.1 ± 3.3 cm, ranging 2.5-16.5 cm. All patients underwent CT perfusion examinations pre-(1 d before TACE) and post-treatment (4 wk after TACE).

CT perfusion imaging technique

CT perfusion was performed with a 64-row multi-detector CT scanner (VCT 64 slices; GE Medical Systems). For initial localization of the tumor, a CT scan of the abdomen was performed without contrast medium during a breath hold at the end of expiration. After tumor localization, a 4-cm tumor region was selected independently for the dynamic study of the tumor maximal diameter. A dynamic study of the selected area was performed in a single breath hold at a static supine position. A total of 40-50 mL of non-ion iodinated contrast medium (Iohexol300, 300 mg of iodine per milliliter) was injected at a rate of 4 mL/s through an 18-gauge intravenous cannula. The following CT parameters were used to acquire dynamic data: 1-second gantry rotation time, 120 kV, 80 mA, acquisition in 264 transverse mode (64 sections per gantry rotation), and 5-mm reconstructed section thickness. The examination was repeated in all patients using the same technique before and 4 wk after TACE.

Imaging data processing

After image acquisition, the data were transferred to an image processing workstation (Advantage Windows 4.3; GE Medical Systems) and analyzed using CT Perfusion 3.0 (GE Medical Systems). For derivation of the functional maps of perfusion parameters, a reference arterial input curve was obtained by placing a region of interest (ROI) in the aorta (range, 10-15 mm^2) manually, and a reference input curve of portal vein was obtained by placing a ROI in the portal vein (range, 8-12 mm^2) manually. A ROI for tumor was hand drawn in a selected section in which the lesion demonstrated the maximal diameter.

The parameters generated by the software included hepatic blood flow (HBF), hepatic blood volume (HBV), mean transit time (MTT), and permeability-surface area product (PS), hepatic arterial fracture (HAF), hepatic artery perfusion (HAP), and portal vein perfusion (PVP).

TACE technique

All TACE procedures were performed by one experienced interventional radiologist. Diagnostic arteriogram of the common, right or left hepatic artery was obtained in all patients prior to TACE. For solitary lesions in liver, TACE with superselective catheterization of the feeding hepatic artery branch was performed. Chemoembolization was performed at the common hepatic artery for multiple lesions involving both lobes of liver. After a combined chemotherapy with an average dose of epirubicin (60 mg), hydroxycamptothecin (20 mg), and fluorouracil (750 mg), an average dose of lipiodol (6 mL) with epirubicin (10 mg) mixture and/or gelfoam particles were used in our study. The total volume of emulsion was judged by the tumor size and achievement of stagnant arterial flow. Follow-up hepatic angiograms were performed in the latter sessions, 4-5 wk and 8-9 wk following the first TACE. Additional chemoembolization would be given at the same session setting if there was evidence of residual hypervascularity in hepatic arteriograms.

Statistical analysis

DSA has been recognized as the gold standard for detection of viable hepatic tumors[5,7]. Therefore, in this study, DSA was used as the assessment method for measuring target lesions responsive to TACE. The first angiogram and follow-up angiograms in the latter sessions 4-5 wk and 8-9 wk following the first TACE were compared. The following response evaluation criteria [complete response (CR): disappearance of all target lesions; partial response (PR): at least a 30% decrease in the sum of the longest diameters (LD) of target lesions; progressive disease (PD): at least a 20% increase in the sum of the LD of target lesions; stable disease (SD): neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD][13] for solid tumors (RECIST) were referred to when it was necessary to assess the therapeutic effect after TACE.

All data were expressed as mean ± SD, and statistical analysis was performed with SPSS 11.0. Wilcoxon-signed ranks test (nonparametric test) of variance was used to compare the differences in CT perfusion parameters pre- and post-TACE for different treatment response groups. $P < 0.05$ was considered statistically significant.

RESULTS

In all patients of our study cohort, only one patient who underwent a period of TACE and CT perfusion examinations pre- and post-treatment had treatment response to CR. His CT perfusion imaging of post-treatment lesion displayed complete absence of blood

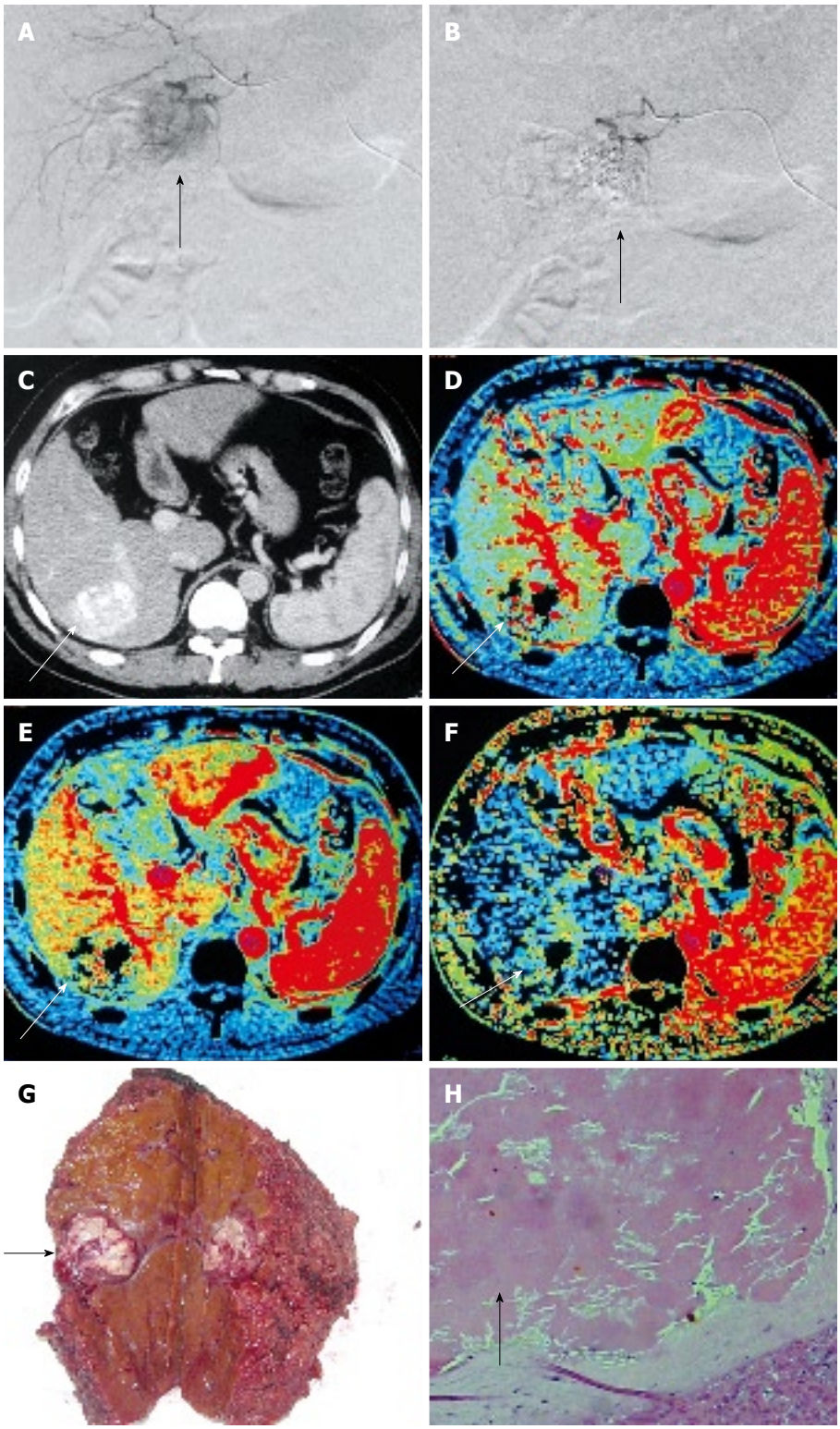


Figure 1 Images obtained in a patient with HCC verified with treatment response of PR. **A**: Pre-TACE angiography showing a tumor with a diameter of 3.5 cm in the right lobe of liver (arrow); **B**: Post-TACE angiography showing a viable region of tumor that was reduced by more than 50% (arrow) compared with pre-TACE; **C**: CT image 4 wk after TACE showing an incomplete lipiodol accumulation in the viable tumor region (arrow); **D**: HBF map of post-TACE CT perfusion showing a significant decrease of HBF in the viable tumor (arrow) compared with pre-TACE; **E**: Post-TACE HBV map showing a significant decrease of HBV in the viable tumor (arrow) compared with pre-TACE; **F**: Post-TACE HAF map showing a significant decrease of HAF in the viable tumor (arrow); **G**: Small necrotic lesions observed (arrow) after two periods of TACE treatment (arrow); **H**: Pathological slides (HE staining, × 40) showing lipiodol embolism in the small vascular cavity (arrow).

perfusion on CT perfusion maps. Therefore, in the CR group, the perfusion parameters of post-treatment tumor could not be generated.

In remaining 38 HCC patients, response to treatment was observed in the PR group (n = 14), SD group (n = 14), and PD group (n = 10).

In the PR treatment response group, some perfusion parameters (HAP, HAF, and HBV) of HCC post-TACE were significantly reduced compared with pre-TACE (Figure 1) and significantly different pre- and post-TACE (P = 0.001, 0.030 and 0.001, respectively), while no significant difference was observed in other perfusion parameters (HBF,MTT,PS, and PVP) pre- and post-TACE (Table 1).

In the SD treatment response group, no significant difference was found in all CT perfusion parameters pre- and post-TACE (Table 1).

In the PD treatment response group, some CT perfusion parameters (HAP, HAF, PVP, and HBF) of HCC post-TACE were significantly increased compared

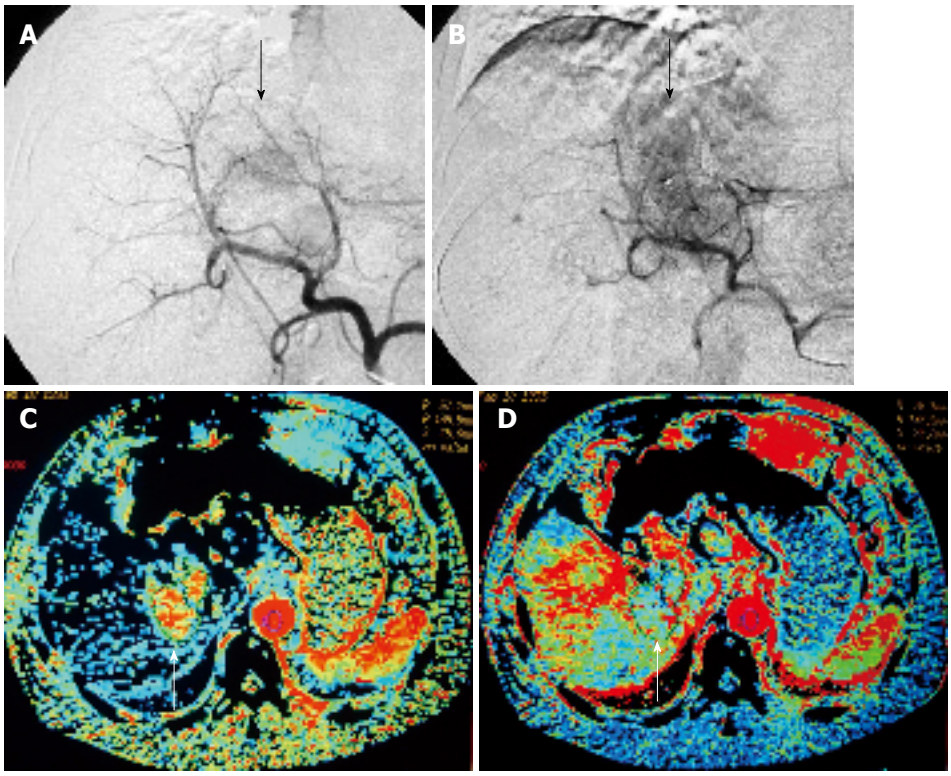


Figure 2 Images obtained from a patient with HCC with treatment respo-nse of PD group. **A**: Pre-TACE angiography showing a tumor in the left lobe of liver (arrow); **B**: Post-TACE angiography showing an increased viable tumor region by more than 25% (arrow) compared with pre-TACE; **C**: HAF map of post-TACE CT perfusion showing significantly increased HAF in the viable tumor (arrow) compared with pre-TACE; **D**: Post-TACE HBF map showing increased HBF in the viable tumor (arrow) compared with pre-TACE.

Table 1 Perfusion parameters in PR and SD group (mean ± SD)

	PR			SD		
n = 14	Pre-treatment	Post-treatment	*P* value	Pre-treatment	Post-treatment	*P* value
HBF (mL/min per 100 mL)	294.594 ± 162.104	203.941 ± 147.717	0.096	133.277 ± 111.124	205.580 ± 217.101	0.054
HBV (mL/100 mL)	37.569 ± 19.818	19.252 ± 11.847	0.001	19.399 ± 17.515	21.558 ± 21.085	0.220
MTT (s)	10.385 ± 4.747	10.687 ± 2.132	0.637	14.097 ± 4.276	12.235 ± 4.276	0.421
PS (mL/min per 100 mL)	20.066 ± 19.398	19.997 ± 22.516	0.638	27.357 ± 21.960	22.504 ± 18.439	0.310
HAF (%)	63.671 ± 20.218	38.154 ± 22.357	0.030	42.343 ± 14.025	33.793 ± 18.575	0.248
HAP (mL/min/100 mL)	201.857 ± 145.608	57.266 ± 33.537	0.001	62.621 ± 65.274	78.230 ± 76.442	0.172
PVP (mL/min/100 mL)	92.590 ± 69.771	146.681 ± 144.414	0.330	70.664 ± 50.880	127.349 ± 167.413	0.054

Table 2 Perfusion parameters in PD group (mean ± SD)

PD (*n* = 10)	Pre-treatment	Post-treatment	*P* value
HBF (mL/min per 100 mL)	178.508 ± 63.650	263.828 ± 91.731	0.005
HBV (mL/100 mL)	28.101 ± 26.862	29.978 ± 9.261	0.385
MTT (S)	9.336 ± 2.517	10.001 ± 2.457	0.798
PS (mL/min/100 mL)	33.103 ± 3.784	26.384 ± 10.544	0.092
HAF (%)	45.856 ± 41.332	69.628 ± 30.126	0.012
HAP (mL/min per 100 mL)	32.686 ± 7.145	94.200 ± 55.928	0.005
PVP (mL/min per 100 mL)	19.820 ± 5.546	33.480 ± 8.765	0.035

to pre-TACE (Figure 2) and their difference showed a statistical significance (P = 0.005, 0.012, 0.035 and 0.005, respectively), while no significant difference was found in other perfusion parameters (HBV, MTT, and PS) pre- and post-TACE (Table 2).

DISCUSSION

CT perfusion is a feasible, reproducible technique for assessing tissue perfusion in locally advanced HCC[14]. Measures of tumor perfusion have been correlated with angiogenesis and microvessel density within the tumor[14,15]. It is logical to speculate that high perfusion values indirectly suggest a high rate of angiogenesis and microvessel density within the tumor.

However, to our knowledge, there are few studies on the role of CT perfusion in evaluating the therapeutic efficacy of TACE, although Tsushima *et al*[11] presented their data on CT perfusion and demonstrated decreased values of HAP and HAF in viable tumors post-treatment compared with pre- treatment, which are consistent with our findings.

In our study, the number of cases collected allowed us to divide them into different treatment response groups for observation. In the CR group, CT perfusion imaging of post-TACE lesion displayed complete absence of signal on the CT perfusion maps. We observed significant differences in some CT perfusion parameters of viable tumors per- and post-TACE in the treatment response PR and PD groups. In the PR group of HCC, after TACE treatment, the change in perfusion parameters

was consistent with previous findings[11,16]. The decreased HBV and HBF within liver tumors indicate the reduction of vascular capacity and microvessel density, which are due to the lipiodol embolism (Figure 1G and H). Kan *et al*[16] reported that post-embolization MTT is elongated in intrahepatic lesions of a rat model, while post-embolization PS is significantly decreased. However, we did not find any significant difference in values of MTT and PS pre- and post-treatment, which may be due to the difference in observational time windows, in which the microvascular function within tumors dynamically changes. In the SD group, the perfusion parameters remained unchanged pre- and post-TACE treatment, suggesting a perfusion recovery within the tumor after treatment. In the PD group, HBF, HAF, and HAP were increased after treatment, reflecting the increased angiogenesis and microvessel density within the liver tumor. The value of PVP was further increased in HCC of the PD group after treatment, suggesting involvement of hepatic portal vein during tumor advancement. In our study, the value of HBV was not further increased during tumor advancement, suggesting that the vascular capacity within the tumor mass has reached its limit and thus, cannot further expand. Further histological research is needed to prove this speculation.

In addition, many CT perfusion analyses were performed using a single arterial input[8,9,15], but the liver has a dual arterial-portal blood supply, and the tumor and portal vein could not be consistently included. In our study, 64-rows multi-detector CT was introduced to offer a greater coverage (up to 4 cm), thus overcoming this limitation by including both the tumor and portal vein in dual-input analysis. The introduction of multi-detector CT has stimulated further interest in perfusion CT techniques and their future implementation in clinical practice[14].

We believe that comparison of the results from different CT perfusion studies has to be made cautiously, as the values measured are dependent on mathematic model and pharmacokinetics of the contrast medium used. Thus, application of different models to the same data may well yield different perfusion values. It should be emphasized that our results were specific to the method of analysis and the software employed in this study.

The limitations of our study are as follows. The grouping of patients did not involve factors such as differentiation of HCC, cirrhosis, invasion of portal vein, dosage of chemotherapeutant and lipiodol, and use of gelfoam particles, all of which could influence the therapeutic response of HCC to TACE and change the CT perfusion parameters after TACE. Because the determinants for therapeutic response of HCC to TACE are complicated and numerous, this study did not take into account these determinants of therapeutic response to TACE, but rather it only correlated the changes in CT perfusion parameters with different treatment responses to TACE.

In conclusion, CT perfusion imaging can be used in the assessment of perfusion changes resulting from TACE therapy. Post-treatment changes in perfusion parameters are correlated with different therapeutic efficacies for HCC. Thus, CT perfusion imaging is a feasible and non-invasive technique for monitoring treatment response to TACE.

COMMENTS

Background

Computed tomography (CT) perfusion imaging is a non-invasive and non-invasive technique for assessing tissue perfusion in locally advanced HCC.

Research frontiers

CT perfusion scan for patients with HCC allows assessment of perfusion changes due to transarterial chemoembolization (TACE). There are few reports on the application of this technique in evaluating the efficacy of TACE based on the quantitative analysis of perfusion parameters.

Innovations and breakthroughs

In this study, changes in parameters of CT perfusion pre- and post-TACEwere observed in different treatment response groups.

Applications

CT perfusion imaging, a feasible and non-invasive technique, can be used for monitoring treatment response to TACE.

Peer review

This study reported the changes of CT perfusion parameters in different treatment response groups and provides a useful imaging modality for monitoring treatment response to HCC.

REFERENCES

1 **Rossi P**, Salvatori FM, D'Erme M, Maradei A, Rossi M, Santoro P, Mastantuono M, Gualdi G. [Therapy of hepatic carcinoma by the intra-arterial injection of lipiodol, antineoplastic agents and gelfoam] *Radiol Med* (Torino) 1989; **77**: 37-43

2 **Nakamura H**, Hashimoto T, Oi H, Sawada S. Transcatheter oily chemoembolization of hepatocellular carcinoma. *Radiology* 1989; **170**: 783-786

3 **Ngan H**, Lai CL, Fan ST, Lai EC, Yuen WK, Tso WK. Treatment of inoperable hepatocellular carcinoma by transcatheter arterial chemoembolization using an emulsion of cisplatin in iodized oil and gelfoam. *Clin Radiol* 1993; **47**: 315-320

4 **Yoshioka H**, Nakagawa K, Shindou H, Ono Y, Kawakami A, Mabuchi N, Arita S, Fujii K, Hamada T, Ishida O. MR imaging of the liver before and after transcatheter hepatic chemo-embolization for hepatocellular carcinoma. *Acta Radiol* 1990; **31**: 63-67

5 **Castrucci M**, Sironi S, De Cobelli F, Salvioni M, Del Maschio A. Plain and gadolinium-DTPA-enhanced MR imaging of hepatocellular carcinoma treated with transarterial chemoembolization. *Abdom Imaging* 1996; **21**: 488-494

6 **Okusaka T**, Okada S, Ueno H, Ikeda M, Yoshimori M, Shimada K, Yamamoto J, Kosuge T, Yamasaki S, Iwata R, Furukawa H, Moriyama N, Sakamoto M, Hirohashi S. Evaluation of the therapeutic effect of transcatheter arterial embolization for hepatocellular carcinoma. *Oncology* 2000; **58**: 293-299

7 **Tsui EY**, Chan JH, Cheung YK, Cheung CC, Tsui WC, Szeto ML, Lau KW, Yuen MK, Luk SH. Evaluation of therapeutic effectiveness of transarterial chemoembolization for hepatocellular carcinoma: correlation of dynamic susceptibility contrast-enhanced echoplanar imaging and hepatic angiography. *Clin Imaging* 2000; **24**: 210-216

8 **Miles KA**, Hayball MP, Dixon AK. Functional images of hepatic perfusion obtained with dynamic CT. *Radiology* 1993; **188**: 405-411

9 **Fournier LS**, Cuenod CA, de Bazelaire C, Siauve N, Rosty C, Tran PL, Frija G, Clement O. Early modifications of hepatic perfusion measured by functional CT in a rat model

of hepatocellular carcinoma using a blood pool contrast agent. *Eur Radiol* 2004; **14**: 2125-2133

10 **Tsushima Y**, Funabasama S, Aoki J, Sanada S, Endo K. Quantitative perfusion map of malignant liver tumors, created from dynamic computed tomography data. *Acad Radiol* 2004; **11**: 215-223

11 **Tsushima Y**, Unno Y, Koizumi J, Kusano S. Hepatic perfusion changes after transcatheter arterial embolization (TAE) of hepatocellular carcinoma: measurement by dynamic computed tomography (CT). *Dig Dis Sci* 1998; **43**: 317-322

12 **Lin WY**, Wang SJ, Yeh SH. Hepatic perfusion index in evaluating treatment effect of transcatheter hepatic artery embolization in patients with hepatocellular carcinoma. *Neoplasma* 1995; **42**: 89-92

13 **Therasse P**, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000; **92**: 205-216

14 **Miles KA**. Perfusion CT for the assessment of tumour vascularity: which protocol? *Br J Radiol* 2003; **76** Spec No 1: S36-S42

15 **Sahani DV**, Holalkere NS, Mueller PR, Zhu AX. Advanced hepatocellular carcinoma: CT perfusion of liver and tumor tissue--initial experience. *Radiology* 2007; **243**: 736-743

16 **Kan Z**, Kobayashi S, Phongkitkarun S, Charnsangavej C. Functional CT quantification of tumor perfusion after transhepatic arterial embolization in a rat model. *Radiology* 2005; **237**: 144-150

S- Editor Zhong XY **L- Editor** Wang XL **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5744

World J Gastroenterol 2008 October 7; 14(37): 5744-5748
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Genetic polymorphism of *MCP-1-2518*, *IL-8-251* and susceptibility to acute pancreatitis: A pilot study in population of Suzhou, China

Wei-Chang Chen, Jin-Shan Nie

Wei-Chang Chen, Department of Gastroenterology, the First Affiliated Hospital, Suzhou University, Suzhou 215006, Jiangsu Province, China
Jin-Shan Nie, Department of Gastroenterology, Taicang Hospital, the Affiliated Hospital of Suzhou University, Taicang 215400, Jiangsu Province, China
Author contributions: Chen WC and Nie JS contributed equally to this work; Chen WC contributed to conception and designed the research; Nie JS performed the research and analyzed data; Nie JS drafted the article and Chen WC instructed and revised the paper.
Correspondence to: Dr. Wei-Chang Chen, Department of Gastroenterology, the First Affiliated Hospital of Suzhou University, 188 Shizi Road, Suzhou 215006, Jiangsu Province, China. weichangchen@126.com
Telephone: +86-512-65238033 Fax: +86-512-65228072
Received: February 13, 2008 Revised: July 22, 2008
Accepted: July 29, 2008
Published online: October 7, 2008

Abstract

AIM: To study the relationship between *MCP-1-2518A/G, IL-8-251A/T* polymorphism and acute pancreatitis (AP) in the Han population of Suzhou, China.

METHODS: A case-control study was conducted to compare the distribution of genotype and genetic frequency of *MCP-1-2518A/G, IL-8-251A/T* gene polymorphism among AP (n = 101), including mild AP (n = 78) and severe AP (n = 23) and control healthy individuals (n = 120) with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and DNA sequencing, and analyze the relationship between the *MCP-1-2518A/G, IL-8-251A/T* gene polymorphism and the susceptibility to AP.

RESULTS: Significant differences were found in the distribution of genotype of *MCP-1-2518A/G* between the healthy control group and mild AP group (χ^2 = 32.015, P < 0.001), the same was evident between the healthy control group and severe AP group (χ^2 = 12.932, P < 0.05) in Suzhou. However, no difference of genotypic distribution was noted between MAP and SAP (χ^2 = 0.006, P = 0.997). The genetic frequencies of G allele in mild AP were 72.4% (113/156) and 76.1% (35/46) in severe AP, both were higher than the controls, 47.1% (113/240) (χ^2 = 24.804; P < 0.001, and χ^2 = 13.005; P < 0.001), but no difference was found between severe AP and mild AP (χ^2 = 0.242; P = 0.623). No difference was found in the distribution of genotype of *IL-8-251A/T* between the healthy control group and AP group neither in the frequency of A and T allele.

CONCLUSION: The *MCP-1-2518* AA genotype of the population in Suzhou may be a protective genotype of AP, while one with higher frequency of G allele is more likely to suffer from pancreatitis. But the genotype of AA and the frequency of G allele could not predict the risk of severe AP. No correlation is found between the *IL-8-251* polymorphism and the liability of AP.

Key words: Acute pancreatitis; *MCP-1* DNA; *IL-8* DNA; Polymorphism

Peer reviewer: Ibrahim A Al Mofleh, Professor, Department of Medicine, College of Medicine, King Saud University, PO Box 2925, Riyadh 11461, Saudi Arabia

Chen WC, Nie JS. Genetic polymorphism of *MCP-1-2518, IL-8-251* and susceptibility to acute pancreatitis: A pilot study in population of Suzhou, China. *World J Gastroenterol* 2008; 14(37): 5744-5748 Available from: URL: http://www.wjgnet.com/1007-9327/14/5744.asp DOI: http://dx.doi.org/10.3748/wjg.14.5744

INTRODUCTION

Chemokines are the cytokines that can activate or chemoattract leukocytes, and provide a stimulus to direct leukocytes to the areas of injury. They play vital roles in inflammatory reaction, infection of causative organism, trauma and renovation, cytotoxic effect, *etc*[1]. They are 70-90 amino acids in length and approximately 8-10 kDa in molecular weight. They are divided into four subfamilies according to the presence of four cysteine residues in conserved locations of primary structure and the two amino terminal cysteine residues are immediately adjacent or separated by one amino acid. Four subfamilies are CXC, CC, C and CX3C[2]. Up to now, the chemokines we have discovered mainly belong to CXC and CC subgroups, and monocyte chemoattractant

protein-1 (*MCP-1*) and interleukin-8 (*IL-8*) are typical examples of those two subgroups.

Recently it was found that chemokines play an important role in the initiation and development of acute pancreatitis (AP), which takes part in systemic inflammatorome reaction syndrome, remote organ complications, and multiple organ dysfunction syndrome[3-6]. *MCP-1* is regarded as a mediator of inflammatory reaction in early-stage of AP[7]. Studies carried by Rau *et al*[8] found that *MCP-1* serum concentrations increase dramatically in patients who developed local complications and/or remote organ failure. A close correlation was found between the severity of remote organ failure and *MCP-1* elevation. *MCP-1* might play a pivotal role in the pathological mechanism of complicated AP. Studies also found that *IL-8*[9-11] increase in early-stage of acute pancreatitis and the serum concentration is correlated with the severity of pathogenetic condition.

American researchers[12] found that the *MCP-1-2518* polymorphism might be related to the severity of AP, and *MCP-1-2518G* allele is a risk factor for severe AP. Moreover, Hofner[13] drew a conclusion that the frequency of IL-8 polymorphism may predict the risk of SAP. But no similar studies have been done in the ethnic group of China. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and DNA sequencing were carried out in our study to assess the possible correlation between the gene polymorphism of *MCP-1-2518*, *IL-8-251 A/T* and AP in the Han population of Suzhou, China.

MATERIALS AND METHODS

Study population

A total of 101 consecutive patients (60 men and 41 women, mean age 51.57 ± 13.39, range 18-80 years) were analyzed in this study, including 78 patients (50 men and 28 women, mean age 52.46 ± 13.04) with mild AP and 23 patients (16 men and 7 women, mean age 56.83 ± 13.14) with severe AP. The patients were treated in the Department of Gastroenterology of the First Affiliated Hospital of Suzhou University. The diagnosis of AP was established based on abdominal pain or abdominal localizing signs and increased amylase levels increased by at least 3 times that of the upper limit of normal, and CT verification of pancreatitis. Pancreatitis was classified as severe when the APACHE Ⅱ score ⩾ 8[14], and the Balthazar CT severity index ⩾ D[15]. One hundred and twenty healthy volunteers (71 men and 49 women, mean age 51.05 ± 9.37 years, range 25-69 years) served as a control group. All subjects were Han Chinese people, who came from Suzhou city.

DNA isolation

For the examination of *MCP-1* and *IL-8* polymorphisms, genomic DNA purified from peripheral blood was used. Blood samples were collected in ethylenediaminetetraace tic acid-containing tubes for DNA extraction. Genomic DNA was isolated from peripheral blood leucocytes using a commercial Kit (The Wizard® Genomic DNA Purification Kit, Promega) and the genomic DNA was stored at -20℃ for further use.

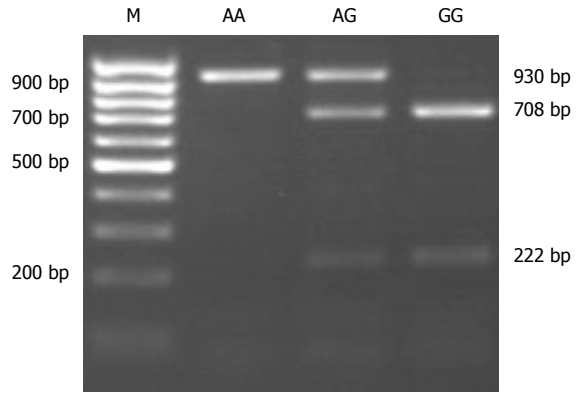


Figure 1 Electrophoresis of MCP-1-2518 polymorphism PCR product cut by *Pvu* Ⅱ. AA sample (930 bp), GG sample (708 bp and 222 bp), and AG sample (930 bp, 708 bp, and 222 bp).

Determination of MCP-1-2518 polymorphism

MCP-1-2518 polymorphism was assessed by means of a polymerase chain reaction procedure using the following primers[16]: sense: 5'-CCAGAGTGTTCCCAGCAC AG-3' and antisense: 5'-CTGCTTTGCTTGTGCCTC TT-3'. The PCR reaction mixture (50 μL) contained 100 ng genomic DNA, 5 μL 10 × Buffer, 1 μL of dNTPs (10 mmol/L each), 0.5 μL of Taq polymerase (Fermentas, 5 U/μL). PCR was performed using a 9700 Gene Amp PCR System Thermal Cycler (Applied Biosystems) under the following conditions: 95℃ for 5 min, followed by 30 cycles of 95℃ for 40 s, 57℃ for 40 s, and 72℃ for 60 s, with a final extension at 72℃ for 10 min. After that the products of PCR were genotyped by restriction fragment length polymorphism (RFLP). Ten μL PCR products were digested with 10 U of *Pvu* Ⅱ in 10 × buffer and H_2O up to a final volume of 20 μL at 37℃ for 16 h. 930 bp PCR product was cut into two bands of 708 bp and 222 bp. These digestion products were visualized in 2% agarose gels, stained with ethidium bromide. Samples showing only a 930 bp band were type AA. Samples showing two bands of 708 bp and 222 bp were considered GG and those showing three bands at 930 bp, 708 bp and 222 bp were designated AG (Figure 1).

Determination of IL-8-251 polymorphism

DNA was extracted from peripheral blood leucocytes, then the IL-8 promoter polymorphism at -251 was amplified with forward primer 5'-ATTGGCTGGCTT ATCTTC-3' and reverse primer 5'-TTCCTGGCTCTTG TCCTA-3', based on GenBank accession No. AF385628. PCR was carried out with the following protocol: 5 min 94℃, 35 cycles of 30 s 94℃, 1 min 52℃, 45 s 72℃, followed by 5 min 72℃, and 10 min 4℃. The product was 485 bp. The sequencing of the PCR products was performed using the M13RP primer and the DNA Big Dye Terminator Sequencing Kit (ABI) on an ABI 377

Table 1 Genotype distribution and allele frequency of *MCP-1-2518 A/G, IL-8-251A/T* polymorphism and Hardy-Weinberg test *n* (%)

	Genotype				Gene frequency		Hardy-Weinberg test
	AA	AG	GG	Total	A	G	
MCP-1-2518 A/G							
Control	29 (24.2)	63 (52.5)	28 (23.3)	120	121 (50.4)	119 (49.6)	$P = 0.583$
MAP	4 (4.5)	35 (39.8)	49 (55.7)	78	43 (27.6)	133 (72.4)	$P = 0.469$
SAP	1 (4.3)	9 (39.1)	13 (56.5)	23	11 (23.9)	35 (76.1)	$P = 0.718$
IL-8-251A/T							
Control	13 (10.8)	64 (53.4)	43 (35.8)	120	90 (37.5)	150 (62.5)	$P = 0.131$
MAP	5 (6.4)	40 (51.3)	33 (42.3)	78	50 (32.1)	106 (67. 0)	$P = 0.117$
SAP	1 (4.3)	14 (60.9)	8 (34.8)	23	16 (34.8)	30 (65.2)	$P = 0.101$

automated sequencer, following the manufacturers' protocols.

Statistical analysis

A comparison of the genotypic and allelic frequencies between the groups was performed using the Fisher exact test or χ^2 test when appropriate. Description data of continuous variables were tested by Student's *t* test. Statistical significance was established at $P < 0.05$. There was relationship between the genotypes or carriage of individual alleles and disease severity if presented as odds ratio (OR), with a 95% confidence interval (CI) of odds ratio (95% CI). All statistical calculations were performed with the SPSS 13.0 statistical program. Hardy-Weinberg equilibrium of the allele distribution was tested.

RESULTS

MCP-1-2518A/G polymorphisms

Three genotypes (AA, AG and GG) of MCP-1-2518 A/G polymorphisms were found in Han people of the Suzhou region. Control group AA 24.2% (29/120), AG 52.5% (63/120) and GG 23.3% (28/120); MAP group AA 4.5% (4/78), AG 39.8% (35/78) and GG 55.7% (49/78); and SAP group AA 4.3% (1/23), AG 39.1% (9/23) and GG 56.5% (13/23) (Table 1). There were significant differences in the distribution of genotype of MCP-1-2518 A/G between the healthy control group and MAP group ($\chi^2 = 32.015$, $P < 0.001$), the same was evident between the healthy control group and SAP group ($\chi^2 = 12.932$, $P < 0.05$) in Suzhou. However, no difference of genotypic distribution was noted between MAP and SAP ($\chi^2 = 0.006$, $P = 0.997$). The genetic frequencies of G allele in MAP 72.4% (113/156) and in SAP 76.1% (35/46) were all higher than the controls 49.6% (119/240) ($\chi^2 = 24.804$; $P < 0.001$, and $\chi^2 = 13.005$; $P < 0.001$), but we have found no difference between SAP and MAP ($\chi^2 = 0.242$; $P = 0.623$).

IL-8-251A/T polymorphism

The distribution of the IL-8-251 A and T alleles was as follows: MAP: AA 6.41%, AT 51.28% and TT 42.31%; SAP: AA 4.3%, AT 60.87% and TT 34.78%; Control: AA 10.83%, AT 53.33% and TT 35.83% (Table 1). There were no significant differences in the distribution of genotype of IL-8-251 A/T between the healthy control group and AP group ($P > 0.05$). There was also no difference in the frequency of A and T allele between the healthy control group and AP group ($P > 0.05$).

Hardy-Weinberg equilibrium

In two polymorphisms, the genotype distributions were studied in the Suzhou population (both AP and control subjects) according to Hardy-Weinberg equilibrium (Table 1).

DISCUSSION

MCP-1 and *IL-8* are known as the members of the family of chemokines. CXC chemokines represented by IL-8 exhibit a potent chemotactic activity for neutrophils while CC chemokines represented by *MCP-1* are implicated in the activation of monocytes, macrophages, and lymphocytes[17]. *MCP-1* and *IL-8* play an important role in the cause and development of AP. Ishibashi[18] found that blocking of MCP-1 activity attenuates the severity of AP in rats, which manifests that MCP-1 may be involved in the progression of severe AP. Grady T[19] found that the level of *MCP-1/JE* mRNA elevated in caerulin-induced experimental pancreatitis. In human AP, *MCP-1* expression was found to up-regulate in pancreatic tissues, and monocytic exudation was considered to be caused by this chemokine[20]. *IL-8* as one of the earliest cytokines appearing in the serum of patients with AP remains persistently elevated and therefore it might be of help in assessing the severity of AP after admission[21]. In severe AP patients, *IL-8* could concentrate and activate neutrophilic leukocyte, which could play a vital role in the development of adult respiratory distress syndrome[22]. Experiments in rat models suggested that inhibiting the release of *IL-8* could lower the severity level and case-fatality rate than inhibiting the other inflammatory factors[23]. All these results provide evidences that *MPC-1* and *IL-8* play a key role in the occurrence and development of AP.

In recent years, so many studies found that there is a relationship between the gene polymorphism and AP. Balog *et al*[24] found that high frequencies of the *HSP70-2 G* and the *TNF-α-308 A* alleles were associated wit the risk of severe AP. Genotype assessments may be important prognostic tools to predict disease severity

and the course of AP. He considered that genotype assessments may also be used to guide treatment or to identify risk populations for severe AP. Rahman *et al*[25] found that the functional *GSTT-1*A* genotype was associated with severe attacks of pancreatitis. Intensified oxidative stress characterized by glutathione depletion may be of importance in mediating the progression from mild to severe pancreatitis. All these studies note that variations in the DNA sequence might predispose individuals to AP.

Our study is designed to illustrate the relationship between *MCP-1-2518A/G* and *IL-8-251A/T* polymorphism and the liability of AP. The result showed that not only in MAP group but also in SAP group, the proportion of AA genotype was obviously lower in *MCP-1-2518* than in the control group. However, the frequency of G allele of MAP and SAP group was both higher than the control group. We therefore can conclude that the patients with the GA and GG genotype in *MCP-1-2518* might have higher risks for AP, but the patients with AA genotype in *MCP-1-2518* might have less liability to AP. It is possible that the AA genotype has a function of protecting the organism from AP. Nevertheless, our study found no difference between the MAP group and the SAP group of the AA genotype in *MCP-1-2518*. The study by Papachristou *et al*[12] using the method of PCR showed that the G allele in *MCP-1-2518A/G* of SAP group is obviously higher than the control group and MAP group, while the frequency of homozygote AA in the SAP group is lower than the MAP group and the control group. Therefore, it was suggested that the obvious increase of the frequency of G allele of the AP patients is possibly related with the high risk of developing SAP, moreover, the AP patients with the AA genotype are less likely to suffer from pancreatitis. Both our group and Papachristou GI's group discovered that there is a significant deviation between the AP group and the control group of the genotype and the frequency of allele in *MCP-1-2518*, but we found no difference between the AA genotype of SAP and MAP groups and the frequency of allele in *MCP-1-2518*.

At the same time, we did not discover the relationship between genetic polymorphism of *MCP-1-251* and the occurrence of AP. And this result is completely diverse from the research by Hofner *et al*[13]. They found that the *IL-8-251A/T* had significantly higher frequency in the patients with severe pancreatitis than in the healthy blood donors, while the frequency of the TT genotype was higher in the patients with mild pancreatitis than in the group with severe pancreatitis. We therefore, conclude that the IL-8 polymorphism may identify populations at risk of severe AP. In their study, the distribution of the IL-8 genotypes in control population of Hungary was: TT 82/200 (41%), AA 34/200 (17%) and AT 84/200 (42%), which is similar to ours ($\chi^2 = 4.537$, $P = 0.103$). The distribution of the IL-8 genotypes in our control subjects is also similar to other studies in Beijing and Taiwan[26,27]. But the genetic polymorphisms of IL-8-251 in healthy populations in our and Honfer's studies were different from that in Denmark[28], German[29], and Spain[30].

All those data indicated that the ethnic difference could be found in the genetic polymorphisms of IL-8-251. Although we and Hofner P gained similar results in the distribution of the IL-8 genotypes, it is possible that various results could be obtained in the polymorphisms of IL-8-251 among different countries. It is well known that only one genetic polymorphism cannot determine the final outcome of the diseases. Since all these studies are lack of larger cohorts, further studies are needed to confirm that the polymorphisms of IL-8-251 could affect the course of AP.

In summary, our study suggested that genetic polymorphism possibly determines the occurrence and development of AP. Moreover, ethnic difference in genetic polymorphisms may determine the functions in different populations. Thus, the relationship between genetic polymorphism and AP will need more thorough researches.

COMMENTS

Background

Chemokines play an important role in the pathogenesis of acute pancreatitis (AP). Monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) are typical chemokines. Many researchers have explored the relationship between gene polymorphism and the occurrence and development of AP to reveal whether polymorphisms of *MCP-1* and *IL-8* genes relate with the severity of AP.

Research frontiers

Recently the polymorphism of gene correlated with the occurrence and development of diseases is a hotspot. New methods, for example, gene chip techniques, for the research of polymorphism and new findings have been found.

Innovations and breakthroughs

The polymorphism of *MCP-1-2518* might affect the course of AP, but the genotype of AA and the frequency of G allele could not predict the risk of SAP, which is different from Papachristou GI's research. And no correlation has been found between the *IL-8-251* polymorphism and the liability of AP. The result is obviously diverse from Hofner P's research.

Applications

This study is aimed to find a new way to monitor and evaluate the course of AP, and guide therapy or to identify populations at risk of severe AP.

Terminology

PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism.

Peer review

The authors have studied MCP-1 and IL-8 polymorphism in MAP and SAP. The recent environmental and genetic researches have added more knowledge in the field of pancreatitis pathophysiology and prognosis. This is an interesting study.

REFERENCES

1 **Rollins BJ**. Chemokines. *Blood* 1997; **90**: 909-928
2 **Laing KJ**, Secombes CJ. Chemokines. *Dev Comp Immunol* 2004; **28**: 443-460
3 **Hotz HG**, Reber HA. Acute pancreatitis. *Curr Opin Gastroenterol* 1999; **15**: 392-397
4 **Sun J**, Bhatia M. Blockade of neurokinin-1 receptor attenuates CC and CXC chemokine production in experimental acute pancreatitis and associated lung injury. *Am J Physiol Gastrointest Liver Physiol* 2007; **292**: G143-G153
5 **Sugita H**, Yamaguchi Y, Ikei S, Yamada S, Ogawa M. Enhanced expression of cytokine-induced neutrophil chemoattractant (CINC) by bronchoalveolar macrophages in cerulein-induced pancreatitis rats. *Dig Dis Sci* 1997; **42**: 154-160

6 **Makhija R**, Kingsnorth AN. Levels of the chemokines growth-related oncogene alpha and epithelial neutrophil-activating protein 78 are raised in patients with severe acute pancreatitis (*Br J Surg* 2002; **89**: 566-572). *Br J Surg* 2002; **89**: 1194

7 **Brady M**, Bhatia M, Christmas S, Boyd MT, Neoptolemos JP, Slavin J. Expression of the chemokines MCP-1/JE and cytokine-induced neutrophil chemoattractant in early acute pancreatitis. *Pancreas* 2002; **25**: 260-269

8 **Rau B**, Baumgart K, Krüger CM, Schilling M, Beger HG. CC-chemokine activation in acute pancreatitis: enhanced release of monocyte chemoattractant protein-1 in patients with local and systemic complications. *Intensive Care Med* 2003; **29**: 622-629

9 **Norman J.** The role of cytokines in the pathogenesis of acute pancreatitis. *Am J Surg* 1998; **175**: 76-83

10 **Gross V**, Andreesen R, Leser HG, Ceska M, Liehl E, Lausen M, Farthmann EH, Schölmerich J. Interleukin-8 and neutrophil activation in acute pancreatitis. *Eur J Clin Invest* 1992; **22**: 200-203

11 **Berney T**, Gasche Y, Robert J, Jenny A, Mensi N, Grau G, Vermeulen B, Morel P. Serum profiles of interleukin-6, interleukin-8, and interleukin-10 in patients with severe and mild acute pancreatitis. *Pancreas* 1999; **18**: 371-377

12 **Papachristou GI**, Sass DA, Avula H, Lamb J, Lokshin A, Barmada MM, Slivka A, Whitcomb DC. Is the monocyte chemotactic protein-1 -2518 G allele a risk factor for severe acute pancreatitis? *Clin Gastroenterol Hepatol* 2005; **3**: 475-481

13 **Hofner P**, Balog A, Gyulai Z, Farkas G, Rakonczay Z, Takács T, Mándi Y. Polymorphism in the IL-8 gene, but not in the TLR4 gene, increases the severity of acute pancreatitis. *Pancreatology* 2006; **6**: 542-548

14 **Domínguez-Muñoz JE**, Carballo F, García MJ, de Diego JM, Campos R, Yangüela J, de la Morena J.Evaluation of the clinical usefulness of APACHE II and SAPS systems in the initial prognostic classification of acute pancreatitis: a multicenter study. *Pancreas* 1993; **8**: 682-686

15 **Balthazar EJ**, Robinson DL, Megibow AJ, Ranson JH. Acute pancreatitis: value of CT in establishing prognosis. *Radiology* 1990; **174**: 331-336

16 **Mühlbauer M**, Bosserhoff AK, Hartmann A, Thasler WE, Weiss TS, Herfarth H, Lock G, Schölmerich J, Hellerbrand C. A novel MCP-1 gene polymorphism is associated with hepatic MCP-1 expression and severity of HCV-related liver disease. *Gastroenterology* 2003; **125**: 1085-93

17 **Mukaida N**, Harada A, Matsushima K. Interleukin-8 (IL-8) and monocyte chemotactic and activating factor (MCAF/MCP-1), chemokines essentially involved in inflammatory and immune reactions. *Cytokine Growth Factor Rev* 1998; **9**: 9-23

18 **Ishibashi T**, Zhao H, Kawabe K, Oono T, Egashira K, Suzuki K, Nawata H, Takayanagi R, Ito T. Blocking of monocyte chemoattractant protein-1 (MCP-1) activity attenuates the severity of acute pancreatitis in rats. *J Gastroenterol* 2008; **43**: 79-85

19 **Grady T**, Liang P, Ernst SA, Logsdon CD. Chemokine gene expression in rat pancreatic acinar cells is an early event associated with acute pancreatitis. *Gastroenterology* 1997; **113**: 1966-1975

20 **Saurer L**, Reber P, Schaffner T, Büchler MW, Buri C, Kappeler A, Walz A, Friess H, Mueller C. Differential expression of chemokines in normal pancreas and in chronic pancreatitis. *Gastroenterology* 2000; **118**: 356-67

21 **Pooran N**, Indaram A, Singh P, Bank S. Cytokines (IL-6, IL-8, TNF): early and reliable predictors of severe acute pancreatitis. *J Clin Gastroenterol* 2003; **37**: 263-266

22 **Donnelly SC**, Strieter RM, Kunkel SL, Walz A, Robertson CR, Carter DC, Grant IS, Pollok AJ, Haslett C. Interleukin-8 and development of adult respiratory distress syndrome in at-risk patient groups. *Lancet* 1993; **341**: 643-647

23 **Abe R**, Shimosegawa T, Kimura K, Abe T, Kashimura J, Koizumi M, Toyota T. The role of endogenous glucocorticoids in rat experimental models of acute pancreatitis. *Gastroenterology* 1995; **109**: 933-943

24 **Balog A**, Gyulai Z, Boros LG, Farkas G, Takács T, Lonovics J, Mándi Y. Polymorphism of the TNF-alpha, HSP70-2, and CD14 genes increases susceptibility to severe acute pancreatitis. *Pancreas* 2005; **30**: e46-e50

25 **Rahman SH**, Ibrahim K, Larvin M, Kingsnorth A, McMahon MJ. Association of antioxidant enzyme gene polymorphisms and glutathione status with severe acute pancreatitis. *Gastroenterology* 2004; **126**: 1312-1322

26 **Lee WP**, Tai DI, Lan KH, Li AF, Hsu HC, Lin EJ, Lin YP, Sheu ML, Li CP, Chang FY, Chao Y, Yen SH, Lee SD. The -251T allele of the interleukin-8 promoter is associated with increased risk of gastric carcinoma featuring diffuse-type histopathology in Chinese population. *Clin Cancer Res* 2005; **11**: 6431-6441

27 **Lu W**, Pan K, Zhang L, Lin D, Miao X, You W. Genetic polymorphisms of interleukin (IL)-1B, IL-1RN, IL-8, IL-10 and tumor necrosis factor {alpha} and risk of gastric cancer in a Chinese population. *Carcinogenesis* 2005; **26**: 631-636

28 **Vogel U**, Christensen J, Wallin H, Friis S, Nexø BA, Tjønneland A. Polymorphisms in COX-2, NSAID use and risk of basal cell carcinoma in a prospective study of Danes. *Mutat Res* 2007; **617**: 138-146

29 **Arinir U**, Klein W, Rohde G, Stemmler S, Epplen JT, Schultze-Werninghaus G. Polymorphisms in the interleukin-8 gene in patients with chronic obstructive pulmonary disease. *Electrophoresis* 2005; **26**: 2888-2891

30 **Infante J**, Llorca J, Berciano J, Combarros O. Interleukin-8, intercellular adhesion molecule-1 and tumour necrosis factor-alpha gene polymorphisms and the risk for multiple system atrophy. *J Neurol Sci* 2005; **228**: 11-13

S- Editor Li DL **L- Editor** Ma JY **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5749

World J Gastroenterol 2008 October 7; 14(37): 5749-5754
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Expression of Livin and vascular endothelial growth factor in different clinical stages of human esophageal carcinoma

Li Chen, Guo-Sheng Ren, Fan Li, Shan-Quan Sun

Li Chen, Department of Thoracic Surgery, the First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China
Guo-Sheng Ren, Fan Li, Department of General Surgery, the First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China
Shan-Quan Sun, Anatomy institution of Chongqing Medical University, Chongqing 400016, China
Author contributions: Ren GS and Chen L contributed equally to this work; Chen L and Sun SQ designed research; Chen L and Li F performed research; Chen L contributed new reagents/analytic tools; Li F analyzed data; and Chen L and Ren GS wrote the paper.
Correspondence to: Guo-Sheng Ren, Department of General Surgery, the First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China. renguosheng726@163.com
Telephone: +86-23-89012301 Fax: +86-23-68120808
Received: July 18, 2008 Revised: August 24, 2008
Accepted: August 31, 2008
Published online: October 7, 2008

Abstract

AIM: To investigate the role of Livin and vascular endothelial growth factor (VEGF) in human esophageal carcinoma, and analyze its relationship to clinical stages.

METHODS: Expression of Livin in fresh esophageal cancer tissues was detected by immunohistochemistry (IHC), Western blotting and reverse transcriptase-polymerase chain reaction (RT-PCR), and VEGF by Western blotting and RT-PCR. All statistical analyses were performed by SPSS version 11.0.

RESULTS: Livin positivity was also significantly correlated with tumor stages, increasing with tumor progression. Expression of Livin and VEGF increased with the process of esophageal carcinoma. In the fourth clinical stage, expression of Livin and VEGF was the most significant. Expression of Livin was positively correlated with VEGF.

CONCLUSION: Over-expression of Livin and VEGF contributes to the pathogenesis of esophageal carcinoma.

Key words: Esophageal carcinoma; Livin; Vascular endothelial growth factor

Peer reviewer: Kam-Meng Tchou-Wong, Assistant Professor, Departments of Environmental Medicine and Medicine, NYU School of Medicine, 57 Old Forge Road, Tuxedo, New York 10987, United States

Chen L, Ren GS, Li F, Sun SQ. Expression of Livin and vascular endothelial growth factor in different clinical stages of human esophageal carcinoma. *World J Gastroenterol* 2008; 14(37): 5749-5754 Available from: URL: http://www.wjgnet.com/1007-9327/14/5749.asp DOI: http://dx.doi.org/10.3748/wjg.14.5749

INTRODUCTION

Livin, also known as inhibitor of apoptosis (IAP) has been identified as a new member of the IAP family proteins[1-3]. Like other IAP family proteins, Livin interacts with downstream caspases, such as caspase-3, caspase-7, and caspase-9, leading to their inactivation and degradation[4,5]. Its overexpression can protect cells from several proapoptotic stimuli. Very importantly, treatment of cancer cells with Livin antisense oligo-DNA causes apoptotic cell death, indicating that Livin expression may be essential for survival of certain cancer cells[6,7]. Vascular endothelial growth factor (VEGF) is a potent mitogen for endothelial cells, and its expression has been correlated with increased tumour angiogenesis[8,9]. VEGF plays a crucial role in tumour expansion by initiating permeabilization of blood vessels, by extravasation of plasma proteins, by invasion of stromal cells, and by causing the sprouting of new blood vessels that supply the tumour with oxygen and nutrients. A number of studies have shown that expression of certain VEGF transcripts are correlated with tumour progression[10]. Although increases of certain VEGF transcripts have been demonstrated to correlate with the progression of various tumours, the actual protein levels of the different VEGF isoforms and their significance during cellular transformation are unknown. Moreover, it has been suggested that elevated protein expression in tumour tissues was mediated by both enhanced transcription and translation. Thus, in order to understand the role of Livin and VEGF in tumour progression, it is important to investigate Livin and VEGF expression of different clinical stages at the protein and mRNA level during tumourigenesis. Esophageal carcinoma is one of the most frequent malignancies in many countries. Despite recent progress in chemotherapeutic, radiotherapeutic, and surgical treatment, the 5-year survival rate of esophageal carcinoma patients is still low, especially in advanced cases. In order to further explore the role of Livin and

VEGF in the development of esophageal carcinoma, we investigated the role of Livin and VEGF in human esophageal carcinoma and analyze its relationship with clinical stages.

MATERIALS AND METHODS

Materials

Specimens of cancer tissues were taken from 67 consecutive patients with esophageal carcinoma from Oct 2004 to Sept 2005 at the Department of Thoracic Surgery, the First Affiliated Hospital of Chongqing Medical University. None of them received irradiation or chemotherapy preoperatively. The patients included 46 men and 21 women with a mean age of 57 ranges from 38-86 years. The clinicopathologic stage was determined according to TNM classification. Six tumors were located in the upper thorax, 6 cases for clinicopathologic stage one, 24 case clinicopathologic stage two, 28 cases clinicopathologic stage three, 9 cases clinicopathologic stage four. All fresh tissues were taken immediately after operation and stored in a nitrogen canister. Informed consent was obtained from all participants, and the study was approved by the ethics committee on human research in Chongqing Medical University, Chongqing, China.

Immunohistochemical (IHC) assay

Tissue samples were collected after surgery and immediately frozen in liquid nitrogen. Prior to IHC assay, frozen sections were prepared with a cryostat (FACS caliber, Becton Dickinson, USA) at -20℃, dried at room temperature, and fixed with acetone. The PBMC were routinely isolated and the slides were prepared with a cytospinner. The ABC immunohistochemical assay was carried out according to the protocols we described before Anti-Livin (Antibody Diagnosis, USA) was prepared in our lab. The second antibody, a goat anti-mouse IgG labeled with biotin, was purchased from Vector Co., USA. Two hundred cells were counted and the intensity of staining for each of those cells was adjusted. Five grades were employed to express the degrees of staining, which represent 5 reaction coefficients respectively. The 5 products of every coefficient and the corresponding cell number were added up, which resulted in the value of a positive score. All slides were measured in duplicate. Those samples with a positive score over 10 or frequency over 5% were considered as positive.

Western blotting

Mouse tissues were dissected and homogenized in T-PER buffer in the presence of protease inhibitors. After homogenization, the lysates were centrifuged at 100 000 × *g*, and the supernatants were saved for Western blot, Ciphergen (BioSource International, Inc., USA) Protein Chip Array. Equal amounts of lysates were subject to SDS-PAGE (Tris-glycine mini gel; 1:2500, BioSource International, Inc., USA) and Western blot analysis using antibodies specific for the following: Livin (1:2500, BioSource International, Inc., USA), VEGF (1:2500, BioSource International, Inc., USA), β-tublin (1:5000; BioSource International, Inc., USA). The optical densities of the specific bands were scanned and measured by image analysis software (HPIAS 2000, Tongji Qianping Company, Wuhan, China).

Table 1 Oligonucleotides used for reverse transcriptase-polymerase chain reaction

Target gene	Primer sequence (5′-3′)	Size (bp)	Annealing temperature (℃)
β-actin	Forward: GTTCGCCATGGA TGACGATATC	266	59
	Reverse: GCCAGATCTTCTC CATGTCGTC		
VEGF	Forward: TGCTCAGCATTC GGACTGACCT	228	61
	Reverse: CAGTATGCATGGA CCATGACGG		
Livin	Forward: CTGGTCAGAGCC AGTGTTCCT	312	61
	Reverse: TCATAGAAGGA GGCCAGACG		

Primers were designed using the Primer Express Program and offered by AuGCT Biotechnology, Beijing, China.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Animals were sacrificed at corresponding time points and total RNA in the treated sections were extracted according to the total RNA extracting kit. 4 μg total RNA was heated at 70℃ for 5 min and then chilled on ice. Samples were incubated at 37℃ for 1 h and the reaction was stopped by heating at 70℃ for 10 min. Specific primers were designed for PCR: *Livin* and *VEGF* (Table 1). PCR was performed using 2 μL cDNA, 2 mmol/L dNTP, a specific pair of primers (20 pmol), 2 U DNA polymerase, 5 × PCR buffer and deionized water were added to the cDNA. The total volume was 25 μL. Amplification was performed for 32 cycles. The PCR products were separated by electrophoresis using a 1.5% (w/v) agarose gel containing 0.5 mg/L of ethidium bromide. Single band corresponding to the predicted size of the amplified product for Livin and VEGF and β-actin were identified under an ultraviolet transilluminator and transferred to a nylon filter membrane, and hybridized with an ECL-labeled probe 10 mL. The probes hybridized only to the bands which corresponded in size to the ethidium bromide stained gels, thereby confirming the amplified PCR products. The band densities were scanned with a densitometer. The relative amount of mRNA in each sample was calculated from the densitometry ratio of Livin and VEGF *A* value/β-actin *A* value.

Statistical analysis

Quantitative data were expressed as mean ± SD. All statistical analyses used the SPSS software for Windows 11.0 (SPSS, Inc., Chicago, IL, USA), using Student's *t*-test for intergroup. For statistical evaluation one-way analysis of variance (ANOVA) were employed. Pearson correlation analysis was also performed to some index. $P < 0.05$ was considered as statistically significant.

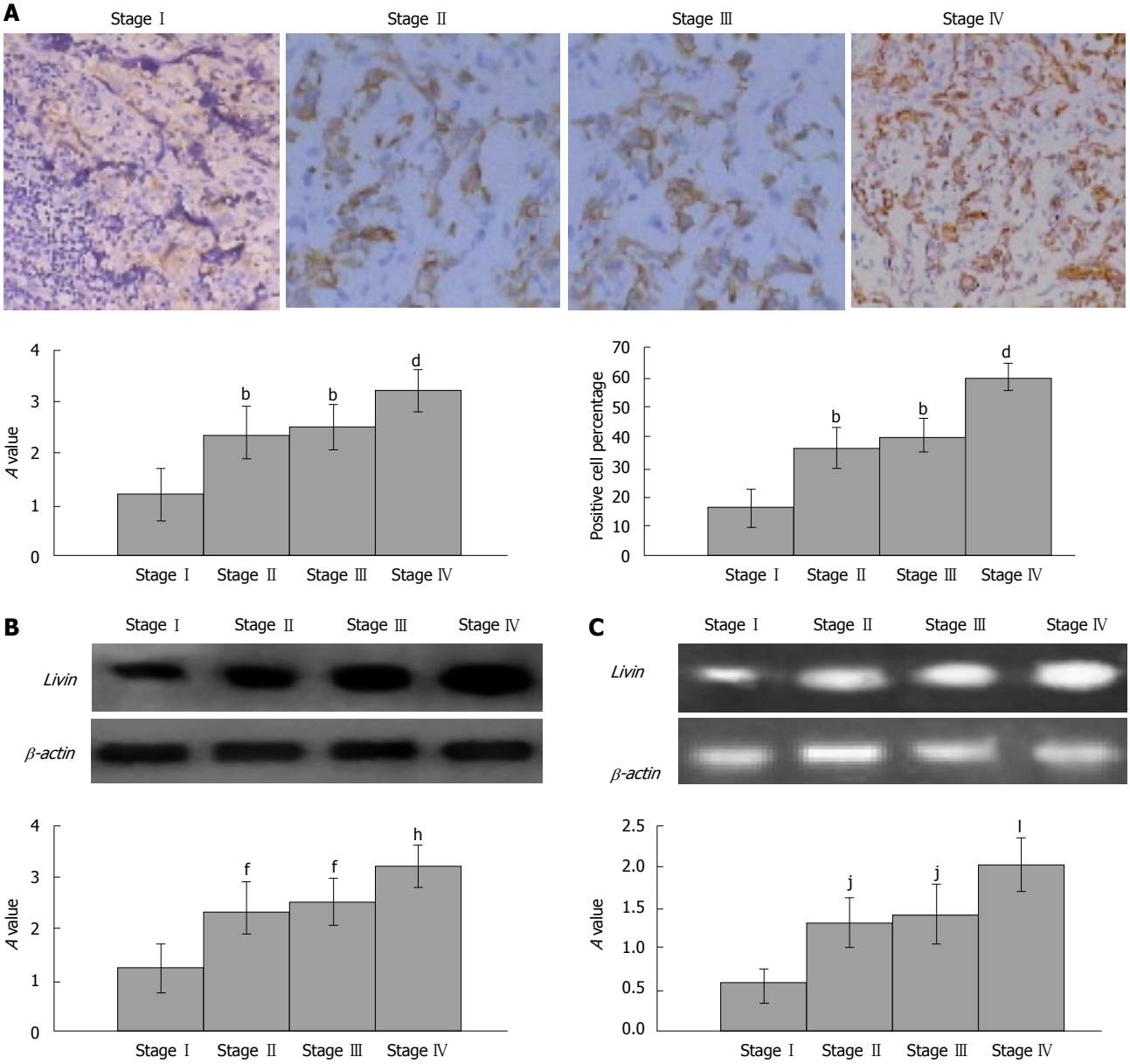


Figure 1 **A**: The expression of Livin was measured by IHC (SP × 400). Optical density value and positive cell percentage in clinicopathologic stage two, three and four was significantly higher than that of stage one ($^{b}P < 0.01$). Furthermore, optical density value and positive cell percentage in clinicopathologic stage four was significantly higher than that of stage two and three ($^{d}P < 0.01$). IHC showed that Livin had significant expression in the cytoplasm and nucleus in the Stage Ⅱ, Ⅲ and Ⅳ (the cytoplasm and nucleus had stained yellow), slight expression in the cytoplasm and nucleus in Stage Ⅰ (the cytoplasm and nucleus had slightly stained yellow); **B**: The expression of Livin by Western blotting showed that expression of Livin in clinicopathologic stage two, three and four was significantly higher than that of stage one ($^{f}P < 0.01$). Optical density value and positive cell percentage in clinicopathologic stage four was significantly higher than that of stage two and three ($^{h}P < 0.01$); **C**: mRNA level of *Livin* was tested by RT-PCR. Up-regulation of *Livin* gene transcription matched with the protein level of Livin that was significantly increased along with the progression of esophageal carcinoma. Optical density value in clinicopathologic stage two, three and four was significantly higher than that of stage one ($^{j}P < 0.01$). Optical density value and positive cell percentage in clinicopathologic stage four was significantly higher than that of stage two and three ($^{l}P < 0.01$).

RESULTS

Expression of Livin in esophageal carcinoma

The expression of Livin measured by IHC showed that expression of Livin in clinicopathologic stage two, three and four was significantly higher than that of stage one ($P < 0.01$). Optical density value and positive cell percentage in clinicopathologic stage two, three and four was significantly higher than that of stage one ($P < 0.01$). Furthermore, optical density value and positive cell percentage in clinicopathologic stage four was significantly higher than that of stage two and three ($P < 0.01$) (Figure 1A). The results by IHC showed that expression of Livin increases along with the progression of esophageal carcinoma. To further determine that Livin contributes to the pathogenesis of esophageal carcinoma, the expression of Livin was tested by Western blotting. In coincidence with IHC results, the expression of Livin by Western blotting showed that expression of Livin in clinicopathologic stage two, three and four was significantly higher than that of stage one ($P < 0.01$). Optical density value in clinicopathologic stage two, three and four was significantly higher than that of stage one ($P < 0.01$). Optical density value and

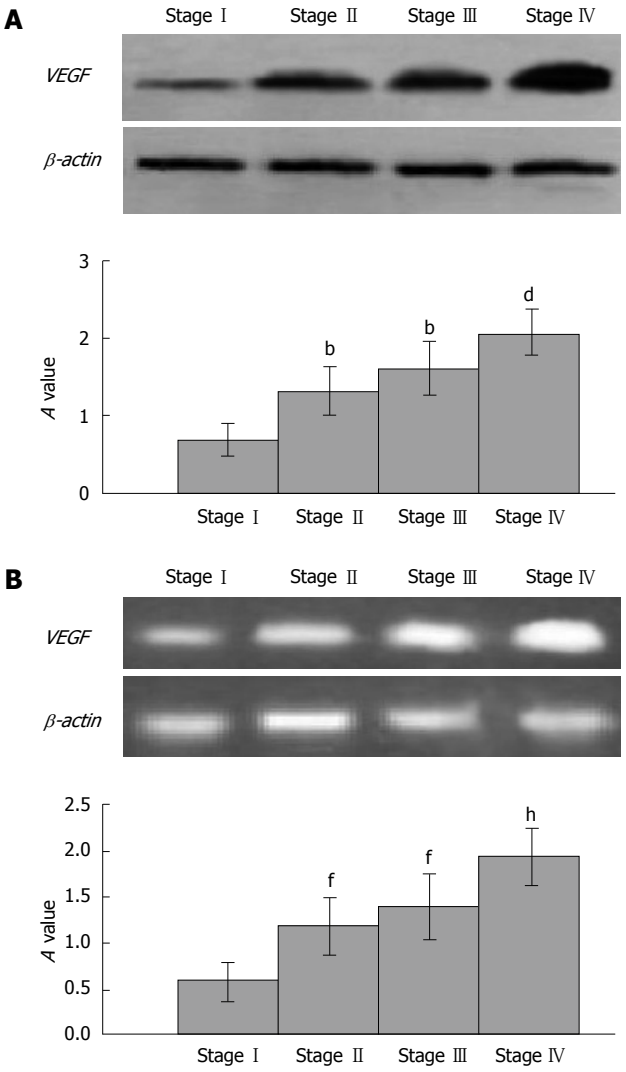


Figure 2 Assay of level of VEGF. **A**: The expression of VEGF by Western blotting showed that expression of VEGF in clinicopathologic stage two, three and four was significantly higher than that of stage one ($^bP < 0.01$). Optical density value in clinicopathologic stage two, three and four was significantly higher than that of stage one ($^bP < 0.01$). Optical density value and positive cell percentage in clinicopathologic stage four was significantly higher than that of stage two and three ($^dP < 0.01$); **B**: Up-regulation of VEGF gene transcription matched with the protein level of VEGF that was significantly increased along with the progression of esophageal carcinoma. Optical density value in clinicopathologic stage two, three and four was significantly higher than that of stage one ($^fP < 0.01$). Optical density value and positive cell percentage in clinicopathologic stage four was significantly higher than that of stage two and three ($^hP < 0.01$).

positive cell percentage in clinicopathologic stage four was significantly higher than that of stage two and three ($P < 0.01$) (Figure 1B). Previously, the protein level of Livin increases the progression of esophageal carcinoma. To further evaluate that Livin contributes to the pathogenesis of esophageal carcinoma, the mRNA level of *Livin* was tested by RT-PCR. Up-regulation of *Livin* gene transcription matched with the protein level of Livin that was significantly increased along with the progression of esophageal carcinoma. Optical density value in clinicopathologic stage two, three and four was significantly higher than that of stage one ($P < 0.01$). Optical density value and positive cell percentage in clinicopathologic stage four was significantly higher than that of stage two and three ($P < 0.01$) (Figure 1C).

Expression of VEGF in esophageal carcinoma

The expression of VEGF measured by Western blotting and RT-PCR showed expression of VEGF increases along with the progression of esophageal carcinoma. The expression of VEGF by Western blotting showed that expression of VEGF in clinicopathologic stage two, three and four was significantly higher than that of stage one ($P < 0.01$). Optical density value in clinicopathologic stage two, three and four was significantly higher than that of stage one ($P < 0.01$). Optical density value and positive cell percentage in clinicopathologic stage four was significantly higher than that of stage two and three ($P < 0.01$) (Figure 2A). Previously, the protein level of VEGF increases the progression of esophageal carcinoma. To further evaluate that VEGF contributes to the pathogenesis of esophageal carcinoma, the mRNA level of *VEGF* was tested by RT-PCR. Up-regulation of *VEGF* gene transcription matched with the protein level of VEGF that was significantly increased along with the progression of esophageal carcinoma. Optical density value in clinicopathologic stage two, three and four was significantly higher than that of stage one ($P < 0.01$). Optical density value and positive cell percentage in clinicopathologic stage four was significantly higher than that of stage two and three ($P < 0.01$) (Figure 2B). Pearson correlation analysis showed that the level of VEGF by Western blotting has a positive correlation with Livin ($r = 0.384$, $P < 0.05$), and VEGF by RT-PCR a positive correlation with Livin ($r = 0.452$, $P < 0.05$) as well. The hypothesis has been made that Livin and VEGF play such an inter-enhancement role in the progress of esophageal carcinoma.

DISCUSSION

Livin may be essential for survival of certain cancer cells[11-13]. Of the IAP family members, CARD-RING domain of cIAP1, CARD-RING domain of cIAP2, X-linked IAP, and NAIP are expressed in normal adult tissues[14,15], whereas Survivin expression is limited to tumor tissues[16-18]. It has been reported that Livin was expressed in some tumor cells and several fetal tissues but not in normal adult tissues. Hence, its expression profiles seem to be very similar to those of Survivin, a cancer-specific IAP family protein. In the present study, we investigated expression of Livin in human esophageal carcinoma and analyze its relationship with clinical stages. The results showed that Livin positivity was also significantly correlated with tumor stages, increasing with tumor progression. Expression of Livin increased with the process of esophageal carcinoma. In the fourth clinical stage, expression of Livin was the most significant. Therefore, over-expression of Livin contributes to the pathogenesis of esophageal carcinoma. Livin is known to play an important role in antiapoptotic cell survival by suppression

of caspase family proteins. The other antiapoptotic proteins, including IAP family and Bcl-2 family[19,20], were also reported to be overexpressed in esophageal carcinoma cells. Namely, Survivin, a member of the IAP family, was overexpressed in esophageal carcinoma specimens, and patients with Survivin expression had significant unfavorable prognosis. Survivin was one of the tumor-associated antigens recognized by both humoral and cellular immunity of cancer patients, and could become a target of CTL. It has been reported that Livin might be involved in the progression of superficial bladder cancer and used as a marker of early recurrence[19,21,22]. Because loss of Livin expression could lead to apoptotic cell death in cervical cancer cells, suppression of Livin should have much advantage in cancer treatment. From this perspective, Livin might be a good candidate as a molecular target for treatment as well as having a prognostic value for esophageal carcinoma.

VEGF plays a crucial role in tumour expansion by initiating permeabilization of blood vessels, by extravasation of plasma proteins, by invasion of stromal cells, and by causing the sprouting of new blood vessels that supply the tumour with oxygen and nutrients. A number of studies have shown that expression of certain VEGF transcripts are correlated with tumour progression[23,24]. Although increases of certain VEGF transcripts have been demonstrated to correlate with the progression of various tumours[25-27], the actual protein levels of the different VEGF isoforms and their significance during cellular transformation are unknown. Moreover, it has been suggested that elevated protein expression in tumour tissues was mediated by both enhanced transcription and translation. Thus, in order to understand the role of VEGF in tumour progression, it is important to investigate VEGF expression of different clinical stages at the protein and mRNA level during tumourigenesis. In the present study, we investigated expression of VEGF in human esophageal carcinoma and analyze its relationship with clinical stages. The results showed that VEGF positivity was also significantly correlated with tumor stages, increasing with tumor progression. Expression of VEGF increased with the process of esophageal carcinoma. In the fourth clinical stage, expression of VEGF was the most significant. Because previous evidence indicated that VEGF plays a crucial role in tumour expansion by initiating permeabilization of blood vessels, our results suggest that over-expression of VEGF contributes to the pathogenesis of esophageal carcinoma. Allowing that VEGF introduces the sprouting of new blood vessels that supply the tumour with oxygen and nutrients, it is a novel strategy for treating esophageal carcinoma, and the results of the ongoing clinical trials in patients with esophageal carcinoma are eagerly awaited. Although encouraging data have emerged to support the use of antiangiogenic therapy in some cancers such as myeloma and glioma, poor tumor response has been reported in others[28-31]. One major problem confronting clinical trials of antiangiogenic therapy is the lack of an established surrogate marker to measure antiangiogenic activity *in vivo* in cancer patients. Tumor response in terms of shrinkage alone might not be an appropriate index of treatment efficacy because of the cytostatic nature of the treatment. Instead, the ability of an antiangiogenic drug to induce prolonged stabilization of the disease and increase survival might be more meaningful end points for clinical trials on antiangiogenic therapy.

Taken together, over-expression of Livin and VEGF contributes to the pathogenesis of esophageal carcinoma. The level of VEGF has a positive correlation with Livin. The hypothesis has been made that Livin and VEGF played such an inter-enhancement role in the progress of esophageal carcinoma. Inhibitors of Livin and VEGF may be potential targets for the prevention or treatment of human esophageal carcinoma.

ACKNOWLEDGMENTS

Thanks are given to Professor Shan-Quan Sun, director of the anatomy institution of Chongqing Medical University, for designing and guiding this study.

COMMENTS

Background

Livin expression may be essential for survival of certain cancer cells. Vascular endothelial growth factor (VEGF) is a potent mitogen for endothelial cells, and its expression has been correlated with increased tumour angiogenesis. It is important to investigate Livin and VEGF expression of different clinical stages at the protein and mRNA level during tumourigenesis.

Research frontiers

In order to further explore the role of Livin and VEGF in the development of esophageal carcinoma, it is critical to investigate the role of Livin and VEGF in human esophageal carcinoma and analyze its relationship with clinical stages.

Innovations and breakthroughs

Over-expression of Livin and VEGF contributes to the pathogenesis of esophageal carcinoma. The level of VEGF has a positive correlation with Livin. The hypothesis has been made that Livin and VEGF have an inter-enhancement role in the progress of esophageal carcinoma.

Applications

Inhibitors of Livin and VEGF may be potential targets for the prevention or treatment of human esophageal carcinoma.

Terminology

Livin may be essential for survival of certain cancer cells. Of the IAP family members, Livin interacts with downstream caspases, such as caspase-3, caspase-7, and caspase-9, leading to their inactivation and degradation. Its overexpression can protect cells from several proapoptotic stimuli. Very importantly, treatment of cancer cells with Livin antisense oligo DNA causes apoptotic cell death, indicating that Livin expression may be essential for survival of certain cancer cells. VEGF is a potent mitogen for endothelial cells, and its expression has been correlated with increased tumour angiogenesis.

Peer review

This is an interesting report showing increased expression of Livin and VEGF in late stages (Ⅱ Ⅲ Ⅳ) of esophageal carcinoma compared to early stage (Ⅰ) of esophageal carcinoma. Since Livin expression may affect apoptosis, correlation of Livin with assays of apoptosis will be of interest. In addition, correlation of Livin expression with patient survival in various stages of esophageal carcinoma will also be interesting.

REFERENCES

1 **Li JH**, He WJ, He YJ. [Expression and clinical significance of Survivin and Livin in DukesoB colorectal cancer] *Ai Zheng* 2007; **26**: 547-551

2 **Hariu H**, Hirohashi Y, Torigoe T, Asanuma H, Hariu M, Tamura Y, Aketa K, Nabeta C, Nakanishi K, Kamiguchi K, Mano Y, Kitamura H, Kobayashi J, Tsukahara T, Shijubo N, Sato N. Aberrant expression and potency as a cancer immunotherapy target of inhibitor of apoptosis protein family, Livin/ML-IAP in lung cancer. *Clin Cancer Res* 2005; **11**: 1000-1009

3 **Kasof GM**, Gomes BC. Livin, a novel inhibitor of apoptosis protein family member. *J Biol Chem* 2001; **276**: 3238-3246

4 **Nachmias B**, Ashhab Y, Bucholtz V, Drize O, Kadouri L, Lotem M, Peretz T, Mandelboim O, Ben-Yehuda D. Caspase-mediated cleavage converts Livin from an antiapoptotic to a proapoptotic factor: implications for drug-resistant melanoma. *Cancer Res* 2003; **63**: 6340-6349

5 **Chang H**, Schimmer AD. Livin/melanoma inhibitor of apoptosis protein as a potential therapeutic target for the treatment of malignancy. *Mol Cancer* Ther 2007; **6**: 24-30

6 **Liu B**, Han M, Wen JK, Wang L. Livin/ML-IAP as a new target for cancer treatment. *Cancer Lett* 2007; **250**: 168-176

7 **Kim DK**, Alvarado CS, Abramowsky CR, Gu L, Zhou M, Soe MM, Sullivan K, George B, Schemankewitz E, Findley HW. Expression of inhibitor-of-apoptosis protein (IAP) livin by neuroblastoma cells: correlation with prognostic factors and outcome. *Pediatr Dev Pathol* 2005; **8**: 621-629

8 **Kamat AA**, Merritt WM, Coffey D, Lin YG, Patel PR, Broaddus R, Nugent E, Han LY, Landen CN Jr, Spannuth WA, Lu C, Coleman RL, Gershenson DM, Sood AK. Clinical and biological significance of vascular endothelial growth factor in endometrial cancer. *Clin Cancer Res* 2007; **13**: 7487-7495

9 **Hervé MA**, Buteau-Lozano H, Vassy R, Bieche I, Velasco G, Pla M, Perret G, Mourah S, Perrot-Applanat M. Overexpression of vascular endothelial growth factor 189 in breast cancer cells leads to delayed tumor uptake with dilated intratumoral vessels. *Am J Pathol* 2008; **172**: 167-178

10 **Zhang B**, Zhao WH, Zhou WY, Yu WS, Yu JM, Li S. Expression of vascular endothelial growth factors-C and -D correlate with evidence of lymphangiogenesis and angiogenesis in pancreatic adenocarcinoma. *Cancer Detect Prev* 2007; **31**: 436-442

11 **Yagihashi A**, Ohmura T, Asanuma K, Kobayashi D, Tsuji N, Torigoe T, Sato N, Hirata K, Watanabe N. Detection of autoantibodies to survivin and livin in sera from patients with breast cancer. *Clin Chim Acta* 2005; **362**: 125-130

12 **Wagener N**, Crnkovic-Mertens I, Vetter C, Macher-Goppinger S, Bedke J, Grone EF, Zentgraf H, Pritsch M, Hoppe-Seyler K, Buse S, Haferkamp A, Autschbach F, Hohenfellner M, Hoppe-Seyler F. Expression of inhibitor of apoptosis protein Livin in renal cell carcinoma and non-tumorous adult kidney. *Br J Cancer* 2007; **97**: 1271-1276

13 **Choi J**, Hwang YK, Sung KW, Lee SH, Yoo KH, Jung HL, Koo HH, Kim HJ, Kang HJ, Shin HY, Ahn HS. Expression of Livin, an antiapoptotic protein, is an independent favorable prognostic factor in childhood acute lymphoblastic leukemia. *Blood* 2007; **109**: 471-477

14 **Ashhab Y**, Alian A, Polliack A, Panet A, Ben Yehuda D. Two splicing variants of a new inhibitor of apoptosis gene with different biological properties and tissue distribution pattern. *FEBS Lett* 2001; **495**: 56-60

15 **Takeuchi H**, Kim J, Fujimoto A, Umetani N, Mori T, Bilchik A, Turner R, Tran A, Kuo C, Hoon DS. X-Linked inhibitor of apoptosis protein expression level in colorectal cancer is regulated by hepatocyte growth factor/C-met pathway via Akt signaling. *Clin Cancer Res* 2005; **11**: 7621-7628

16 **Kleinberg L**, Lie AK, Fløenes VA, Nesland JM, Davidson B. Expression of inhibitor-of-apoptosis protein family members in malignant mesothelioma. *Hum Pathol* 2007; **38**: 986-994

17 **Kleinberg L**, Fløenes VA, Nesland JM, Davidson B. Survivin, a member of the inhibitors of apoptosis family, is down-regulated in breast carcinoma effusions. *Am J Clin Pathol* 2007; **128**: 389-397

18 **Yin W**, Chen N, Zhang Y, Zeng H, Chen X, He Y, Wang X, Zhou Q. Survivin nuclear labeling index: a superior biomarker in superficial urothelial carcinoma of human urinary bladder. *Mod Pathol* 2006; **19**: 1487-1497

19 **Gazzaniga P**, Gradilone A, Giuliani L, Gandini O, Silvestri I, Nofroni I, Saccani G, Frati L, Aglianò AM. Expression and prognostic significance of LIVIN, SURVIVIN and other apoptosis-related genes in the progression of superficial bladder cancer. *Ann Oncol* 2003; **14**: 85-90

20 **Nedelcu T**, Kubista B, Koller A, Sulzbacher I, Mosberger I, Arrich F, Trieb K, Kotz R, Toma CD. Livin and Bcl-2 expression in high-grade osteosarcoma. *J Cancer Res Clin Oncol* 2008; **134**: 237-244

21 **Liu HB**, Kong CZ, Zeng Y, Liu XK, Bi JB, Jiang YJ, Han S. Livin may serve as a marker for prognosis of bladder cancer relapse and a target of bladder cancer treatment. *Urol Oncol* 2009; **27:** 277-283

22 **Zhu Z**, Wang Y, Ding X, Zeng F, Xu K. Expression and prognostic significance of Livin in the progression of bladder cancer. *J Huazhong Univ Sci Technolog Med Sci* 2008; **28**: 90-92

23 **Eichhorn ME**, Kleespies A, Angele MK, Jauch KW, Bruns. Angiogenesis in cancer: molecular mechanisms, clinical impact. *Langenbecks Arch Surg* 2007; **392**: 371-379

24 **Zhang J**, Jia Z, Li Q, Wang L, Rashid A, Zhu Z, Evans DB, Vauthey JN, Xie K, Yao JC. Elevated expression of vascular endothelial growth factor correlates with increased angiogenesis and decreased progression-free survival among patients with low-grade neuroendocrine tumors. *Cancer* 2007; **109**: 1478-1486

25 **Das K**, Zhao Y, Sugiono M, Lau W, Tan P, Cheng C. Differential expression of vascular endothelial growth factor165b in transitional cell carcinoma of the bladder. *Urol Oncol* 2007; **25**: 317-321

26 **Raspollini MR**, Asirelli G, Taddei GL. The role of angiogenesis and COX-2 expression in the evolution of vulvar lichen sclerosus to squamous cell carcinoma of the vulva. *Gynecol Oncol* 2007; **106**: 567-571

27 **Baritaki S**, Sifakis S, Huerta-Yepez S, Neonakis IK, Soufla G, Bonavida B, Spandidos DA. Overexpression of VEGF and TGF-beta1 mRNA in Pap smears correlates with progression of cervical intraepithelial neoplasia to cancer: implication of YY1 in cervical tumorigenesis and HPV infection. *Int J Oncol* 2007; **31**: 69-79

28 **Pouessel D**, Culine S, Verhoest G, Patard JJ. [Renal cell carcinoma and antiangiogenic therapies] *Presse Med* 2008; **37**: 628-633

29 **Pouessel D**, Culine S. [Angiogenesis targeting in renal carcinomas] *Bull Cancer* 2007; **94**: F223-F226

30 **Yilmaz A**, Ernam D, Unsal E, Demirag F, Atikcan S, Taştepe I. Vascular endothelial growth factor immunostaining correlates with postoperative relapse and survival in non-small cell lung cancer. *Arch Med Res* 2007; **38**: 764-768

31 **Giaccone G**. The potential of antiangiogenic therapy in non-small cell lung cancer. *Clin Cancer Res* 2007; **13**: 1961-1970

S- Editor Li DL **L- Editor** Alpini GD **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5755

World J Gastroenterol 2008 October 7; 14(37): 5755-5759
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Gastrointestinal polyposis with esophageal polyposis is useful for early diagnosis of Cowden's disease

Ken Umemura, Sho Takagi, Yasushi Ishigaki, Masahiro Iwabuchi, Shigeru Kuroki, Yoshitaka Kinouchi, Tooru Shimosegawa

Ken Umemura, Sho Takagi, Yoshitaka Kinouchi, Tooru Shimosegawa, Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan
Yasushi Ishigaki, Division of Molecular Metabolism and Diabetes, Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan
Masahiro Iwabuchi, Department of Gastroenterology, Sendai Medical Center, Sendai 980-8574, Japan
Shigeru Kuroki, Department of Dermatology, Kurihara Central Hospital, Kurihara 228-0014, Japan
Author contributions: Umemura K, Takagi S, Ishigaki Y, Iwabuchi M and Kuroki S contributed equally to this work; Umemura K, Takagi S and Iwabuchi M designed the research; Umemura K, Ishigaki Y and Kuroki S performed the research; Ishigaki Y analyzed the data; Umemura K, Takagi S, Kinouchi Y and Shimosegawa T wrote the paper.
Correspondence to: Ken Umemura, Division of Gastroenterology, Tohoku University Graduate School of Medicine, 1-1 Seiryo, Aoba, Sendai 980-8574, Japan. umekun@nyc.odn.ne.jp
Telephone: +81-22-7177171 Fax: +81-22-7177177
Received: May 27, 2008 Revised: August 18, 2008
Accepted: August 25, 2008
Published online: October 7, 2008

Abstract

Cowden's disease, one of the several hamartoma syndromes, is characterized by hyperplastic lesions and hamartomas distributed in the whole body. About thirty percent of patients with Cowden's disease have been reported to be complicated by malignant tumors. Based on the criteria of the International Cowden Consortium, this disease is mainly diagnosed as trichilemmoma of the face and oral mucosal papillomatosis. However, Cowden's disease patients themselves often do not recognize trichilemmoma of the face and oral mucosal papillomatosis. We report a case of Cowden's disease in a 33-year-old female patient who was diagnosed based on the characteristic findings at gastrointestinal endoscopy. Clinically, the patient was aware of having bloody stools. Multiple polyps found endoscopically in the esophagus, stomach, ileum, colon and rectum showed histopathologically hamartomatous changes and epithelial hyperplasia. Physical examination revealed oral papillomatosis and facial trichilemmomas. A germline mutation in exon 8 of the phosphatase and tensin homolog deleted on chromosome ten (*PTEN*) gene was found in this case. It was a point mutation of C to T at codon 1003 (CGA→TGA, arginine→stop codon). The characteristic findings on gastrointestinal endoscopy led us to a diagnosis of Cowden's disease. It has been reported that gastrointestinal polyposis with esophageal polyposis is found in about 85.7% of Japanese patients with Cowden's disease. The characteristic findings on gastrointestinal endoscopy can be a useful diagnostic clue to Cowden's disease.

Key words: Cowden's disease; Gastrointestinal polyposis; *PTEN*; Early diagnosis; Hamartoma

Peer reviewer: Damian Casadesus Rodriguez, MD, PhD, Calixto Garcia University Hospital, J and University, Vedado, Havana City, Cuba

Umemura K, Takagi S, Ishigaki Y, Iwabuchi M, Kuroki S, Kinouchi Y, Shimosegawa T. Gastrointestinal polyposis with esophageal polyposis is useful for early diagnosis of Cowden's disease. *World J Gastroenterol* 2008; 14(37): 5755-5759 Available from: URL: http://www.wjgnet.com/1007-9327/14/5755.asp DOI: http://dx.doi.org/10.3748/wjg.14.5755

INTRODUCTION

Cowden's disease was reported for the first time by Lloyd and Dennis in 1963[1]. There have been more than 200 case reports in Japan. Cowden's disease, one of the several hamartoma syndromes, is characterized by hyperplastic lesions and hamartomas distributed on the whole body[2]. About 30% of patients with Cowden's disease have been reported to be complicated by malignant tumors[3]. It was reported that this disease is mainly diagnosed mainly as facial papules and oral mucosal papillomatosis[4] (Table 1). Recently, the criteria of the International Cowden Consortium are commonly used for the diagnosis of Cowden's disease[5] (Table 2). Ninety-nine percent of individuals with Cowden's disease are believed to have developed mucocutaneous lesions at the age of about 30 years[5]. However, Cowden's disease is rarely diagnosed based on the physical findings of typical skin lesions, and the diagnosis of typical

trichilemmoma requires many biopsy specimens[6,7]. Cowden's disease patients themselves often do not recognize the characteristic dermatological findings of Cowden's disease. Furthermore, it is possible that they are not checked for the specific findings of Cowden's disease even when they do notice the lesions[8]. Gastrointestinal polyposis has been reported in about 40% of patients with Cowden's disease in Western countries[9], but it has been reported that this disease is frequently accompanied with gastrointestinal polyposis in Japan[10]. We, here, report a case of Cowden's disease diagnosed based on the characteristic findings at gastrointestinal endoscopy.

CASE REPORT

A 33-year-old female patient had a medical examination at the Department of Internal Medicine, Kurihara Central Hospital, because of bloody stools. The patient underwent total thyroidectomy at the age of 20 years. Her mother died of gastric and breast cancer after she underwent the same operation. The patient was obese with no abnormality in the laboratory data. Colonoscopy showed multiple polyps in the terminal ileum, colon and rectum, which gave the macroscopic appearance of smooth lesions. Moreover, all polyps were within 5 mm in size. All biopsy specimens of these lesions showed epithelial hyperplasia and were diagnosed histopathologically as hyperplastic polyps (Figure 1). Double contrast X-ray study showed multiple small polypoid lesions in the ileum, colon and rectum (Figure 2). Endoscopy of the upper digestive tract showed the presence of whitish polypoid lesions in the esophagus as well as in the stomach and duodenum. Specimens of these polyps were resected by polypectomy. These polyps also had the macroscopic appearance of smooth lesions. Moreover, the size of all these polyps was within 10 mm. The gastric polyps showed hamartomatous changes and epithelial hyperplasia. A specimen was diagnosed as hamartoma by histopathological examination (Figure 3). The esophageal polyps were diagnosed histopathologically as glycogenic acanthosis (Figure 4). Otherwise, no abnormal findings were found both at computed tomography (CT) scan of the abdomen or breast and at abdominal ultrasonography. Papillomatosis of the gingiva was found (Figure 5) in addition to a small papule on the face. It was diagnosed histopathologically as a trichilemmoma by skin biopsy (Figure 6). Genetic analyses performed with informed consent clarified a germline mutation of the phosphatase and tensin homolog was deleted on chromosome ten (*PTEN*) gene (Figure 7). A germline mutation in exon 8 of the *PTEN* gene was found. It was a point mutation of C to T at codon 1003 (CGA→TGA, arginine→stop codon). We diagnosed this patient as Cowden's disease based on the characteristic physical findings and the result of the genetic test described above. This study was approved by the Institutional Ethics Committee of Kurihara Central Hospital.

Table 1 Proposed diagnostic criteria for Cowden's disease

Proposed diagnostic criteria for Cowden's disease
Major clinical criteria
Cutaneous facial papules
Oral mucosal papillomatosis
Minor clinical criteria
Acral keratosis
Palmoplantar keratoses
Family history of Cowden's disease
Definite: 1a+1b,(1a or 1b)+(2a or 2b)
(1a or 1b)+3, 2a+2b+3
Probable: 1a or 1b, (2a or 2b)+3
Possible: 2a and/or 2b

Table 2 Criteria of international Cowden Consortium (Verion 2000) for Cowden's disease

Pathognomonic criteria
Mucocutaneous lesions
Trichilemmomas, facial
Acral keratoses
Papillomatous papules
Mucosal lesions
Major criteria
Breast carcinoma
Thyroid carcinoma (non-medullary), especially follicular thyroid carcinoma
Macrocephaly (megalencephaly) (say, 95th centile)
Lhermitte-Duclos disease (LDD)
Endometrial carcinoma
Minor criteria
Other thyroid lesions (eg, adenoma or multinodular goitre)
Mental retardation (say, IQ 75)
GI hamartomas
Fibrocystic disease of the breast
Lipomas
Fibromas
GU tumours (e.g. renal cell carcinoma, uterine fibroids) or malformation
Operational diagnosis in a person
Mucocutaneous lesions alone if:
There are 6 or more facial papules, of which 3 or more must be trichilemmoma, or
Cutaneous facial papules and oral mucosal papillomatosis, or
Oral mucosal papillomatosis and acral keratoses, or
Palmoplantar keratoses, 6 or more
2 major criteria but one must include macrocephaly or LDD
1 major and 3 minor criteria
4 minor criteria
Operational diagnosis in a family where one person is diagnostic for Cowden syndrome
The pathognomonic criterion/ia
Any one major criterion with or without minor criteria
Two minor criteria

DISCUSSION

Cowden's disease, reported for the first time by Lloyd and Dennis in 1963[1], belongs to a multiple hamartoma syndrome[2], and there have been more than 200 case reports in Japan. The diagnosis of Cowden's disease was originally made b based on the examination of skin and a family history of Cowden's disease[4] (Table 1).

However, the original diagnostic criteria for Cowden's disease were based on dermatological findings. The criteria of the International Cowden Consortium are commonly used for the diagnosis[5] (Table 2). The patient

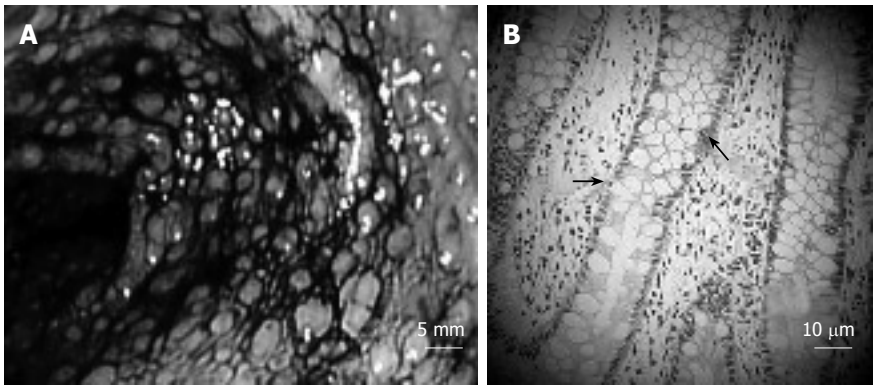


Figure 1 Polyposis in the rectum. **A**: Colonoscopy showing multiple polyps in the terminal ileum, colon and rectum, which gave the macroscopic appearance of smooth lesions. Moreover, the size of all polyps was within 5 mm or smaller; **B**: Pathological appearance of multiple polyps with all biopsy specimens of these lesions showing epithelial hyperplasia. These specimens were diagnosed histopathologically as hyperplastic polyps (arrow).

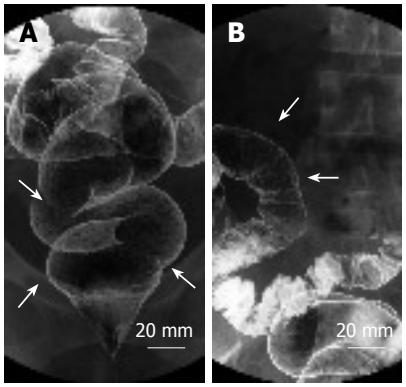


Figure 2 Double contrast X-ray study (**A**) and double contrast X-ray study (**B**) showing multiple small polypoid lesions in the ileum, colon and rectum.

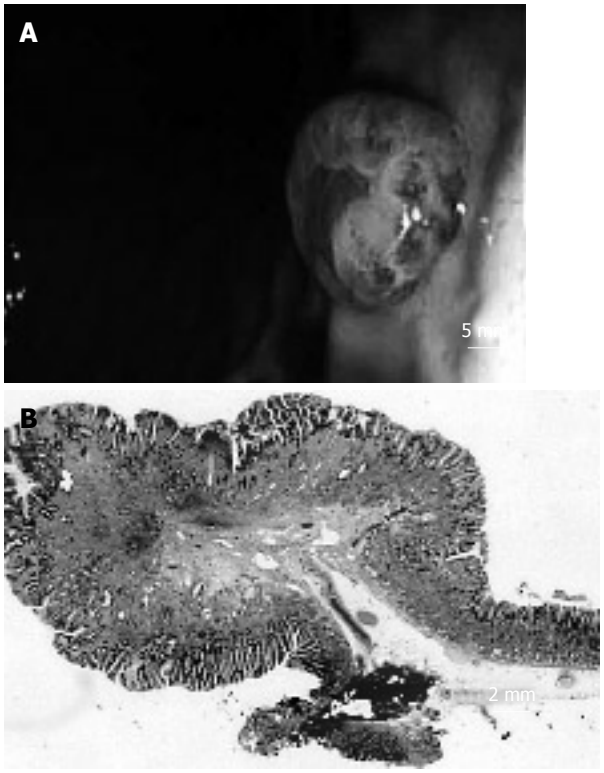


Figure 3 Polypoid lesions in the stomach. **A**: Endoscopy of the upper digestive tract showing whitish polypoid lesions in the stomach. The size of all these polyps was within 10 mm; **B**: Gastric polyps showing histopathologically hamartomatous changes and epithelial hyperplasia. A specimen was diagnosed as a hamartoma by histopathological examination.

was diagnosed as trichilemmoma and oral papillomatosis, thyroid tumor and gastrointestinal hamartoma. This case fulfilled both diagnostic criteria for this disease. Moreover, the patient's mother also had a history of total thyroidectomy and died of gastric and breast cancer. We think that her mother suffered from Cowden's disease, based on the criteria of the International Cowden Consortium.

The criteria of the International Cowden Consortium are useful for diagnosis of Cowden's disease, but such criteria may not be useful for its early diagnosis. Although we diagnosed a case of Cowden's disease based on the criteria of the International Cowden Consortium, we did not recognize the facial papules and oral mucosal papillomatosis at the first examination. The patient also did not recognize them. At first, we considered the possibility that this case was Cowden's disease because of her history of thyroid goiter and the finding of gastrointestinal polyposis including esophagus at gastrointestinal endoscopy. Later, detailed physical examination revealed oral papillomatosis and a small papule of the face. We made a histopathological diagnosis of trichilemmoma. Ninety-nine percent of individuals with Cowden's disease are believed to have mucocutaneous lesions at the age of about 30 years[9]. However, it has been reported that it is difficult to diagnose Cowden's disease only based on the physical finding of a typical trichilemmoma and in fact that it requires many biopsy specimens[6,7]. Cowden's disease patients themselves often do not recognize the characteristic dermatological findings of Cowden's disease. Furthermore, it is possible that they are not checked for the specific findings of Cowden's disease even when they do notice them[8,11].

Gastrointestinal endoscopy is more frequently performed in Japan. Recently, we have often diagnosed Cowden's disease based on the characteristic findings at gastrointestinal endoscopy[12]. It was reported that gastrointestinal polyposis occurs in about 40% of Cowden's disease patients in Western countries[9], but in Japan it occurs in about 95% of Cowden's disease patients[10]. Gastrointestinal endoscopy and double contrast X-ray study endoscopy and double contrast X-ray study can detect gastrointestinal polyposis showing histopathologically hamartomatous changes and

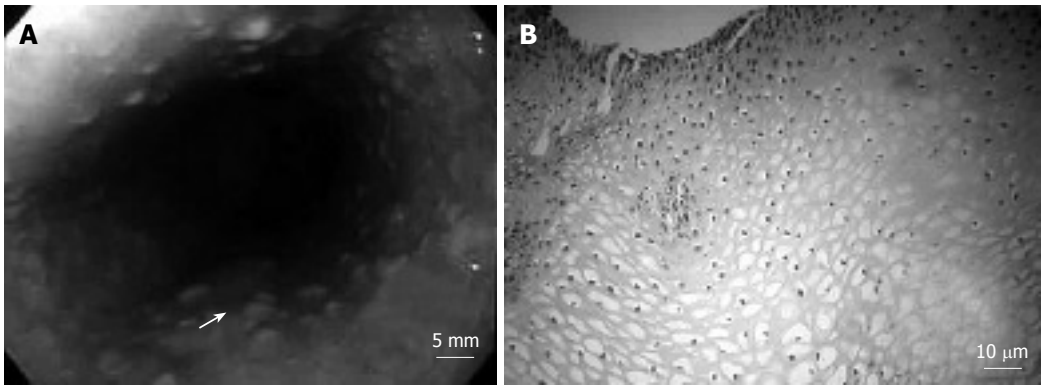


Figure 4 Polypoid lesions in the esophagus. **A**: Endoscopy of the upper digestive tract showing whitish polypoid lesions in the esophagus and macroscopic appearance of smooth lesions. The size of all these polyps was within 5 mm; **B**: Pathological appearance of the esophagus. Histlogically, a specimen confirmed the diagnosis of glycogenic acanthosis.

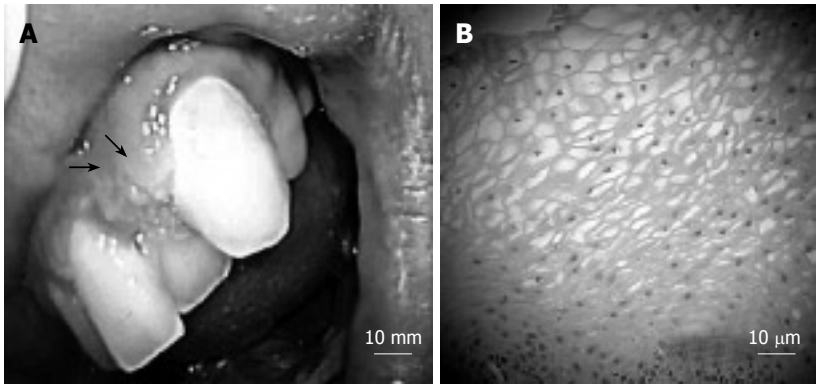


Figure 5 Many polyps in the gingiva (**A**) and pathological appearance of the gingiva confirming the diagnosis of papilloma(**B**).

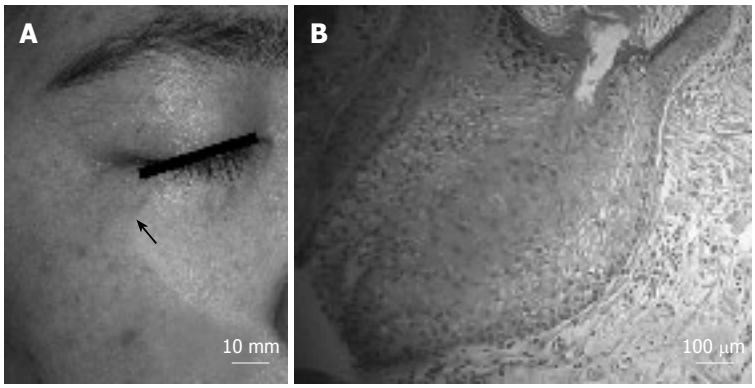


Figure 6 Small papules found on face with their size within 5 mm (**A**) and pathological appearance of small papules confirming the diagnosis of trichilemmoma (**B**).

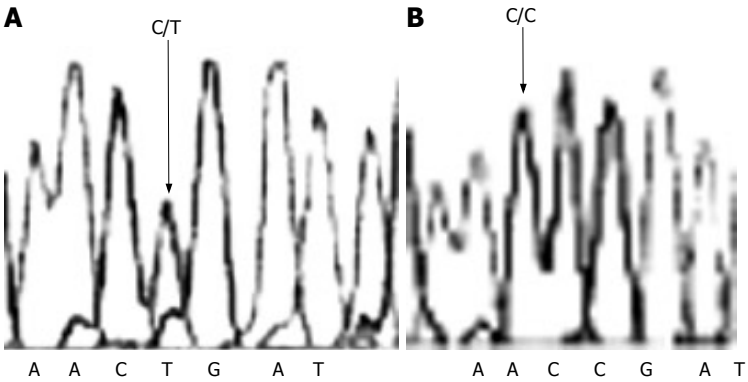


Figure 7 A germline mutation of phosphatase and tensin homolog deleted on chromosome ten (*PTEN*) gene. **A**: Genetic analyses performed with informed consent clarified a germline mutation of the *PTEN* gene in exon 8 of the *PTEN* gene, which was a point mutation of C to T at codon 1003 (CGA→TGA, arginine→stop codon); **B**: Control.

epithelial hyperplasia. Esophageal polyposis found in 85.7% of Cowden's disease patients[10], is a characteristic finding in young patients with Cowden's disease[13,14]. Esophageal polyposis shows histopathologically glycogenic acanthosis[14]. In addition to Cowden's disease, other types of gastrointestinal polyposis

include familial adenomatous polyposis, Peutz-Jeghers syndrome and Juvenile polyposis, which, however, do not show esophageal polyposis[13,14]. Multiple polyps found endoscopically in the esophagus, stomach, ileum, colon and rectum of the present case, showed histopathologically hamartomatous changes and epithelial hyperplasia, suggesting that this case fulfills the diagnostic criteria for Cowden's disease. Gastrointestinal and esophageal polyposis is not described in the criteria of the International Cowden Consortium. However, the characteristic gastrointestinal findings are useful for early diagnosis of Cowden's disease.

In the present case, a germline mutation found in exon 8 of the *PTEN* gene was a point mutation of C to T at codon 1003 (CGA→TGA, arginine→stop codon). Germline *PTEN* mutations were first described in Cowden's disease. The *PTEN* gene encodes a lipid phosphatase on 10q23 that mediates cell cycle arrest and apoptosis. Germline *PTEN* mutations are not described in the criteria of the International Cowden Consortium, but are found in 80% of Cowden's disease patients[12,15-17], indicating that Germline *PTEN* mutations may be useful for the surveillance of Cowden's disease.

In the present case, we could not find any malignant diseases. However, because about 30% of Cowden's disease patients have been reported to be complicated by malignant tumors, early diagnosis of Cowden's disease is necessary[3,9,18]. Recently, 18-fluoro-deoxyglucose positron emission tomography has become useful for cancer surveillance in Cowden's disease patients. We expect that sensitive molecular diagnostic tests for mutations in appropriate genes will become clinically available in the setting of cancer genetics consultation[19].

In summary, we report a case of Cowden's disease diagnosed based on the characteristic findings at gastrointestinal endoscopy. Gastrointestinal and esophageal polyposis is useful for the early diagnosis of Cowden's disease, and the characteristic findings at gastrointestinal endoscopy can be considered useful diagnostic clues to Cowden's disease.

REFERENCES

1 **Lloyd KM 2nd**, Dennis M. Cowden's disease. A possible new symptom complex with multiple system involvement. *Ann Intern Med* 1963; **58**: 136-142

2 **Weary PE**, Gorlin RJ, Gentry WC Jr, Comer JE, Greer KE. Multiple hamartoma syndrome (Cowden's disease). *Arch Dermatol* 1972; **106**: 682-690

3 **Ushio K**, Ishikawa T, Hukutomi T. Cowden's disease (multiple hamartoma syndorome)-Recent knowledge and problems. *Clinical oncology* 1998; **44** : 1024-1032

4 **Salem OS**, Steck WD. Cowden's disease (multiple hamartoma and neoplasia syndrome). A case report and review of the English literature. *J Am Acad Dermatol* 1983; **8**: 686-696

5 **Eng C**. Will the real Cowden syndrome please stand up: revised diagnostic criteria. *J Med Genet* 2000; **37**: 828-830

6 **Brownstein MH**, Wolf M, Bikowski JB. Cowden's disease: a cutaneous marker of breast cancer. *Cancer* 1978; **41**: 2393-2398

7 **Kobayashi T**, Tukuda H, Inoue T. A case of Cowden disease diagnosed with the assistance of characteristic findings on gastrointestinal endoscopy. *Gastroenterological Endoscopy* 1999; **41** : 1438-1444

8 **Takahashi M**, Umeki K, Harada K. A case of Cowden's disease. *Clinical Dermatology* 1995; **49**: 736-738

9 **Starink TM**, van der Veen JP, Arwert F, de Waal LP, de Lange GG, Gille JJ, Eriksson AW. The Cowden syndrome: a clinical and genetic study in 21 patients. *Clin Genet* 1986; **29**: 222-233

10 **Sumioka M**, Watanabe C, Yamada H, Fujii Y, Koike N, Hata J, Hiraoka T, Hirata K, Imagawa M, Ishida M. [Two cases of Cowden's disease] *Nippon Shokakibyo Gakkai Zasshi* 1994; **91**: 2219-2224

11 **Kimura K**, Katou N, Aoyagi T. Cowden's disease determined germline mutation of PTEN gene. *Jpn J Clin Dermatol* 2000; **54** : 585-589

12 **Harada N**, Sugimura T, Yoshimura R, Motomura S, Shirahama S, Naramoto J, Chijiiwa Y, Nakamura K, Ito K, Nawata H. Novel germline mutation of the PTEN gene in a Japanese family with Cowden disease. *J Gastroenterol* 2003; **38**: 87-91

13 **Kobayashi M**, Kurachi K, Inoue T. A case of Cowden's disease to show gastrointestinal polyposis and to merge a colon cancer. *Dermatology* 1996; **38**: 243-248

14 **Ushio K**, Ino A, Iwasa I. A clinical characteristic of Cowden' s disease (multiple i.hamartoma syndrome). *Medical Science Digest* 2002; **28**: 366-369

15 **Waite KA**, Eng C. Protean PTEN: form and function. *Am J Hum Genet* 2002; **70**: 829-844

16 **Eng C**. PTEN: one gene, many syndromes. *Hum Mutat* 2003; **22**: 183-198

17 **Negoro K**, Takahashi S, Kinouchi Y, Takagi S, Hiwatashi N, Ichinohasama R, Shimosegawa T, Toyota T. Analysis of the PTEN gene mutation in polyposis syndromes and sporadic gastrointestinal tumors in Japanese patients. *Dis Colon Rectum* 2000; **43**: S29-S33

18 **Hizawa K**, Iida M, Yao T. A Clinicopathological Comparative Study of Clinical Features in Cowden's Disease and Tuberous Sclerosis Complex. *Stom Intest* 1993; **28** : 1279-1293

19 **McGarrity TJ**, Wagner Baker MJ, Ruggiero FM, Thiboutot DM, Hampel H, Zhou XP, Eng C. GI polyposis and glycogenic acanthosis of the esophagus associated with PTEN mutation positive Cowden syndrome in the absence of cutaneous manifestations. *Am J Gastroenterol* 2003; **98**: 1429-1434

S- Editor Zhong XY **L- Editor** Wang XL **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5760

World J Gastroenterol 2008 October 7; 14(37): 5760-5762
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Colonic lymphangiomatosis associated with anemia

Woo Chul Chung, Hye-Kang Kim, Jin Young Yoo, Jeong Rok Lee, Kang-Moon Lee, Chang Nyol Paik, U-Im Jang, Jin Mo Yang

Woo Chul Chung, Hye-Kang Kim, Jeong Rok Lee, Kang-Moon Lee, Chang Nyol Paik, U-Im Jang, Jin Mo Yang, Department of Internal Medicine, College of Medicine, the Catholic University of Korea, Seoul 137-701, South Korea
Jin Young Yoo, Department of Pathology, College of Medicine, the Catholic University of Korea, Seoul 137-701, South Korea
Author contributions: Paik CN and Chung WC collected the data and designed the research; Paik CN, Kim HK, Chung WC, Lee KM, You JY and Yang JM contributed to the conception and carried out the literature research; Paik CN, Lee JR, Jang UM, and Lee KM prepared the manuscript; Paik CN and Chung WC wrote the paper.
Correspondence to: Chang Nyol Paik, MD, Department of Internal Medicine, College of Medicine, the Catholic University of Korea, St. Vincent's Hospital Ji-dong, Suwon Si, Paldal-gu, Gyeonggi-Do 442-723, South Korea. cmcu@catholic.ac.kr
Telephone: +82-31-2497138 Fax: +82-31-2538898
Received: July 25, 2008 Revised: August 27, 2008
Accepted: September 4, 2008
Published online: October 7, 2008

Abstract

Lymphangioma is an uncommon malformation of lymphatic system. Multiple colonic lymphangioma named as lymphangiomatosis is considered an extremely rare disease. Although lymphangioma is a benign tumor and most colonic lymphangiomas do not cause symptoms and do not require treatment, resection of lymphangioma is necessary in the presence of symptoms such as abdominal pain, bleeding, intussusceptions. We report a case of colonic lymphangiomatosis in a man who presented with abdominal discomfort and anemia, which was diagnosed and treated with endoscopic snare polypectomy.

Key words: Colonic lymphangiomatosis; Anemia; Snare polypectomy

Peer reviewer: Zvi Fireman, Professor, Department of Gastroenterology, Hillel-yaffe Med. Ctr., POB 169, Gastroenterology Department, Hadera 38100, Israel

Chung WC, Kim HK, Yoo JY, Lee JR, Lee KM, Paik CN, Jang UI, Yang JM. Colonic lymphangiomatosis associated with anemia. *World J Gastroenterol* 2008; 14(37): 5760-5762 Available from: URL: http://www.wjgnet.com/1007-9327/14/5760.asp DOI: http://dx.doi.org/10.3748/wjg.14.5760

INTRODUCTION

Lymphangioma is a lymphatic malformation which shows benign proliferation of lymph vessels, with the characteristics of submucosal tumors covered with normal mucosa[1-5]. It may occur anywhere but rarely in the colon. However, it has been reported more frequently with the increasing prevalence of endoscopy. Multiple colonic lymphangiomas reported as "colonic lymphangiomatosis", are even rarer. We report a case of colonic lymphangiomatosis suspicious of submucosal tumor in a man who presented with anemia and abdominal discomfort, which was treated with endoscopic snare polypectomy.

CASE REPORT

A 48-year-old man with chief complaints of abdominal discomfort and anemia was admitted to our hospital. He had no significant past medical history. Physical examination revealed no remarkable abnormality in the abdomen. Laboratory rests showed hypochromic microcytic anemia, 6.3 g/dL hemoglobin, 24.2% hematocrit, 66.5 fL mean corpuscular volume (normal = 85-99), 17.3 pg mean corpuscular hemoglobin (normal= 26-34), 26.0% mean corpuscular hemoglobin concentration (normal = 32%-36%), 12 ug/dL iron (normal = 65-157), and 3.59 ng/mL ferritin (normal = 16.4-323). Tumor marker levels (CEA, CA19-9), urinalysis and blood chemistry tests were within normal ranges.

Colonoscopy revealed several protruding mucosal lesions covered with normal mucosa raging 10-20 mm in diameter at the proximal transverse colon. These lesions were semi-transparent, and the cushion sign was positive. Some indigested food materials were got in between most protruding lesion and colonic wall (Figure 1A). The most prominent lesion underwent snare polypectomy (Figure 1B), and inner surface of the lesion was filled with seroanguious fluid and yellowish fibroadipose tissue-like materials (Figure 1C). Abdomen computer tomography (CT) was unremarkable. Histopathologic examination showed cystic lumen covered with a single layer of flat endothelial cells (Figure 2). Based on the clinical and histopathologic findings, a diagnosis of colonic lymphangiomatosis was made. Considering that the patient did not show any bleeding signs and symptoms after the mucosectomy, we decided to follow up on an out-patient

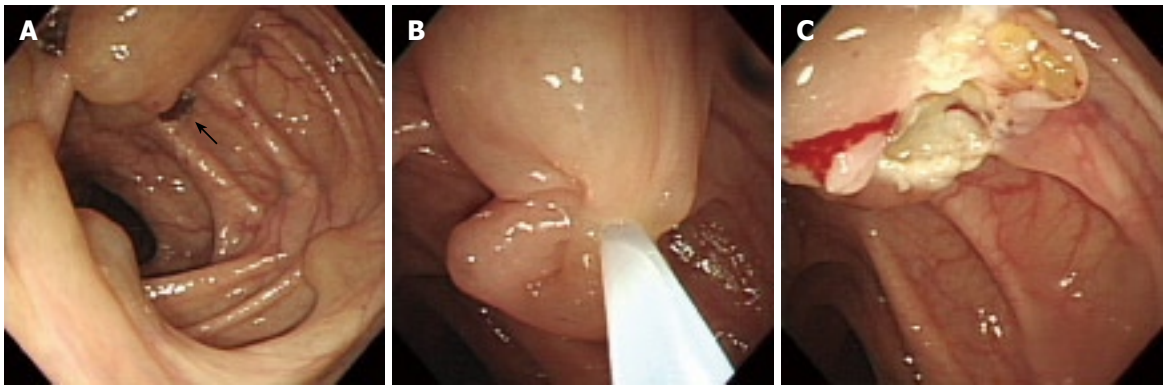


Figure 1 Colonoscopy revealing a semiparent, protruding mucosal lesion covered with normal mucosa with indigested food materials between the lesion and colonic wall (**A**), which underwent snare polypectomy (**B**), and inner surface of the lesion filled with seroanguious fluid and yellowish fibroadipose tissue-like materials (**C**) after the procedure.

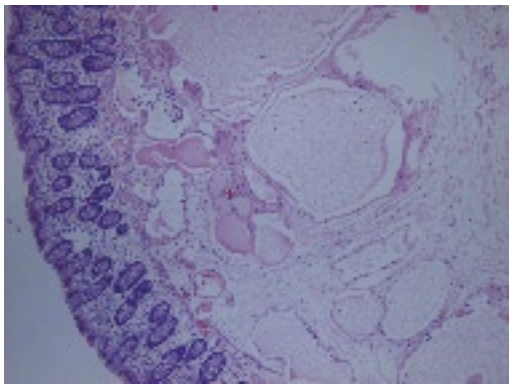


Figure 2 Histopathology showing a cystic lumen covered with a single layer of flat endothelial cells (HE, × 100).

basis. The patient had abdominal pain and anemia when he was followed up 3 mo after mucosectomy.

DISCUSSION

Lymphangioma, a benign tumor of lymphatics, rarely occurs in the abdomin, and most of them arise in the mesentery, omentum, mesocolon and retroperitoneum[6-9]. The incidence of lymphangioma in the intestinal wall is very low, and few cases of multiple colonic lymphangioma have been reported[1-4]. Barium enema, colonoscopy, abdomen CT, endoscopic ultrasonography are useful in its diagnosis[10-12]. Colonoscopic findings of lymphagioma are characterized by a steep rising margin and a somewhat narrow base, the presence of properties of submucosal tumors covered with normal colon mucosa, as well as a smoother, glassier, and more translucent surface than that of adenoma, and soft lesions with their shape changed on postural alterations or compression[1]. Our case also had such characteristics. Imaging studies may be needed to evaluate the wall structure and coexisting extracolonic lesions. Our case did not have any extracolonic lesions.

Because lymphangioma is a benign tumor and most colonic lymphangiomas are asymptomatic, and do not need treatment[2]. However, symptoms such as abdominal pain, bleeding, intussusception or protein losing enteropathy can occur[1,2,13-16]. The cardinal sign of our case was anemia, which may be due to the seroanguinous cystic contents of lymphangiomatosis from the internal bleeding. Endoscopic resection has been recently performed for the diagnosis and treatment of colonic lymphangiomas[1]. Endoscopic mucosectomy has been recommended for pedunculated tumors less than 2 cm in diameter[1,5]. We report a colonic lymphangiomatosis associated with anemia and abdominal discomfort, which was treated with endoscopic mucosal resection.

REFERENCES

1 **Matsuda T**, Matsutani T, Tsuchiya Y, Okihama Y, Egami K, Yoshioka M, Maeda S, Onda M. A clinical evaluation of lymphangioma of the large intestine: a case presentation of lymphangioma of the descending colon and a review of 279 Japanese cases. *J Nippon Med Sch* 2001; **68**: 262-265
2 **Kim J**, Han D, Hong CH, Lee HL, Kim JP, Sohn JH, Hahm JS. Colonic lymphangiomatosis associated with protein-losing enteropathy. *Dig Dis Sci* 2005; **50**: 1747-1753
3 **Watanabe T**, Kato K, Sugitani M, Hasunuma O, Sawada T, Hoshino N, Kaneda N, Kawamura F, Arakawa Y, Hirota T. A case of multiple lymphangiomas of the colon suggesting colonic lymphangiomatosis. *Gastrointest Endosc* 2000; **52**: 781-784
4 **Wildhaber BE**, Chardot C, Coultre CL, Genin B. Total Laparoscopic Excision of Retroperitoneal Cystic Lymphangioma. *J Laparoendosc Adv Surg Tech A* 2006; **16**: 530-533
5 **Sato K**, Maekawa T, Yabuki K, Tomita N, Eguchi M, Matsumoto M, Sugiyama N. Cystic lymphangiomas of the colon. *J Gastroenterol* 1999; **34**: 520-524
6 **Fujishiro M**, Kamoshida T, Hotra S, Hirai S, Oka Y, Sato M, Okumura M, Inadome Y, Takahashi A. Retroperitoneal lymphangioma with a duodenal lesion in an adult. *J Gastroenterol* 2002; **37**: 381-386
7 **Rieker RJ**, Quentmeier A, Weiss C, Kretzschmar U, Amann K, Mechtersheimer G, Blaker H, Herwart OF. Cystic lymphangioma of the small-bowel mesentery: case report and a review of the literature. *Pathol Oncol Res* 2000; **6**: 146-148
8 **Takiff H**, Calabria R, Yin L, Stabile BE. Mesenteric cysts and intra-abdominal cystic lymphangiomas. *Arch Surg* 1985; **120**: 1266-1269
9 **Tsukada H**, Takaori K, Ishiguro S, Tsuda T, Ota S, Yamamoto T. Giant cystic lymphangioma of the small bowel mesentery: report of a case. *Surg Today* 2002; **32**: 734-737
10 **Kawamoto K**, Ueyama T, Iwashita I, Utsunomiya T, Honda H, Onitsuka H, Haraguchi Y, Kojima N, Takano H, Masuda

K. Colonic submucosal tumors: comparison of endoscopic US and target air-enema CT with barium enema study and colonoscopy. *Radiology* 1994; **192**: 697-702

11 **Fujimura Y**, Nishishita C, Iida M, Kajihara Y. Lymphangioma of the colon diagnosed with an endoscopic ultrasound probe and dynamic CT. *Gastrointest Endosc* 1995; **41**: 252-254

12 **Kochman ML**, Wiersema MJ, Hawes RH, Canal D, Wiersema L. Preoperative diagnosis of cystic lymphangioma of the colon by endoscopic ultrasound. *Gastrointest Endosc* 1997; **45**: 204-206

13 **Zilko PJ**, Laurence BH, Sheiner H, Pollard J. Cystic lymphangiomyoma of the colon causing protein-losing enteropathy. *Am J Dig Dis* 1975; **20**: 1076-1080

14 **Iwabuchi A**, Otaka M, Okuyama A, Jin M, Otani S, Itoh S, Sasahara H, Odashima M, Kotanagi H, Satoh M, Masuda H, Masamune O. Disseminated intra-abdominal cystic lymphangiomatosis with severe intestinal bleeding. A case report. *J Clin Gastroenterol* 1997; **25**: 383-386

15 **Kok KY**, Mathew VV, Yapp SK. Lymphangioma of the small-bowel mesentery: unusual cause of intestinal obstruction. *J Clin Gastroenterol* 1997; **24**: 186-187

16 **Matsuba Y**, Mizuiri H, Murata T, Niimi K. Adult intussusception due to lymphangioma of the colon. *J Gastroenterol* 2003; **38**: 181-185

S- Editor Li DL **L- Editor** Wang XL **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5763

World J Gastroenterol 2008 October 7; 14(37): 5763-5768
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Primary signet ring cell carcinoma of the appendix: A rare case report and our 18-year experience

Yoon Ho Ko, Chan-Kwon Jung, Soon Nam Oh, Tae Hee Kim, Hye Sung Won, Jin Hyoung Kang, Hyung Jin Kim, Won Kyung Kang, Seong Taek Oh, Young Seon Hong

Yoon Ho Ko, Tae Hee Kim, Hye Sung Won, Jin Hyoung Kang, Young Seon Hong, Division of Oncology, Department of Internal Medicine, Kangnam St. Mary's hospital, Catholic University, Seoul 137-701, South Korea
Chan-Kwon Jung, Department of Hospital Pathology, Kangnam St. Mary's hospital, Catholic University, Seoul 137-701, South Korea
Soon Nam Oh, Department of Radiology, Kangnam St. Mary's hospital, Catholic University, Seoul 137-701, South Korea
Hyung Jin Kim, Won Kyung Kang, Seong Taek Oh, Department of General Surgery, Kangnam St. Mary's hospital, Catholic University, Seoul 137-701, South Korea
Author contributions: Ko YH and Hong YS contributed equally to this work; Jung CK reviewed the pathological findings; Oh SN reviewed the radiological findings; Ko YH, Kim TH, Won HS, Kang JH and Hong YS performed the palliative chemotherapy and management; Kim HJ, Kang WK and Oh ST performed the surgical operation; Ko YH and Hong YS wrote the paper.
Correspondence to: Young Seon Hong, MD, Division of Oncology, Department of Internal Medicine, Kangnam St. Mary's hospital, Catholic University, Banpo-dong 505, Seocho-gu, Seoul 137-701, South Korea. y331@catholic.ac.kr
Telephone: +82-2-5901348 Fax: +82-2-5338450
Received: May 27, 2008 Revised: August 18, 2008
Accepted: August 25, 2008
Published online: October 7, 2008

Abstract

Primary adenocarcinoma of the appendix is a rare malignancy that constitutes < 0.5% of all gastrointestinal neoplasms. Moreover, primary signet ring cell carcinoma of the appendix is an exceedingly rare entity. We have encountered 15 cases of primary appendiceal cancer among 3389 patients who underwent appendectomy over the past 18 years. In the present report, we describe a rare case of primary signet ring cell carcinoma of the appendix with ovarian metastases and unresectable peritoneal dissemination occurring in a 67-year-old female patient. She underwent appendectomy and bilateral salpingo-oophorectomy with a laparoscopy procedure. She then received palliative systemic chemotherapy with 12 cycles of oxaliplatin, 5-fluorouracil, and leucovorin (FOLFOX-4). The patient currently is well without progression of disease 12 mo after beginning chemotherapy.

Key words: Appendiceal neoplasms; Carcinoma; Signet ring cell; Chemotherapy

Peer reviewer: Toru Ishikawa, MD, Department of Gastroenterology, Saiseikai Niigata Second Hospital, Teraji 280-7, Niigata, Niigata 950-1104, Japan

Ko YH, Jung CK, Oh SN, Kim TH, Won HS, Kang JH, Kim HJ, Kang WK, Oh ST, Hong YS. Primary signet ring cell carcinoma of the appendix: A rare case report and our 18-year experience. *World J Gastroenterol* 2008; 14(37): 5763-5768 Available from: URL: http://www.wjgnet.com/1007-9327/14/5763.asp DOI: http://dx.doi.org/10.3748/wjg.14.5763

INTRODUCTION

Primary adenocarcinoma of the appendix, first described in 1882, is an uncommon malignancy of the gastrointestinal tract that constitutes approximately 0.12 cases per one million people per year[1]. Moreover, primary signet ring cell carcinoma of the appendix is an exceedingly rare entity and little information on the discrete characteristics of this tumor has been published[2]. The clinical presentation is usually non-specific, although right lower abdominal pain is the most common symptom, which is indistinguishable from acute appendicitis[3,4]. Therefore, it is invariably difficult to diagnose primary signet ring cell carcinoma of the appendix preoperatively. Usually the diagnosis is made only after histologic examination of a surgically-removed inflamed appendix.

The treatment options for metastatic disease include systemic chemotherapy alone, hyperthermic intraoperative intraperitoneal chemotherapy, cytoreductive surgery with a peritonectomy, and a combination of treatments. However, whether debulking surgery and intraperitoneal chemotherapy are worthwhile for all aggressive, advanced disease cases remains controversial.

There are a few reported cases of primary signet ring cell appendiceal carcinoma[5]. In the case reports published, the patients underwent cytoreductive surgery and intraperitoneal chemotherapy. However, this case report documents the use of systemic chemotherapy with oxaliplatin, and 5-fluorouracil (5-FU) chemotherapy (FOLFOX-4) for the female patient with primary signet ring cell appendiceal carcinoma with ovarian metastases

and unresectable peritoneal dissemination. The indications for systemic chemotherapy for aggressive primary appendiceal carcinoma were discussed. A case report is presented with a review of our 18-year experience at Kangnam St. Mary's Hospital.

CASE REPORT

A 67-year-old female visited her primary physician because of an increasing abdominal distention for 3 mo. She was then referred to our hospital for further evaluation and treatment. Cytology of ascitic fluid showed atypical cells. Her past history was unremarkable, as was her family history.

On examination, the patient appeared well with no signs of anemia. Physical examination performed on admission showed moderate distension of the abdomen without hepatomegaly. Gynecological examination was completely unremarkable. Gastrofibroscopy revealed a gastric ulcer and a biopsy showed chronic gastritis. Colonoscopy showed no positive findings. Abdominal computed tomography showed a moderate amount of fluid collection in the abdomen and pelvis with diffuse omental thickening (Figure 1). However, no other evidence of masses was seen within the abdomen and pelvis. The following tumor markers measured were 13 U/mL carbohydrate antigen (CA) 125, 12 U/mL CA19-9, and 2.55 mg/mL carcinoembryonic antigen (CEA). The results of tumor markers were normal. A diagnostic laparoscopy was performed and intra-operative frozen section analysis of a tumour of the appendix showed a signet ring cell carcinoma. The metastatic deposits extended from the pelvic cavity into the upper abdomen along the surface of the liver and diaphragm. The patient underwent appendectomy and bilateral salpingo-oophorectomy. The final pathologic examination was reported as a signet ring cell carcinoma infiltrating the vermiform appendix and metastasizing to the ovaries. Diffuse, strong immunoreactivity against CEA, cytokeratin 20, MUC2, and CDX-2 was observed in the tumor cells. Focal immunopositivity for MUC-5AC also was detected (Figure 2).

The patient received twelve cycles of FOLFOX-4 and is currently well without progression of disease, 12 mo after beginning chemotherapy.

Over an 18-year period from January 1990 to December 2007, there were 15 cases (an incidence rate of 0.4%) of primary appendiceal carcinoma among 3389 appendectomies performed in our hospital (Table 1). The mean age of patients with mucinous adenocarcinoma and patients with colonic type carcinomas was 58.0 years (range, 43-68 years) and 61 years (range, 42-73 years), respectively. Among the 15 patients, abdominal pain was the most common symptom of primary appendiceal cancer in 9 patients, and 7 of these 9 patients underwent emergency surgery. Histologically, 2 patients had low grade mucinous epithelial tumors, 8 patients had mucinous adenocarcinomas, 4 patients had colonic adenocarcinomas, and 1 patient had a signet ring cell carcinoma. In our series of 10 patients with regular

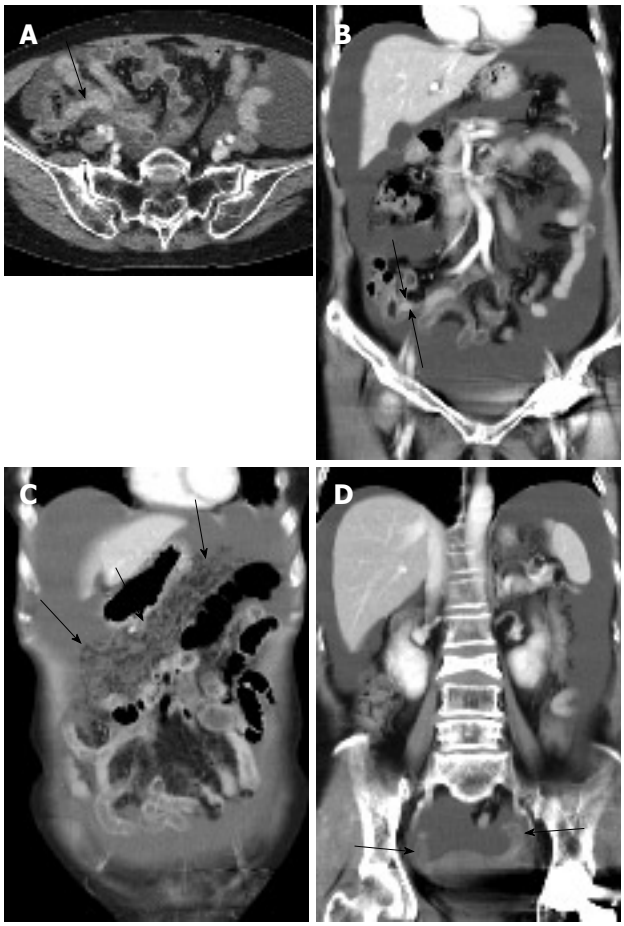


Figure 1 Contrast enhanced CT axial scan (**A**) and coronal reformatted image (**B**) showing massive ascites in abdominal cavity. Appendix is prominently seen with mild thickening (arrowed). Coronal reformatted images (**C** and **D**) showing increased reticulonodular densities (arrows in **C**) along the omentum representing carcinomatosis peritonei with no definite evidence of mass like lesion in adnexa (arrows in **D**).

follow-up, 4 patients developed relapses after debulking surgery. Of the four relapsed patients, two had a high grade colonic type adenocarcinoma and one had a mucinous adenocarcinoma with distant metastases on initial presentation. Ovarian metastases occurred in four of the 7 female cases, including the present case. Extra-abdominal metastatic sites were documented in two patients. Patient 8 had a mucinous adenocarcinoma (well-differentiated) and developed lung metastases and patient 11 had a colonic type adenocarcinoma (poorly differentiated) with liver metastases to the abdominal cavity. Of the six patients with peritoneal metastases, four underwent cytoreductive surgery, including right hemicolectomy. Palliative 5-FU-based chemotherapy was administered in three of seven cases with distant metastases.

DISCUSSION

Primary adenocarcinoma of the appendix is a rare malignancy of the gastrointestinal tract[1]. Smeenk *et al*[6] reported a 0.3% prevalence of primary appendiceal mucinous epithelial neoplasms identified from 167 744 patients who underwent appendectomy. Our 18-year-experience showed a similar incidence rate (0.4%) of primary ap-

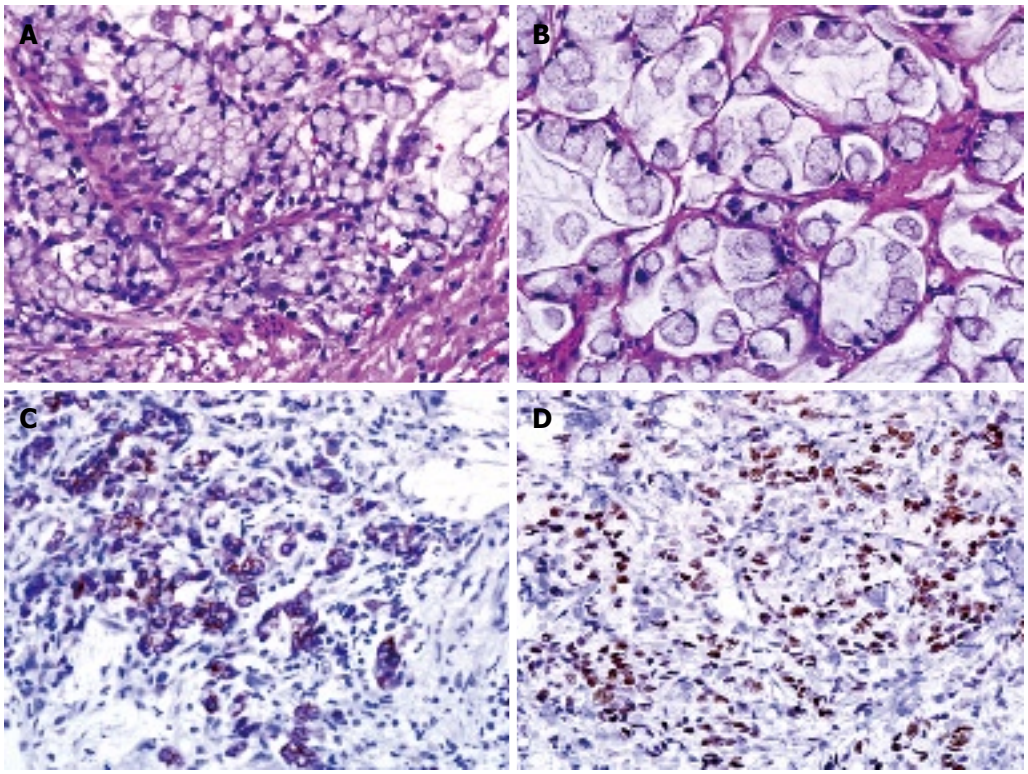


Figure 2 Histologic examination showing a signet ring cell carcinoma infiltrating vermiform appendix (**A**) and metastasizing to the ovary (**B**) (HE, × 400) with diffuse, strong immunoreactivity against cytokeratin 20 (**C**) and CDX-2 (**D**) in the tumor cells.

pendiceal carcinoma after appendectomy. Moreover, a primary signet ring cell carcinoma of the appendix (the case reported herein, was the first of the 15 cases) is an exceedingly rare entity, comprising only 4% of all appendiceal neoplasms[2].

The demographic characteristics of patients with cancer of the appendix vary by histology. According to report of McCusker *et al*[1], those diagnosed with malignant carcinoids are significantly younger (mean age, 38 years) than those diagnosed with any of the other cancer types. The mean age of patients at diagnosis of mucinous adenocarcinoma, colonic type adenocarcinoma, and signet ring cell carcinoma is approximately 60 years, 62 years, and 58 years, respectively, and an equal number of males and females developed goblet cell carcinoid, mucinous adenocarcinoma, and signet ring cell carcinoma, except for colonic adenocarcinoma which had a male predominance[1]. Our results also showed similar demographic findings.

Reported series of appendiceal adenocarcinoma are difficult to compare, as terminology and classification of these lesions are not consistent[7,8]. The International Classification of Diseases for Oncology (ICD-O) divides the tumors of appendix into five categories: colonic type adenocarcinoma, mucinous adenocarcinoma, signet ring cell carcinoma, goblet cell carcinoma, and malignant carcinoid/adenocarcinoid[1,9]. These carcinomas arise in preexisting adenomas, either by a cystic and colonic growth pattern[10,11]. On the other hand, signet ring cell carcinomas, usually frequent in stomach and intestine, are adenocarcinomas with mucus-producing tumor cells. In this case, the tumor cells showed diffuse, strong immunoreactivity against cytokeratin 20, CDX-2, MUC-2, and CEA, and focal immunopositivity for MUC-5AC (Figure 2). CDX-2 is a useful marker to confirm an appendiceal origin of pseudomyxoma peritonei, particularly when used in conjunction with CK20, MUC-2, and MUC-5AC[12].

Most appendiceal cancers are low-grade neoplasms that are typically relatively indolent. The overall 5-year survival rate for mucinous appendiceal adenocarcinomas reported by Park *et al*[13] is 20.5%. According to report of McCusker[1], except for signet ring cell carcinoma and malignant carcinoid, the histologic type does not have a significant impact on survival. In addition, the extent of disease at diagnosis is a more important predictor of survival than histology. Ronnett *et al*[7,14] reported that the long-term survival of patients with diffuse, peritoneal metastases arising from these carcinomas is poor, with a reported 5-year survival as low as 6.7%-14%. Moreover, in a study by McGory *et al*[2], poorly differentiated adenocarcinoma and signet ring cell carcinoma of the appendix had the highest proportion of distant disease with a 5-year survival rate of 7%. Therefore, signet ring cell carcinoma may be a separate tumor type in the appendix that should be considered apart from other carcinomas, largely because of its poor prognosis.

Right hemicolectomy is considered the optimal treatment for most histologic types of primary appendiceal carcinoma even in the presence of perforation and in Dukes A tumors[15]. The treatment options for metastatic disease include systemic chemotherapy alone, hyperthermic intraoperative intraperitoneal chemotherapy, cytoreductive surgery with peritonectomy, and combination of treatments. Of those treatment options, cytoreductive surgery and hyperthermic intraperitoneal chemotherapy have recently become the treatment of choice for metastatic diseases at most large centers[16]. In the present case, laparoscopic exploration allowed assessment of the

Table 1 Clinical characteristics, tumor features, treatment, and survival of 15 out of 3389 patients who underwent appendectomy over an 18-year period

Patient's No.	Age /sex	Symptom	Type of treatment	Histology	Grade	Stage	Tumor marker elevated	Surgery	Additional treatment	Metastatic sites	Relapse
1	62/F	No	EL	Mucinous epithelial tumor	Low grade	D	CEA	RHC, TAH, BSO, Appendicectomy	Chemotherapy (CP)	Ovary, Peritoneum, Omentum	No
2	58/F	Pain	EM	Mucinous epithelial tumor	Low grade	D	-	RHC, Appendicectomy	No	Rupture of appendix	No
3	43/F	Pain	EL	Mucinous adenocarcinoma	WD	L	NA	BSO, Appendicectomy	No	No	Loss
4	43/M	Pain	EL	Mucinous adenocarcinoma	Low grade	L	NA	BSO, Appendicectomy	No	No	No
5	69/F	Pain	EL	Mucinous adenocarcinoma	WD	D	NA	TAH, BSO, Appendicectomy	No	Ovary, Peritoneum	Loss
6	68/M	Pain	EM	Mucinous adenocarcinoma	WD	L	-	RHC, Appendicectomy	No	No	No
7	58/M	Pain	EM	Mucinous adenocarcinoma	WD	D	-	Appendicectomy Omentectomy	No	Omentum	Yes
8	61/M	Pain	EM	Mucinous adenocarcinoma	WD	L	-	RHC, Appendicectomy	Chemotherapy (FL)	Lung	Yes
9	63/F	No	EL	Mucinous adenocarcinoma	WD	D	-	TAH, BSO, Appendicectomy	Chemotherapy (FOLFOX)	Ovary, Peritoneum, Omentum	No
10	59/M	No	EL	Mucinous adenocarcinoma	WD	L	-	RHC, Appendicectomy	No	No	No
11	42/M	Pain	EM	Colonic type adenocarcinoma	PD	L	NA	RHC, Appendicectomy	No	Liver, Peritoneum	Yes
12	73/M	Pain	EM	Colonic type adenocarcinoma	MD	R	CEA	RHC, Appendicectomy	No	No	Yes (colon cancer)
13	62/F	Abdominal distension	EL	Colonic type adenocarcinoma	MD	L	CEA	RHC, Appendicectomy	No	No	No
14	67/M	Pain	EM	Colonic type adenocarcinoma	MD	L	NA	RHC, Appendicectomy	No	No	No
15	67/F	Abdominal distension	EL	Signet ring cell carcinoma		D	-	Appendicectomy, BSO	Chemotherapy (FOLFOX)	Ovary, Peritoneum, Omentum	-

EL: Elective; EM: Emergency; WD: Well differentiated; MD: Moderately differentiated; L: Localized; R: Regional; D: Distant; NA: Not available; RHC: Right hemicolectomy; TAH: Transabdominal abdominal hysterectomy; BSO: Bilateral salpingo-oophorectomy; CP: Cyclophosphamide/cisplatin; FL: 5-fluorouracil/leucovorin; FOLFOLX: Oxaliplatin/5-fluorouracil/leucovorin.

pelvic metastasis which was unresectable and the final pathologic diagnosis was a signet ring cell carcinoma, with aggressive biologic behaviors. Therefore, we did not perform cytoreduction or intraperitoneal chemotherapy, but proceeded with an appendicectomy and bilateral salpingo-oophorectomy followed by systemic chemotherapy. Routine oophorectomy should be considered in all females, especially if post-menopausal. An oophorectomy itself may assist with tumour staging, reduce the likelihood of symptomatic metastases and also provide a survival advantage[17].

Given the predilection for exclusive peritoneal metastasis of mucinous adenocarcinomas of the appendix, in the case of low-grade tumors, cytoreductive surgery and regional chemotherapy are very important. Aggressive cytoreductive surgery for mucinous-type tumours is known to improve the survival rate and reduce the recurrence rate in patients with generalized pseudomyxoma peritonei compared with simple appendectomy[18]. In addition, the most consistent prognostic factor for survival is the completeness of cytoreduction[19,20]. However, whether debulking surgery for all patients with aggressive, advanced disease is worthwhile has not been elucidated. In fact, only limited data are available on surgical debulking for diffuse, peritoneal dissemination from aggressive appendiceal carcinomas, in part because the disease is so rare. Incomplete cytoreduction plus perioperative intraperitoneal chemotherapy for peritoneal dissemination from aggressive appendiceal malignancies can achieve a limited long-term survival[5,20]. Furthermore, Gonzalez-Moreno and Sugarbaker demonstrated that right hemicolectomy does not confer a survival advantage in patients with mucinous appendiceal tumors with peritoneal seeding[21]. These data suggest that in patients with aggressive appendiceal malignancy, routine right hemicolectomy should not be performed unless in conjunction with complete cytoreduction in combination with intraperitoneal chemotherapy.

Considering systemic chemotherapy, an alternative option to metastatic appendiceal carcinomas, although limited, the available data favor integration of systemic chemotherapy, including mitomycin C, fluoropyrimidines and platinum compounds, which has been used as an intraperitoneal chemotherapy[20,22]. Ishibashi *et al*[22] described a case of pseudomyxoma peritonei caused by carcinoma of the appendix, which was successfully treated with multidisciplinary treatment, including oxaliplatin, 5-FU, and leucovorin (modified FOLFOX6 regimen) combination systemic chemotherapy followed by cytoreduction. In a recent randomized phase Ⅲ trial comparing debulking alone to debulking with continuous hyperthermic peritoneal perfusion of cisplatin in patients with low-grade, mucinous tumors of the gastrointestinal tract, all patients who had surgical debulking received four courses of postoperative intravenous chemotherapy (5-FU, leucovorin, and oxaliplatin)[23].

Recently, many targeted drugs have been used as a combination therapy to improve the survival of patients with gastrointestinal malignancies[24]. Mucin genes, which play an important role in the pathogenesis of pseudomyxoma peritonei, are regulated in part by epidermal growth factor receptor (EGFR) signaling[25,26]. Andreopoulou *et al*[27] recently presented that heavily pretreated patients who underwent cetuximab monotherapy showed a transient decrease in tumor markers, with an encouraging time-to-progression (3 mo) in a phase Ⅱ trial of cetuximab in mucinous peritoneal carcinomatosis. In addition, Logan-Collins *et al*[28] demonstrated that in patients who undergo cytoreductive surgery and intraperitoneal hyperthermic perfusion for mucinous adenocarcinoma of the appendix, (n = 32), the average vascular endothelial growth factor (VEGF) counts correlate with survival (P = 0.017), and for patients with recurrence, this correlation is stronger (P = 0.002), indicating that VEGF may predict the survival of patients with peritoneal surface metastases from mucinous adenocarcinomas and that anti-angiogenic therapies may be effective in patients with this devastating disease. In the future, novel targeted therapies, including EGFR inhibitor or anti-angiogenic agents, in combination with regional treatment or systemic chemotherapy should be evaluated.

REFERENCES

1 **McCusker ME**, Cote TR, Clegg LX, Sobin LH. Primary malignant neoplasms of the appendix: a population-based study from the surveillance, epidemiology and end-results program, 1973-1998. *Cancer* 2002; **94**: 3307-3312
2 **McGory ML**, Maggard MA, Kang H, O'Connell JB, Ko CY. Malignancies of the appendix: beyond case series reports. *Dis Colon Rectum* 2005; **48**: 2264-2271
3 **Connor SJ**, Hanna GB, Frizelle FA. Appendiceal tumors: retrospective clinicopathologic analysis of appendiceal tumors from 7,970 appendectomies. *Dis Colon Rectum* 1998; **41**: 75-80
4 **O'Donnell ME**, Badger SA, Beattie GC, Carson J, Garstin WI. Malignant neoplasms of the appendix. *Int J Colorectal Dis* 2007; **22**: 1239-1248
5 **Glehen O**, Mohamed F, Sugarbaker PH. Incomplete cytoreduction in 174 patients with peritoneal carcinomatosis from appendiceal malignancy. *Ann Surg* 2004; **240**: 278-285
6 **Smeenk RM**, van Velthuysen ML, Verwaal VJ, Zoetmulder FA. Appendiceal neoplasms and pseudomyxoma peritonei: a population based study. *Eur J Surg Oncol* 2008; **34**: 196-201
7 **Ronnett BM**, Zahn CM, Kurman RJ, Kass ME, Sugarbaker PH, Shmookler BM. Disseminated peritoneal adenomucinosis and peritoneal mucinous carcinomatosis. A clinicopathologic analysis of 109 cases with emphasis on distinguishing pathologic features, site of origin, prognosis, and relationship to "pseudomyxoma peritonei". *Am J Surg Pathol* 1995; **19**: 1390-1408
8 **Misdraji J**, Young RH. Primary epithelial neoplasms and other epithelial lesions of the appendix (excluding carcinoid tumors). *Semin Diagn Pathol* 2004; **21**: 120-133
9 **Percy C**, Van Holten V, Muir C, editors. International Classification of Diseases for Oncology (ICD-O). 2nd edition. Geneva: World Health Organization, 1990. Available from: URL: http://www.who.int/classifications/icd/en/
10 **Higa E**, Rosai J, Pizzimbono CA, Wise L. Mucosal hyperplasia, mucinous cystadenoma, and mucinous cystadenocarcinoma of the appendix. A re-evaluation of appendiceal "mucocele". *Cancer* 1973; **32**: 1525-1541
11 **Djuranovic SP**, Spuran MM, Kovacevic NV, Ugljesic MB, Kecmanovic DM, Micev MT. Mucinous cystadenoma of the appendix associated with adenocarcinoma of the sigmoid colon and hepatocellular carcinoma of the liver: report of a case. *World J Gastroenterol* 2006; **12**: 1975-1977
12 **Nonaka D**, Kusamura S, Baratti D, Casali P, Younan R, Deraco M. CDX-2 expression in pseudomyxoma peritonei: a clinicopathological study of 42 cases. *Histopathology* 2006; **49**: 381-387
13 **Park IJ**, Yu CS, Kim HC, Kim JC. [Clinical features and prognostic factors in primary adenocarcinoma of the appendix] *Korean J Gastroenterol* 2004; **43**: 29-34
14 **Ronnett BM**, Yan H, Kurman RJ, Shmookler BM, Wu L, Sugarbaker PH. Patients with pseudomyxoma peritonei associated with disseminated peritoneal adenomucinosis have a significantly more favorable prognosis than patients with peritoneal mucinous carcinomatosis. *Cancer* 2001; **92**: 85-91
15 **Nitecki SS**, Wolff BG, Schlinkert R, Sarr MG. The natural history of surgically treated primary adenocarcinoma of the appendix. *Ann Surg* 1994; **219**: 51-57
16 **Murphy EM**, Farquharson SM, Moran BJ. Management of an unexpected appendiceal neoplasm. *Br J Surg* 2006; **93**: 783-792
17 **Cortina R**, McCormick J, Kolm P, Perry RR. Management and prognosis of adenocarcinoma of the appendix. *Dis Colon Rectum* 1995; **38**: 848-852
18 **Sugarbaker PH**. Cytoreductive surgery and intraperitoneal chemotherapy with peritoneal spread of cystadenocarcinoma. *Eur J Surg Suppl* 1991; 75-82
19 **Sugarbaker PH**. Cytoreductive surgery and peri-operative intraperitoneal chemotherapy as a curative approach to pseudomyxoma peritonei syndrome. *Eur J Surg Oncol* 2001; **27**: 239-243
20 **Yan TD**, Bijelic L, Sugarbaker PH. Critical analysis of treatment failure after complete cytoreductive surgery and perioperative intraperitoneal chemotherapy for peritoneal dissemination from appendiceal mucinous neoplasms. *Ann Surg Oncol* 2007; **14**: 2289-2299
21 **Gonzalez-Moreno S**, Sugarbaker PH. Right hemicolectomy does not confer a survival advantage in patients with mucinous carcinoma of the appendix and peritoneal seeding. *Br J Surg* 2004; **91**: 304-311
22 **Ishibashi K**, Sobajima J, Okada N, Ishizuka N, Yokoyama M, Mitsuhashi T, Miyazaki T, Nakada H, Gonda T, Ishida H. [A case of pseudomyxoma peritonei successfully treated with multidisciplinary treatment including modified FOLFOX6 regimen] *Gan To Kagaku Ryoho* 2007; **34**: 2047-2049

23 **James P**. Phase III randomized study of operative debulking and systemic chemotherapy with or without intra- and peri-operative intraperitoneal chemotherapy in patients with peritoneal carcinomatosis from low-grade gastrointestinal adenocarcinoma. Available from: URL: http://www.cancer.gov/clinicaltrials/NCI-03-C-0085
24 **Rosen LS**. VEGF-targeted therapy: therapeutic potential and recent advances. *Oncologist* 2005; **10**: 382-391
25 **O'Connell JT**, Tomlinson JS, Roberts AA, McGonigle KF, Barsky SH. Pseudomyxoma peritonei is a disease of MUC2-expressing goblet cells. *Am J Pathol* 2002; **161**: 551-564
26 **Perrais M**, Pigny P, Copin MC, Aubert JP, Van Seuningen I. Induction of MUC2 and MUC5AC mucins by factors of the epidermal growth factor (EGF) family is mediated by EGF receptor/Ras/Raf/extracellular signal-regulated kinase cascade and Sp1. *J Biol Chem* 2002; **277**: 32258-32267
27 **Andreopoulou E**, Muggia F, Safa M, Escalon J, Downey A, Newman E, Hochster H, Wojtaszek C, Mauro D, Lowy A. Phase II study of cetuximab in mucinous peritoneal carcinomatosis. Journal of Clinical Oncology, ASCO Annual Meeting Proceedings Part I. Vol 24, No. 18S (June 20 Supplement), 2006: 14020. org/cgi/content/abstract/24/18_suppl/14020
28 **Logan-Collins JM**, Lowy AM, Robinson-Smith TM, Kumar S, Sussman JJ, James LE, Ahmad SA. VEGF expression predicts survival in patients with peritoneal surface metastases from mucinous adenocarcinoma of the appendix and colon. *Ann Surg Oncol* 2008; **15**: 738-744

S- Editor Li DL **L- Editor** Wang XL **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5769

World J Gastroenterol 2008 October 7; 14(37): 5769-5772
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Primary retroperitoneal mucinous cystadenoma: Report of a case and review of the literature

Sheng-Lei Yan, Ho Lin, Chien-Long Kuo, Hurng-Sheng Wu, Ming-Ho Huang, Yueh-Tsung Lee

Sheng-Lei Yan, Division of Gastroenterology, Department of Internal Medicine, Chang Bing Show Chwan Memorial Hospital, Changhua County 505, Taiwan, China
Ho Lin, Yueh-Tsung Lee, Department of Life Sciences, National Chung Hsing University, Taichung City 402, Taiwan, China
Chien-Long Kuo, Department of Pathology, Show Chwan Memorial Hospital, Changhua City 500, Taiwan, China
Hurng-Sheng Wu, Ming-Ho Huang, Division of General Surgery, Department of Surgery, Show Chwan Memorial Hospital, Changhua City500, Taiwan, China
Yueh-Tsung Lee, Division of General Surgery, Department of Surgery, Chang Bing Show Chwan Memorial Hospital, Changhua County 505, Taiwan, China
Author contributions: Yan SL and Lee YT organized the whole manuscript and supervised the process; Lin H helped revise the manuscript; Kuo CL examined the resected tumor; Wu HS organized the patient data and figures; Huang MH revised and approved the final manuscript.
Correspondence to: Yueh-Tsung Lee, MD, Division of General Surgery, Department of Surgery, Chang Bing Show Chwan Memorial Hospital, No. 6, Lugong Rd, Lugang Township, Changhua County 505, Taiwan, China. yslcsmu@yahoo.com
Telephone: +886-4-7813888-72221 Fax: +886-4-7812401
Received: April 30, 2008 Revised: June 16, 2008
Accepted: June 23, 2008
Published online: October 7, 2008

Abstract

Primary retroperitoneal mucinous cystadenomas (RMCs) are very rare and their biological behavior and histogenesis remain speculative. An accurate preoperative diagnosis of these tumors is difficult because no effective diagnostic measures have been established. We describe a 29-year-old woman with abdominal pain and a palpable mass. Computed tomography (CT) of the abdomen revealed a retroperitoneal cystic mass which was resected successfully at laparotomy. Histopathological examination of the resected mass confirmed the diagnosis of RMC. There was no evidence of disease 2 years after surgery.

Key words: Retroperitoneal; Mucinous cystadenoma; Cystic mass; Clinical features

Peer reviewer: Chung-Mau Lo, Professor, Department of Surgery, The University of Hong Kong, Queen Mary Hospital, No. 102 Pokfulam Road, Hong Kong, China

Yan SL, Lin H, Kuo CL, Wu HS, Huang MH, Lee YT. Primary retroperitoneal mucinous cystadenoma: Report of a case and review of the literature. *World J Gastroenterol* 2008; 14(37): 5769-5772 Available from: URL: http://www.wjgnet.com/1007-9327/14/5769.asp DOI: http://dx.doi.org/10.3748/wjg.14.5769

INTRODUCTION

Primary retroperitoneal mucinous cystadenomas (RMCs) are very rare, even though mucinous cystadenomas are frequent ovarian tumors[1]. Like most retroperitoneal masses, they cause symptoms when growing large enough to exert pressure or obstructive effect on adjacent organs[2]. Because of the limited number of reported cases, the biological behavior and histogenesis of such tumors remain speculative[3]. Laboratory studies and imaging methods cannot achieve an accurate preoperative diagnosis[1]. We describe here a case of a huge RMC presenting with a palpable abdominal mass and a literature review is also given together with discussion.

CASE REPORT

A 29-year-old woman presented at our institution with abdominal pain for 1 wk and a palpable mass on the left side of her abdomen. The abdominal pain was described as intermittently cramping and the intensity of pain was exacerbated as the palpable mass grew. She denied any systemic disease or history of drug abuse. Physical examination and pelvic examination showed no remarkable findings except for a large mobile mass which was palpable over her left flank region. The laboratory data were within reference ranges. Tumor markers, including CA199, CA125 and CEA were normal. The KUB film demonstrated a large hazy mass located over the left side of the abdomen displacing the left-side colon medially. Contrast-enhanced computed tomography (CT) of the abdomen showed a well-defined homogenous hypodense mass, which measured 12 cm × 6.5 cm in size, occupying the left retroperitoneal space with medial displacement of the

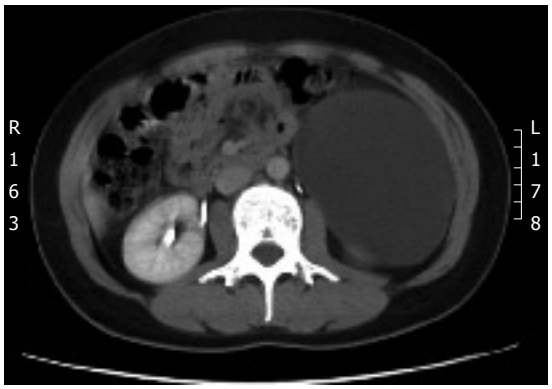


Figure 1 Contrast-enhanced CT of the abdomen showing a 12 cm × 6.5 cm homogenous cystic mass in the retroperitoneal space with medial displacement of the descending colon.

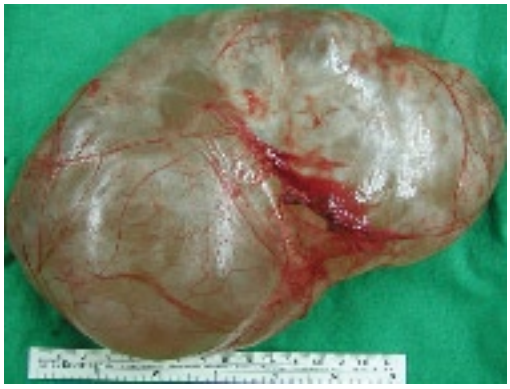


Figure 2 Photograph of the resected mass measuring 20 cm × 14 cm × 6 cm in size.

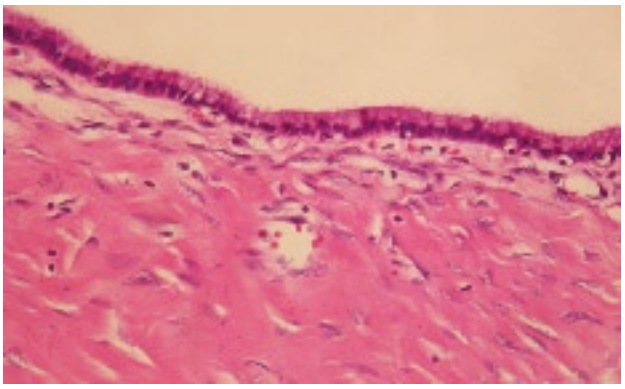


Figure 3 Photomicrograph showing a single layer of mucin-producing columnar epithelium with underlying fibrous connective tissue (HE, × 100).

descending colon (Figure 1). Because a retroperitoneal cystic mass was considered, the patient underwent surgical excision of the tumor. At laparotomy, the mass was located behind the descending colon. The white line of Toldt was divided to expose the cystic mass. A huge retroperitoneal cystic mass was resected, measuring 20 cm × 14 cm × 6 cm in size and 900 mg in weight (Figure 2). The uterus and both ovaries were normal. There was no spread of the tumor in the abdominal cavity. Histopathological assessment of the resected mass disclosed a picture of mucinous cystadenoma lined by a single layer of columnar epithelium with mucin production and surrounded by dense fibrous tissues (Figure 3). A diagnosis of primary RMC was made. The postoperative course was uneventful and she remained asymptomatic with ensuing two years of follow-up.

DISCUSSION

Primary retroperitoneal mucinous tumors are rare. The most common type of retroperitoneal mucinous tumors is the RMC, which shares a histological similarity to ovarian mucinous cystadenomas but can arise at any location in the retroperitoneum without attachment to the ovary [4]. The histogenesis of primary RMCs remains unclear. Three main theories have been proposed to explain the histogenic origin of mucinous cystadenomas in the retroperitoneum. These tumors can arise from teratoma[5,6], heterotopic ovarian tissue[4,5], or mucinous metaplasia of the mesothelial lining cells[4,5,7-9]. Primary RMCs occur exclusively in women[1,4], although three cases of RMC in men have been reported in the literature[10]. Some authors questioned the diagnosis of these lesions as benign because all three patients died of the disease[11]. Furthermore, Subramony *et al*[4] reported that the estrogen receptor is positive in stromal cells of a RMC, which could explain the exclusive occurrence of these tumors in women.

In the present study, we performed a literature review using Medline starting in 1970, and found a total of 19 cases of primary RMCs in the English literature. Based on these cases, including the present one, it was found that all cases were women, with an age range of 14 to 85 years (Table 1). The size of reported tumors ranged from 7 cm to 30 cm. There was no relationship between the age of patients and the size of tumors. The symptoms were nonspecific and most of the patients complained of asymptomatic mass or abdominal discomfort. The preoperative diagnosis was mesenteric cyst in 4 cases, ovarian cyst in 3 cases, and retroperitoneal cystic tumor in one case. Interestingly, preoperative diagnosis of renal cyst was considered in 2 cases. Serum levels of tumor markers were normal in 4 cases. However, two cases demonstrated a slight elevation of CA199 and CA125 levels, respectively. There was no evidence of recurrence after surgical management in 9 patients.

Preoperative diagnosis of primary RMCs is reportedly very difficult due to a lack of pathognomonic clinical features. Based on the review of cases reported in the English literature (Table 1), most patients presented with asymptomatic mass and vague abdominal discomfort. Reported tumors were relatively large, which may be large enough to evoke clinical symptoms or perceived by the patients. However, no cases examined presented with severe abdominal pain. Laboratory studies, including serum tumor markers and cytology study of cystic fluid are not helpful in making diagnosis of the tumors[1,14,20]. However, Motoyama *et al*[21] reported

Table 1 Cases of primary RMC in the English literature since 1970

Reference	Sex	Age	Symptom	Tumor size (cm)	Preoperative diagnosis	Tumor markers	Operation	Outcome (mo)
1	Woman	35	Fullness	14	ND	ND	Right retroperitoneal approach	ND
2	Woman	39	Asymptomatic mass	10 × 9 × 5	ND	ND	Exploratory laparoscopy	ND
3	Woman	18	Flatulence	11 × 8 × 7	ND	Elevated CA199	Laparotomy	ND
	Woman	85	Periumbilical pain	21 × 14 × 8	Ovarian cyst	ND	Laparotomy	ND
4	Woman	25	Asymptomatic mass	30 × 25 × 10	Ovarian cystic mass	ND	Laparotomy	ND
5	Woman	19	Asymptomatic mass	6 × 10	Mesenteric cyst	ND	Celiotomy	NR (18)
6	Woman	26	Asymptomatic mass	22 × 16 × 10	ND	ND	Laparotomy	ND
7	Woman	58	Asymptomatic mass	7	ND	ND	Laparotomy	ND
8	Woman	44	Asymptomatic mass	30 × 20 × 14	ND	CA125:75 IU/mL	Laparotomy	ND
9	Woman	48	Fullness	15 × 13 × 9	Mesenteric cyst	ND	Laparoscopic resection	NR (8)
11	Woman	68	Asymptomatic mass	8 × 8	Renal cyst	ND	Laparotomy	ND
12	Woman	43	Backache	12 × 11 × 6.5	ND	ND	Laparotomy	NR (16)
	Woman	25	Abdominal pain	10	ND	ND	Gastrectomy Splenectomy, nephrectomy	Died 17 d postop
14	Woman	45	Asymptomatic mass	9 × 6.5	ND	Normal	celiotomy	NR (3)
15	Woman	14	Asymptomatic	13 × 9 × 15	Mesenteric cyst Cystic teratoma	ND	Laparotomy	NR (13)
16	Woman	38	Vague abdominal discomfort	10.0 × 7.5 × 5.5	ND	Normal	Celiotomy	NR (12)
17	Woman	32	Asymptomatic mass	11 × 7	ND	ND	Celiotomy	NR (19)
18	Woman	21	Abdominal discomfort	10 × 5.5 × 6	ND	ND	Laparotomy	ND
19	Woman	28	Left flank pain	9 × 10 × 10.9	Renal cyst	ND	ND	ND
Present case	Woman	29	Abdominal pain, mass	20 × 14 × 6	Retroperitoneal cystic mass	Normal	Laparotomy	NR (24)

ND: Not described; NR: No recurrence.

that measurement of CEA level in the cystic fluid may be useful in making the diagnosis. With regard to the imaging characteristics of RMCs, these tumors usually manifest as homogenous unilocular cystic masses at CT of the abdomen[20]. Furthermore, displacement of colon, kidney or ureter may suggest the retroperitoneal location of tumors[1,20]. As for the management of primary RMCs, complete surgical excision is recommended to eliminate the risk of infection, recurrence, and malignant degeneration[1,2], although these tumors seem to behave in a benign fashion with no recurrences after surgical removal, as demonstrated in our study. Exploratory laparotomy with complete enucleation of the cyst is traditionally indicated, although successful laparoscopic excision of a primary RMC has been reported[9].

In conclusion, when confronted with a cystic mass in the retroperitoneum, a primary RMC should be included in the list of differential diagnosis. Complete surgical removal of the tumor is recommended because of high risk of infection, recurrence and malignant potential.

REFERENCES

1 **Lai EC**, Chung KM, Lau WY. Primary retroperitoneal mucinous cystadenoma. *ANZ J Surg* 2006; **76**: 537

2 **Arribas D**, Cay A, Latorre A, Cordoba E, Martinez F, Lagos J. Retroperitoneal mucinous cystadenoma. *Arch Gynecol Obstet* 2004; **270**: 292-293

3 **Isse K**, Harada K, Suzuki Y, Ishiguro K, Sasaki M, Kajiura S, Nakanuma Y. Retroperitoneal mucinous cystadenoma: report of two cases and review of the literature. *Pathol Int* 2004; **54**: 132-138

4 **Subramony C**, Habibpour S, Hashimoto LA. Retroperitoneal mucinous cystadenoma. *Arch Pathol Lab Med* 2001; **125**: 691-694

5 **Pennell TC**, Gusdon JP Jr. Retroperitoneal mucinous cystadenoma. *Am J Obstet Gynecol* 1989; **160**: 1229-1231

6 **Williams PP**, Gall SA, Prem KA. Ectopic mucinous cystadenoma. A case report. *Obstet Gynecol* 1971; **38**: 831-837

7 **Banerjee R**, Gough J. Cystic mucinous tumours of the mesentery and retroperitoneum: report of three cases. *Histopathology* 1988; **12**: 527-532

8 **Balat O**, Aydin A, Sirikci A, Kutlar I, Aksoy F. Huge primary mucinous cystadenoma of the retroperitoneum mimicking a left ovarian tumor. *Eur J Gynaecol Oncol* 2001; **22**: 454-455

9 **Chen JS**, Lee WJ, Chang YJ, Wu MZ, Chiu KM. Laparoscopic resection of a primary retroperitoneal mucinous cystadenoma: report of a case. *Surg Today* 1998; **28**: 343-345

10 **Hansmann G**, Budd J. Massive Unattached Retroperitoneal Tumors: An Explanation of Unattached Retroperitonel Tumors Based on Remnants of the Embryonic Urogenital Apparatus. *Am J Pathol* 1931; **7**: 631–674

11 **de Peralta MN**, Delahoussaye PM, Tornos CS, Silva EG. Benign retroperitoneal cysts of mullerian type: a clinicopathologic study of three cases and review of the literature. *Int J Gynecol Pathol* 1994; **13**: 273-278

12 **Rothacker D**, Knolle J, Stiller D, Borchard F. Primary retroperitoneal mucinous cystadenomas with gastric epithelial differentiation. *Pathol Res Pract* 1993; **189**: 1195-1204

13 **Bortolozzi G**, Grasso A, Zasso B. Mucinous cystadenoma of the retroperitoneum. A case report and review. *Eur J Gynaecol Oncol* 1995; **16**: 65-68

14 **Yunoki Y**, Oshima Y, Murakami I, Takeuchi H, Yasui Y, Tanakaya K, Konaga E. Primary retroperitoneal mucinous cystadenoma. *Acta Obstet Gynecol Scand* 1998; **77**: 357-358
15 **Tamura T**, Yamataka A, Murakami T, Okada Y, Kobayashi H, Ohshiro K, Lane GJ, Miyano T. Primary mucinous cystadenoma arising from behind the posterior peritoneum of the descending colon in a child: a case report. *Asian J Surg* 2003; **26**: 237-239
16 **Min BW**, Kim JM, Um JW, Lee ES, Son GS, Kim SJ, Moon HY. The first case of primary retroperitoneal mucinous cystadenoma in Korea: a case report. *Korean J Intern Med* 2004; **19**: 282-284
17 **Sheen-Chen SM**, Eng HL. Retroperitoneal mucinous cystadenoma. *Dig Dis Sci* 2006; **51**: 752-753
18 **Kehagias DT**, Karvounis EE, Fotopoulos A, Gouliamos AD. Retroperitoneal mucinous cystadenoma. *Eur J Obstet Gynecol Reprod Biol* 1999; **82**: 213-215
19 **Ginsburg G**, Fraser J, Saltzman B. Retroperitoneal mucinous cystadenoma presenting as a renal cyst. *J Urol* 1997; **158**: 2232
20 **Yang DM**, Jung DH, Kim H, Kang JH, Kim SH, Kim JH, Hwang HY. Retroperitoneal cystic masses: CT, clinical, and pathologic findings and literature review. *Radiographics* 2004; **24**: 1353-1365
21 **Motoyama T**, Chida T, Fujiwara T, Watanabe H. Mucinous cystic tumor of the retroperitoneum. A report of two cases. *Acta Cytol* 1994; **38**: 261-266

S- Editor Li DL **L- Editor** Wang XL **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5773

World J Gastroenterol 2008 October 7; 14(37): 5773-5775
World Journal of Gastroenterology ISSN 1007-9327

Intestinal preparation prior to capsule endoscopy administration

Vicente Pons Beltrán, Cristina Carretero, Begoña Gonzalez-Suárez, Iñaqui Fernández-Urien, Miguel Muñoz-Navas

Vicente Pons Beltrán, Gastroenterology Department, Hospital La Fe, Valencia 46009, Spain
Cristina Carretero, Iñaqui Fernandez-Urien, Miguel Muñoz-Navas, Gastroenterology Department, University of Navarra, Pamplona 31008, Spain
Begoña Gonzalez-Suarez, Gastroenterology Department, Hospital del Mar, Barcelona 08036, Spain
Author contributions: All the authors contributed equally to this work.
Correspondence to: Dr. Vicente Pons Beltrán, MD, PhD, Servicio de Medicina Digestiva, Hospital Universitari La Fe, Avda. Campanar 21, Valencia 46009, Spain. pons_vicbel@gva.es
Telephone: +34-96-1973233 Fax: +34-96-1973118
Received: February 15, 2008 Revised: August 26, 2008
Accepted: September 2, 2008
Published online: October 7, 2008

Abstract

In order to have an adequate view of the whole small intestine during capsule endoscopy, the preparation recommended consists of a clear liquid diet and an overnight fast. However, visualization of the small bowel during video capsule endoscopy can be impaired by intestinal contents. To improve mucosal visualization, some authors have evaluated different regimens of preparation. There is no consensus about the necessity of intestinal preparation for capsule endoscopy and it should be interesting to develop adequate guidelines to improve its efficacy and tolerability. Moreover, the effect of preparation type (purgative) on intestinal transit time is not clear. Since a bowel preparation cannot definitively improve its visibility (and theoretically the yield of the test), it is not routinely recommended.

Key words: Capsule endoscopy; Intestinal preparation; Polyethylene glycol; Aqueous sodium phosphate

Peer reviewer: Arno Josef Dormann, PhD, Kliniken der Stadt Köln gGmbH, Neufelder Str. 32, Koeln 51067, Germany

Pons Beltrán V, Carretero C, Gonzalez-Suárez B, Fernández-Urien I, Muñoz-Navas M. Intestinal preparation prior to capsule endoscopy administration. *World J Gastroenterol* 2008; 14(37): 5773-5775 Available from: URL: http://www.wjgnet.com/1007-9327/14/5773.asp DOI: http://dx.doi.org/10.3748/wjg.14.5773

TO THE EDITOR

In order to have an adequate view of the whole small intestine during capsule endoscopy, it was initially thought that fasting for 12 h and a clear liquid diet 24 h prior to the procedure were an effective preparation. Based on this consensus, each group initiated this new technique, adapting it according to its own experience. Before long, it became evident that the capsule had two problems. One is the percentage of incomplete examinations is up to 15% of all those underwent capsule endoscopy due to a prolonged gastric or intestinal transit time[1], the other is the relatively frequent existence of intestinal content, particularly in its most distal parts of the small intestine. For these two reasons, it was thought that a preparation involving the cleaning of the small intestine prior to an examination would improve the quality of the endoscopic view and, in turn, the diagnostic yield of the technique. Thus, proposals were put forward based on the preparations carried out for other types of exploration, such as colonoscopy[2]. The application of such preparations has led to undesired effects such as the prolongation of gastric and/or intestinal transit time and a consequential rise in the proportion of incomplete examinations. To avoid such a negative outcome, trials have been carried out with prokinetics such as erythromycin, but achieved little effect[3,4]. In another prospective study, in which the prokinetic agent metoclopramide (10 mg) was administered orally 15 min before the capsule was swallowed, the percentage of complete studies was greater[5]. An extended transit time represents a less problem for the new generations of capsules whose batteries last longer. However, the use of metoclopramide could still offer advantages in diabetic patients and those confined to the bed, in whom intestinal transit time is usually longer, insuring that the capsule photographs the entire small intestine. Similarly, a better view of the proximal intestine has been described following administration of 300 mg simethicone 20 min before capsule endoscopy[6,7].

With respect to the preparation of the intestine before an examination, Viazis *et al*[8] carried out a prospective study of 80 patients to whom 2 L polyethylene glycol-based electrolyte solution (PEG) *vs* clear liquids was randomly administrated during the entire day prior to the procedure. No effect on the gastric or intestinal

transit of the endoscopic capsule was detected, though there was a higher level of cleanliness among those patients who received the aforementioned preparation, with a statistically significant improvement in the diagnostic yield. Niv *et al*[9] published a retrospective analysis of the use of oral sodium phosphate in 46 *vs* 23 patients prepared only with overnight fasting. The authors recommended the preparation of oral sodium phosphate after observing a higher level of cleanliness. However, it must be said that their study was retrospective and based on a relatively small series of patients whose results do not warrant a generalisation of the use of said preparation. Similarly, Dai *et al*[10] compared the levels of cleanliness obtained after ingestion of a 4 L PEG solution *vs* a 12 h period of fasting. They observed an acceleration of intestinal transit in patients receiving the PEG preparation, and a better view during the examination as a result of a greater intestinal cleanliness.

Ben-Soussan *et al*[11] did not observe a difference between a preparation of 2 L PEG solution and 12 h fasting with respect to the results achieved. Indeed, they reported that the PEG preparation increased the time of gastric emptying, which does not favour a complete small bowel examination.

Some studies have demonstrated that bowel preparation has a negative influence on gastric emptying and intestinal transit time, though there is a lack of uniformity among their results. Moreover, there is also evidence for the absence of any influence over these parameters. In an attempt to establish some common guidelines, different proposals have been made regarding the best procedure to follow. In this way, a review of the related literature by de Franchis *et al*[12] drew attention to the fact that studies on the subject are scarce and provide inconsistent results. Their analysis highlighted the lack of uniformity both in the methodology of the studies and in the results obtained, and considered it was necessary to develop a large, multi-centre, random, prospective study that would confirm, definitively, the best procedure to follow with respect to preparation prior to capsule endoscopy.

We must not ignore the disadvantages of all types of preparation for cleaning the intestine prior to capsule endoscopy, given the low tolerance of patients to such procedures and the often consequential rejection of a test that, without said interference, would normally be perfectly tolerated. On the other hand, there are no validated scales that allow us to accurately quantify and compare the levels of intestinal cleanliness. This makes any comparison of results difficult and can often invalidate them.

Results such as those of Pons *et al*[13] and Lapalus *et al*[14] tilt the balance in favour of no prior preparation. Lapalus *et al*[14] compared the administration of aqueous sodium phosphate (ASP) *vs* clear liquids and did not observe any difference in the level of cleanliness or in visibility.

The results of the aforementioned Spanish group[13] are a fruit of a multi-centre, random, prospective study that compared the efficacy and tolerability of three different preparations applied prior to capsule endoscopy in a large sample of patients. Observers were blind to the type of preparation employed. The 291 patients included in the study were randomly divided into three groups. All were fasted for 10 h prior to capsule endoscopy. Group A received a liquid diet (CL) (4 L) that excluded strongly-coloured liquids and those with residues. Group B received 90 mL of ASP as part of an abundant diet of liquids. Group C received a solution of 4 L PEG. The examinations were evaluated globally according to four categories: poor, fair, good, and excellent (Table 1). No statistically significant difference was found between the qualities of the examinations following the three preparations. The CL preparation was the best tolerated of the three preparations, followed by that of PEG and, finally, that of ASP ($P < 0.001$). The type of preparation did not influence the diagnostic yield of capsule endoscopy. Next, 32 examinations were randomly selected and re-evaluated by two researchers who were blind to the first result of a medium concordance between the first and second results (kappa 0.45).

Table 1 Score used to evaluate the level of intestinal cleanliness

Categories	Evaluate the level of intestinal cleanliness
Poor	Intestinal content impeding evaluation
Fair	Liquid or solid intestinal content allowing evaluation
Good	No intestinal content or some located in the terminal ileum and/or cecum
Excellent	No intestinal content in any part of the small intestinal tract (including ileum) or the cecum

This is the first study (currently being prepared for publication) that resolved the problem of preparation for capsule endoscopy. Based on the results, and pending verification by other studies, we can affirm that a liquid diet during the day prior to administration of the endoscopic capsule, together with fasting, constitutes a sufficient preparation for achieving a good level of cleanliness in the small intestine. Furthermore, this procedure is well tolerated and, thus, better accepted by the patient.

However, if in the future a product is developed that is capable of maintaining an adequate level of cleanliness in both the small intestine and colon, and which is also well tolerated by the patient, the recommended protocol for carrying out these diagnostic tests will no doubt be modified in order to incorporate the said product, not only in the case of capsule endoscopy but also in colonoscopy, in which preparation continues to be a stumbling block.

REFERENCES

1 **Rondonotti E**, Herrerias JM, Pennazio M, Caunedo A, Mascarenhas-Saraiva M, de Franchis R. Complications, limitations, and failures of capsule endoscopy: a review of 733 cases. *Gastrointest Endosc* 2005; **62**: 712-716; quiz 752, 754
2 **Burke CA**, Church JM. Enhancing the quality of colonoscopy: the importance of bowel purgatives. *Gastrointest Endosc* 2007; **66**: 565-573
3 **Fireman Z**, Paz D, Kopelman Y. Capsule endoscopy:

improving transit time and image view. *World J Gastroenterol* 2005; **11**: 5863-5866
4 **Caddy GR**, Moran L, Chong AK, Miller AM, Taylor AC, Desmond PV. The effect of erythromycin on video capsule endoscopy intestinal-transit time. *Gastrointest Endosc* 2006; **63**: 262-266
5 **Selby W**. Complete small-bowel transit in patients undergoing capsule endoscopy: determining factors and improvement with metoclopramide. *Gastrointest Endosc* 2005; **61**: 80-85
6 **Ge ZZ**, Chen HY, Gao YJ, Hu YB, Xiao SD. The role of simeticone in small-bowel preparation for capsule endoscopy. *Endoscopy* 2006; **38**: 836-840
7 **Albert J**, Gobel CM, Lesske J, Lotterer E, Nietsch H, Fleig WE. Simethicone for small bowel preparation for capsule endoscopy: a systematic, single-blinded, controlled study. *Gastrointest Endosc* 2004; **59**: 487-491
8 **Viazis N**, Sgouros S, Papaxoinis K, Vlachogiannakos J, Bergele C, Sklavos P, Panani A, Avgerinos A. Bowel preparation increases the diagnostic yield of capsule endoscopy: a prospective, randomized, controlled study. *Gastrointest Endosc* 2004; **60**: 534-538
9 **Niv Y**, Niv G, Wiser K, Demarco DC. Capsule endoscopy - comparison of two strategies of bowel preparation. *Aliment Pharmacol Ther* 2005; **22**: 957-962
10 **Dai N**, Gubler C, Hengstler P, Meyenberger C, Bauerfeind P. Improved capsule endoscopy after bowel preparation. *Gastrointest Endosc* 2005; **61**: 28-31
11 **Ben-Soussan E**, Savoye G, Antonietti M, Ramirez S, Ducrotte P, Lerebours E. Is a 2-liter PEG preparation useful before capsule endoscopy? *J Clin Gastroenterol* 2005; **39**: 381-384
12 **de Franchis R**, Avgerinos A, Barkin J, Cave D, Filoche B. ICCE consensus for bowel preparation and prokinetics. *Endoscopy* 2005; **37**: 1040-1045
13 Pons V, Gonzalez B, Gonzalez C, Perez-Cuadrado E, Fernandez-Diez S, Fernandez-Urien I, Mata A, Espinos J, Perez-Grueso MJ, Arguello L, Valle J, Carballo F, Munoz-Navas M, Llach J, Ramirez-Armengol JA, Balanzo J, Sala T, Menchen P. Evaluation of Different Bowel Preparations for the Study with Capsule Endoscopy: A Prospective, Randomized, Controlled Study. *Gastrointest Endosc* 2006; **63**: AB161
14 **Lapalus MG**, Ben Soussan E, Saurin JC, Favre O, D'Halluin PN, Coumaros D, Gaudric M, Fumex F, Antonietti M, Gaudin JL, Jacob P, Heresbach D, Pilichos C, Fan R, Mozer M, Heyries L, Dumortier J, Ponchon T. Capsule endoscopy and bowel preparation with oral sodium phosphate: a prospective randomized controlled trial. *Gastrointest Endosc* 2008; **67**: 1091-1096

S- Editor Li DL **L- Editor** Wang XL **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2008 October 7; 14(37): 5776
World Journal of Gastroenterology ISSN 1007-9327

ACKNOWLEDGMENTS

Acknowledgments to Reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

Dr. Sk Md Fazle Akbar, Assistant Professor
Third Department of Internal Medicine, Ehime University School of Medicine, Shigenobu-Cho, Ehime 791-0295, Japan

Bettaieb Ali, PhD, Professor
Immunology and Immunotherapy of Cancer, INSERM U517, 7 Bd Jeanne d'Arc, Dijon 21079, France

Ramsey Chi-man Cheung, MD, Professor
Division of GI & Hepatology, VAPAHCS(154C), 3801 Miranda Ave, Stanford University School of Medicine, Palo Alto, CA 94304,
United States

Dr. Adrian G Cummins
Department of Gastroenterology and Hepatology, (DX 465384), 28 Woodville Road, Woodville South, 5011, South Australia, Australia

Dr. Olivier Detry
Department of Abdominal Surgery and Transplantation, University of Liège, CHU Sart Tilman B35, B-4000 Liège, Belgium

Dr. William Dickey
Altnagelvin Hospital, Londonderry, BT47 6SB, Northern Ireland, United Kingdom

Valentin Fuhrmann, MD
Department of Internal Medicine 4, Intensive Care Unit, Medical University Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria

Nikolaus Gassler, Professor
Institute of Pathology, University Hospital RWTH Aachen, Pauwelsstrasse 30, 52074 Aachen, Germany

Jin Gu, Professor
Peking University School of Oncology, Beijing Cancer Hospital, Beijing 100036, China

Aydin Karabacakoglu, Dr, Assistant Professor
Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey

Peter L Lakatos, MD, PhD, Assistant Professor
1st Department of Medicine, Semmelweis University, Koranyi S 2A, Budapest H1083, Hungary

Robin G Lorenz, Associate Professor
Department of Pathology, University of Alabama at Birmingham, 845 19th Street South BBRB 730, Birmingham, AL 35294-2170, United States

Peter J Mannon, MD
Mucosal Immunity Section, Laboratory of Host Defense, National Institute of Allergy, Laboratory of Clinical Investigation, Building 10/CRC, Room 6-3742, 9000 Rockville Pike, Bethesda, Maryland 20892, United States

Javier San Martín, Chief
Gastroenterology and Endoscopy, Sanatorio Cantegril, Av. Roosevelt y P 13, Punta del Este 20100, Uruguay

Dr. Lynne V McFarland
Department of Health Services Research and Development, Puget Sound VA, 1100 Olive Street, Suite #1400, Seattle, Washington, WA 98101, United States

Stephan Menne, Assistant Professor of Virology
Department of Clinical Sciences/GI Unit, College of Veterinary Medicine, Cornell University , C2-005 Veterinary Medical Center, Ithaca, NY 14853, United States

Giuseppe Montalto, Professor
Medicina Clinica e delle Patologie Emergenti, University of Palermo, via del Vespro, 141, Palermo 90100, Italy

Francis Seow-Choen, Professor
Seow-Choen Colorectal Centre, Mt Elizabeth Medical Centre, Singapore, 3 Mt Elizabeth Medical Centre #09-10 , 228510, Singapore

Shivendra Shukla, Professor
Department of Medical Pharmacology and Physiology, University of Missouri School of Medicine, 1 Hospital Drive, M530 Medical Sciences Bldg., Columbia MO 65212, United States

Seyed A Taghavi, Associate Professor
Department of Internal Medicine, Nemazee Hospital, No.23, 59th Alley, Ghasrodasht St., Shiraz 71838-95453, Iran

Emmanuel Tiret, Professor
Centre de Chirurgie Digestive, Hôpital Saint-Antoine, 184 rue du faubourg Saint-Antoine, Paris 75012, France

Trotter James F Trotter, MD, Associate Professor
University of Colorado, Division of Gastroenterology, 4200 E. 9th Avenue, b-154, Denver, CO 80262, United States

Akihito Tsubota, Assistant Professor
Institute of Clinical Medicine and Research , Jikei University School of Medicine, 163-1 Kashiwa-shita, Kashiwa, Chiba 277-8567, Japan

Takayuki Yamamoto, MD
Inflammatory Bowel Disease Center, Yokkaichi Social Insurance Hospital, 10-8 Hazuyamacho, Yokkaichi 510-0016, Japan

World J Gastroenterol 2008 October 7; 14(37): 5777
World Journal of Gastroenterology ISSN 1007-9327

Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
January 24-25, Frankfurt, Germany
Falk Workshop: Perspectives in Liver
Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
E-mail: education@asge.org

June 4-7, Helsinki, Finland
The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
ESGAR 2008 19th Annual Meeting and Postgraduate Course
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden
16th International Congress of the European Association for Endoscopic Surgery
E-mail: info@eaes-eur.org

June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
10th World Congress on Gastrointestinal Cancer
Imedex and ESMO
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland
Joint Meeting of the European Pancreatic Club (EPC)
and the International Association of Pancreatology (IAP)
E-mail: office@epc-iap2008.org
www.e-p-c.org
www.pancreatology.org

June 26-28, Bratislava, Slovakia
5th Central European Gastroenterology Meeting
www.ceurgem2008.cz

July 9-12, Paris, France
ILTS 14th Annual International Congress
www.ilts.org

September 10-13, Budapest, Hungary
11th World Congress of the International Society for Diseases of the Esophagus
E-mail: isde@isde.net

September 13-16, New Delhi, India
Asia Pacific Digestive Week
E-mail: apdw@apdw2008.net

III FALK GASTRO-CONFERENCE
September 17, Mainz, Germany
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
Falk Symposium 166:
GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
Falk Symposium 167:
Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
Third Annual Meeting
European Society of Coloproctology
www.escp.eu.com

October 8-11, Istanbul, Turkey
18th World Congress of the International Association of Surgeons,
Gastroenterologists and Oncologists
E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria
16th United European Gastroenterology Week
www.negf.org
www.acv.at

October 22-25, Minnesota, USA
Anstralian Gastroenterology Week 2008
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia
71st Annual Colon and Rectal Surgery Conference
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA
59th AASLD Annual Meeting and Postgraduate Course
The Liver Meeting
Information: www.aasld.org

November 6-9, Lucerne, Switzerland
Neurogastroenterology & Motility Joint International Meeting 2008
E-mail: ngm2008@mci-group.com
www.ngm2008.com

November 12, Santiago de Chile, Chile
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt
1st Hepatology and Gastroenterology Post Graduate Course
www.egyptgastrohep.com

December 7-9, Seoul, Korea
6th International Meeting
Hepatocellular Carcinoma: Eastern and Western Experiences
E-mail: sglee@amc.seoul.kr

INFORMATION FOR ALL
FALK FOUNDATION e.V.
E-mail: symposia@falkfoundation.de
www.falkfoundation.de

Advanced Courses - European Institute of Telesurgery EITS - 2008
Strasbourg, France
January 18-19, March 28-29, June 6-7, October 3-4

N.O.T.E.S
April 3-5, November 27-29
Laparoscopic Digestive Surgery

June 27-28, November 7-8
Laparoscopic Colorectal Surgery

July 3-5
Interventional GI Endoscopy Techniques
Contact address for all courses:
E-mail: info@eits.fr

International Gastroenterological Congresses 2009
March 23-26, Glasgow, Scotland
Meeting of the British Society of Gastroenterology (BSG)
E-mail: bsg@mailbox.ulcc.ac.uk

May 17-20, Denver, Colorado, USA
Digestive Disease Week 2009

November 21-25, London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2008 October 7; 14(37): 5778-5780
World Journal of Gastroenterology ISSN 1007-9327

Instructions to authors

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol* ISSN 1007-9327 CN 14-1219/R) is a weekly open access peer-reviewed journal supported by an editorial board consisting of 1208 experts in gastroenterology and hepatology from 60 countries. The aim of the journal is to deliver the most clinically relevant original and commentary articles to readers, and to make the full text publicly available to all clinicians, scientists, patients and biomedical students on an unrestricted platform, so that they can access and learn about the most recent key advances in the field.

In addition to the open access nature, another key characteristic of *WJG* is its reading guidance for each article which includes background, research frontier, related reports, breakthroughs, applications, terminology, and comments of peer reviewers for the general readers.

WJG publishes articles on esophageal, gastrointestinal, hepatobiliary and pancreatic tumors, and other esophageal, gastrointestinal, hepatic-biliary and pancreatic diseases in relation to epidermiology, immunology, microbiology, motility & nerve-gut interaction, endocrinology, nutrition & obesity, endoscopy, imaging and advanced hi-technology.

The main goal of *WJG* is to publish high quality commentary articles contributed by leading experts in gastroenterology and hepatology and original articles that combine the clinical practice and advanced basic research, to provide an interactive platform for clinicians and researchers in internal medicine, surgery, infectious diseases, traditional Chinese medicine, oncology, integrated Chinese and Western medicine, imaging, endoscopy, interventional therapy, pathology and other basic medical specialities, and thus eventually improving the clinical practice and healthcare for patients.

Indexed and abstracted in

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, Index Medicus, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology* and *Hepatology*, CAB Abstracts and Global Health. ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

Published by

The WJG Press

SUBMISSION OF MANUSCRIPTS

Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of The WJG Press, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the International Committee of Medical Journal Editors to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now available, to our knowledge, is http://www. clinicaltrials.gov sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

Authors should retain one copy of the text, tables, photographs and illustrations because rejected manuscripts will not be returned to the author(s) and the editors will not be responsible for loss or damage to photographs and illustrations sustained during mailing.

Online submissions

Manuscripts should be submitted through the Online Submission System at: http://wjg.wjgnet.com/wjg. Authors are highly recommended to consult the ONLINE INSTRUCTIONS TO AUTHORS (http://www.wjgnet.com/wjg/help/instructions.jsp) before attempting to submit online. For assistance, authors encountering problems with the Online Submission System may send an email describing the problem to submission@wjgnet.com, or by telephone: +86-10-85381892. If you submit your manuscript online, do not make a postal contribution. Repeated online submission for the same manuscript is strictly prohibited.

MANUSCRIPT PREPARATION

All contributions should be written in English. All articles must be submitted using word-processing software. All submissions must be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Style should conform to our house format. Required information for each of the manuscript sections is as follows:

Title page

Full manuscript title, running title, all author(s) name(s), affiliations, institution(s) and/or department(s) where the work was carried out; author contributions; disclosure of any financial support for the research; and the name, full address, telephone and fax numbers and email address of the corresponding author should be included. Titles should be concise and informative (remove all unnecessary words), emphasize what is new, and avoid abbreviations. A short running title of less than 40 letters should be provided. List the author(s)' name(s) as follows: initial and/or first name, middle name or initial(s), and full family name.

Author controbutions: The format of this section should be like this: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed research; Wang CL, Zou CC, Hong F and Wu XM performed research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed data; and Wang CL, Liang L and Fu JF wrote the paper.

Peer reviewers: All articles received are subject to peer review. Normally, three experts are invited for each article. Decision for acceptance is made only when at least two experts recommend an article for publication. Reviewers for accepted manuscripts are acknowledged in each manuscript, and reviewers of articles which were not accepted will be acknowledged at the end of each issue. To ensure the quality of the articles published in *WJG*, reviewers of accepted manuscripts will be announced by publishing the name, title/position and institution of the reviewer in the footnote accompanying the printed article. For example, reviewers: Professor Jing-Yuan Fang, Shanghai Institute of Digestive Disease, Shanghai, Affiliated Renji Hospital, Medical Faculty, Shanghai Jiaotong University, Shanghai, China; Professor Xin-Wei Han, Department of Radiology, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan Province, China; and Professor Anren Kuang, Department of Nuclear Medicine, Huaxi Hospital, Sichuan University, Chengdu, Sichuan Province, China.

Abstract

An informative, structured abstract of no more than 350 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections: AIM: Only the purpose should be included. METHODS: The materials, techniques, instruments and equipment, and the experimental procedures should be included. RESULTS: The observed and experimental results, including data, effects, outcome, *etc.* should be included. Authors should present *P* value where necessary, and also include any significant data. CONCLUSION: Accurate view and the value of the results should be included.

The format for structured abstracts can be found at: http://www.wjgnet.com/wjg/help/11.doc.

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles, rapid communication

and case reports, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the body text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, should be found at: http://www.wjgnet.com/wjg/help/instructions.jsp.

Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: …*etc.* It is our principle to publish high resolution-figures for the printed and E-versions.

Tables

Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of P values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscripts and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

Coding system

The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability[1,2]". If references are cited directly in the text, they should be put together within the text, for example, "From references[19,22-24], we know that..."

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

PMID requirement

PMID roots in the abstract serial number indexed by PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed). The author should supply the PMID for journal citation. For those references that have not been indexed by PubMed, a printed copy of the first page of the full reference should be submitted.

The accuracy of the information for journal citations is very important. Using the reference testing system, the authors and editor should check the authors name, title, journal title, publication date, volume number, start page, and end page. We will interlink all references with PubMed in an ASP file so that the readers can immediately access the abstract of the citations online.

DOI requirement

A CrossRef DOI® (Digital Object Identifier) name is a unique string created to identify a piece of scholarly content in the online environment. The author should supply the DOIs for journal citation(doi:10.3748/wjg.13.6458). This link (http://www.crossref.org/SimpleTextQuery/) allows you to retrieve Digital Object Identifiers (DOIs) for journal articles, books, and chapters by simply cutting and pasting the reference list into the box. You may use the form with any reference style, although the tool works most reliably if references are formatted in a standard style such as shown in this example: Assimakopoulos SF, Scopa CD, Vagianos CE. Pathophysiology of increased intestinal permeability in obstructive jaundice. *World J Gastroenterol* 2007; 13(48): 6458-6464

The accuracy of the information of journal citations is very important. We will interlink all references with DOI in ASP file so that readers can access the abstracts of cited articles online immediately.

Style for journal references

Authors: the name of the first author should be typed in bold-faced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

Style for book references

Authors: the name of the first author should be typed in bold-faced letters. The surname of all authors should be typed with the initial letter capitalized, followed by their abbreviated middle and first initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR) Book title. Publication number. Publication place: Publication press, Year: start page and end page.

Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Inappropriate references

Authors should always cite references that are relevant to their article, and avoid any inappropriate references. Inappropriate references include those linked with a hyphen when the difference between the two numbers is greater than five. For example, [1-6], [2-14] and [1, 3, 4-10, 22] are all considered inappropriate references. Authors should not cite their own unrelated published articles.

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/wjg/help/15.doc.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H pylori*, *E coli*, *etc.*

SUBMISSION OF THE REVISED MANUSCRIPTS AFTER ACCEPTED

Please revise your article according to the revision policies of *WJG*. The revised version including manuscript and high-resolution image figures (if any) should be copied on a floppy or compact disk. The author should send the revised manuscript, along with printed high-resolution color or black and white photos, copyright transfer letter, and responses to the reviewers by courier (such as EMS/DHL).

Editorial Office

World Journal of Gastroenterology
Editorial Department: Room 903
Ocean International Center, Building D
No. 62 Dongsihuan Zhonglu
Chaoyang District, Beijing 100025, China
E-mail: wjg@wjgnet.com
http://www.wjgnet.com
Telephone: +86-10-59080039
Fax: +86-10-85381893

Language evaluation

The language of a manuscript will be graded before it is sent for revision. (1) Grade A: priority publishing; (2) Grade B: minor language polishing; (3) Grade C: a great deal of language polishing needed; (4) Grade D: rejected. Revised articles should reach Grade A or B.

Copyright assignment form

Please download a Copyright assignment form from http://www.wjgnet.com/wjg/help/9.doc.

Responses to reviewers

Please revise your article according to the comments/suggestions provided by the reviewers. The format for responses to the reviewers' comments can be found at: http://www.wjgnet.com/wjg/help/10.doc.

Proof of financial support

For paper supported by a foundation, authors should provide a copy of the document and serial number of the foundation.

Links to documents related to the manuscript

WJG will be initiating a platform to promote dynamic interactions between the editors, peer reviewers, readers and authors. After a manuscript is published online, links to the PDF version of the submitted manuscript, the peer-reviewers' report and the revised manuscript will be put on-line. Readers can make comments on the peer reviewer's report, authors' responses to peer reviewers, and the revised manuscript. We hope that authors will benefit from this feedback and be able to revise the manuscript accordingly in a timely manner.

Science news releases

Authors of accepted manuscripts are suggested to write a science news item to promote their articles. The news will be released rapidly at EurekAlert/AAAS (http://www.eurekalert.org). The title for news items should be less than 90 characters; the summary should be less than 75 words; and main body less than 500 words. Science news items should be lawful, ethical, and strictly based on your original content with an attractive title and interesting pictures.

Publication fee

Authors of accepted articles must pay a publication fee.
EDITORIAL, TOPIC HIGHLIGHTS, BOOK REVIEWS and LETTERS TO THE EDITOR are published free of charge.

ISSN 1007-9327
CN 14-1219/R

WJG

World Journal of Gastroenterology®

Indexed and Abstracted in:

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, *Index Medicus*, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology and Hepatology*, CAB Abstracts and Global Health.
ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

Volume 14 Number 38
October 14, 2008
World J Gastroenterol
2008 October 14; 14(38): 5781-5944

Online Submissions
wjg.wjgnet.com
www.wjgnet.com
Printed on Acid-free Paper
世界胃肠病学杂志

A Weekly Journal of Gastroenterology and Hepatology

World Journal of Gastroenterology®

Editorial Board

2007-2009

Published by The WJG Press and Baishideng
Room 903, Ocean International Center, Building D
No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Fax: +86-10-8538-1893 E-mail: wjg@wjgnet.com http://www.wjgnet.com

The World Journal of Gastroenterology Editorial Board consists of 1208 members, representing a team of worldwide experts in gastroenterology and hepatology. They are from 60 countries, including Albania (1), Argentina (4), Australia (39), Austria (10), Belarus (1), Belgium (15), Brazil (2), Bulgaria (1), Canada (28), Chile (1), China (60), Croatia (2), Cuba (1), Czech (2), Denmark (7), Egypt (4), Estonia (1), Finland (4), France (44), Germany (108), Greece (9), Hungary (2), Iceland (1), India (12), Iran (3), Ireland (4), Israel (8), Italy (96), Japan (176), Lebanon (3), Lithuania (1), Macedonia (1), Malaysia (3), Mexico (6), Monaco (1), Morocco (1), The Netherlands (26), New Zealand (1), Nigeria (1), Norway (3), Pakistan (2), Peru (1), Poland (6), Portugal (1), Russia (3), Saudi Arabia (2), Serbia (1), Singapore (4), Slovakia (2), Slovenia (1), South Africa (2), South Korea (14), Spain (38), Sweden (15), Switzerland (13), Turkey (8), United Arab Emirates (1), United Kingdom (83), United States (316) and Uruguay (2).

Andrew V Biankin, *Sydney*
Filip Braet, *Sydney*
Andrew D Clouston, *Sydney*
Graham Cooksley, *Queensland*
Darrell HG Crawford, *Brisbane*
Adrian G Cummins, *Woodville South*
Guy D Eslick, *Sydney*
Michael A Fink, *Melbourne*
Robert JL Fraser, *Daw Park*
Peter Raymond Gibson, *Victoria*
Jacob George, *Westmead*
Mark D Gorrell, *Sydney*
Yik-Hong Ho, *Townsville*
Gerald J Holtmann, *Adelaide*
Michael Horowitz, *Adelaide*
John E Kellow, *Sydney*
Rupert Leong, *Concord*
Geoffrey W McCaughan, *Sydney*
Finlay A Macrae, *Victoria*
Daniel Markovich, *Brisbane*
Phillip S Oates, *Perth*
Jacqui Richmond, *Victoria*
Stephen M Riordan, *Sydney*
Ian C Roberts-Thomson, *Adelaide*
Devanshi Seth, *Camperdown*
Arthur Shulkes, *Melbourne*
Ross C Smith, *Sydney*
Kevin J Spring, *Brisbane*
Huy A Tran, *New South Wales*
Debbie Trinder, *Fremantle*
Martin J Veysey, *Gosford*
Daniel L Worthley, *Bedford*

Austria

Peter Ferenci, *Vienna*
Valentin Fuhrmann, *Vienna*
Alfred Gangl, *Vienna*
Christoph Gasche, *Vienna*
Kurt Lenz, *Linz*
Markus Peck-Radosavljevic, *Vienna*
Rudolf E Stauber, *Auenbruggerplatz*
Herbert Tilg, *Innsbruck*
Michael Trauner, *Graz*
Harald Vogelsang, *Vienna*
Guenter Weiss, *Innsbruck*

Belarus

Yury K Marakhouski, *Minsk*

Belgium

Rudi Beyaert, *Gent*
Bart Rik De Geest, *Leuven*
Inge I Depoortere, *Leuven*
Olivier Detry, *Liège*
Benedicte Y De Winter, *Antwerp*
Karel Geboes, *Leuven*
Thierry Gustot, *Brussels*
Yves J Horsmans, *Brussels*
Geert G Leroux-Roels, *Ghent*
Louis Libbrecht, *Leuven*
Etienne M Sokal, *Brussels*
Marc Peeters, *De Pintelaan*
Gert A Van Assche, *Leuven*
Yvan Vandenplas, *Brussels*
Eddie Wisse, *Keerbergen*

Brazil

Heitor Rosa, *Goiania*
Ana Cristina Simões e Silva, *Belo Horizonte*

Bulgaria

Zahariy Krastev, *Sofia*

Canada

Fernando Alvarez, *Québec*
David Armstrong, *Ontario*
Jeffrey P Baker, *Toronto*
Olivier Barbier, *Québec*
Nancy Baxter, *Toronto*
Matthew Bjerknes, *Toronto*
Frank J Burczynski, *Manitoba*
Michael F Byrne, *Vancouver*
Wang-Xue Chen, *Ottawa*
Chantal Guillemette, *Québec*
Samuel S Lee, *Calgary*
Gary A Levy, *Toronto*
Andrew L Mason, *Alberta*
John K Marshall, *Ontario*
Donna-Marie McCafferty, *Calgary*
Thomas I Michalak, *St. John's*
Gerald Y Minuk, *Manitoba*
Paul Moayyedi, *Hamilton*
Kostas Pantopoulos, *Québec*
William G Paterson, *Kingston*
Eldon Shaffer, *Calgary*
Morris Sherman, *Toronto*
Martin Storr, *Calgary*
Elena F Verdu, *Ontario*
John L Wallace, *Calgary*
Eric M Yoshida, *Vancouver*

Chile

Silvana Zanlungo, *Santiago*

China

Henry LY Chan, *Hongkong*
Xiao-Ping Chen, *Wuhan*
Zong-Jie Cui, *Beijing*
Da-Jun Deng, *Beijing*
Er-Dan Dong, *Beijing*
Sheung-Tat Fan, *Hong Kong*
Jin Gu, *Beijing*
Xin-Yuan Guan, *Pokfulam*
De-Wu Han, *Taiyuan*
Ming-Liang He, *Hong Kong*
Wayne HC Hu, *Hong Kong*
Chee-Kin Hui, *Hong Kong*
Ching-Lung Lai, *Hong Kong*
Kam Chuen Lai, *Hong Kong*
James YW Lau, *Hong Kong*
Yuk-Tong Lee, *Hong Kong*
Suet-Yi Leung, *Hong Kong*
Wai-Keung Leung, *Hong Kong*
John M Luk, *Pokfulam*
Chung-Mau Lo, *Hong Kong*
Jing-Yun Ma, *Beijing*
Ronnie Tung Ping Poon, *Hong Kong*
Lun-Xiu Qin, *Shanghai*
Yu-Gang Song, *Guangzhou*
Qin Su, *Beijing*
Wai-Man Wong, *Hong Kong*
Hong Xiao, *Shanghai*
Dong-Liang Yang, *Wuhan*
Winnie Yeo, *Hong Kong*
Yuan Yuan, *Shenyang*
Man-Fung Yuen, *Hong Kong*
Jian-Zhong Zhang, *Beijing*
Xin-Xin Zhang, *Shanghai*
Bo-Jian Zheng, *Hong Kong*
Shu Zheng, *Hangzhou*

Croatia

Tamara Cacev, *Zagreb*
Marko Duvnjak, *Zagreb*

Cuba

Damian C Rodriguez, *Havana*

Czech

Milan Jirsa, *Praha*
Pavel Trunečka, *Prague*

Denmark

Peter Bytzer, *Copenhagen*
Asbjørn M Drewes, *Aalborg*
Hans Gregersen, *Aalborg*
Jens H Henriksen, *Hvidovre*
Claus P Hovendal, *Odense*
Fin S Larsen, *Copenhagen*
Søren Møller, *Hvidovre*

Egypt

Abdel-Rahman El-Zayadi, *Giza*
Amr M Helmy, *Cairo*
Ayman Yosry, *Cairo*

Estonia

Riina Salupere, *Tartu*

Finland

Irma E Jarvela, *Helsinki*
Katri M Kaukinen, *Tampere*
Minna Nyström, *Helsinki*
Pentti Sipponen, *Espoo*

France

Bettaieb Ali, *Dijon*
Corlu Anne, *Rennes*
Denis Ardid, *Clermont-Ferrand*
Charles P Balabaud, *Bordeaux*
Soumeya Bekri, *Rouen*
Jacques Belghiti, *Clichy*
Jacques Bernuau, *Clichy Cedex*
Pierre Brissot, *Rennes*
Patrice P Cacoub, *Paris*
Franck Carbonnel, *Besancon*
Laurent Castera, *Pessac*
Bruno Clément, *Rennes*
Benoit Coffin, *Colombes*
Jacques Cosnes, *Paris*
Thomas Decaens, *Cedex*

Francoise L Fabiani, *Angers*
Gérard Feldmann, *Paris*
Jean Fioramonti, *Toulouse*
Jean-Noël Freund, *Strasbourg*
Jean-Paul Galmiche, *Nantes*
Catherine Guettier, *Villejuif*
Chantal Housset, *Paris*
Juan L Iovanna, *Marseille*
Rene Lambert, *Lyon*
Patrick Marcellin, *Paris*
Philippe Mathurin, *Lille*
Tamara Matysiak–Budnik, *Paris*
Francis Mégraud, *Bordeaux*
Richard Moreau, *Clichy*
Thierry Piche, *Nice*
Raoul Poupon, *Paris*
Jean Rosenbaum, *Bordeaux*
Dominique Marie Roulot, *Bobigny*
Thierry Poynard, *Paris*
Jean-Philippe Salier, *Rouen*
Didier Samuel, *Villejuif*
Jean-Yves Scoazec, *Lyon*
Khalid A Tazi, *Clichy*
Emmanuel Tiret, *Paris*
Baumert F Thomas, *Strasbourg*
Marie-Catherine Vozenin-brotons, *Villejuif*
Jean-Pierre H Zarski, *Grenoble*
Jessica Zucman-Rossi, *Paris*

Germany

Hans-Dieter Allescher, *G-Partenkirchen*
Martin Anlauf, *Kiel*
Rudolf Arnold, *Marburg*
Max G Bachem, *Ulm*
Thomas F Baumert, *Freiburg*
Daniel C Baumgart, *Berlin*
Hubert Blum, *Freiburg*
Thomas Bock, *Tuebingen*
Katja Breitkopf, *Mannheim*
Dunja Bruder, *Braunschweig*
Markus W Büchler, *Heidelberg*
Christa Buechler, *Regensburg*
Reinhard Buettner, *Bonn*
Elke Cario, *Essen*
Uta Dahmen, *Essen*
Christoph F Dietrich, *Bad Mergentheim*
Arno J Dormann, *Koeln*
Rainer J Duchmann, *Berlin*
Volker F Eckardt, *Wiesbaden*
Paul Enck, *Tuebingen*
Fred Fändrich, *Kiel*
Ulrich R Fölsch, *Kiel*
Helmut Friess, *Heidelberg*
Peter R Galle, *Mainz*
Nikolaus Gassler, *Aachen*
Andreas Geier, *Aachen*
Markus Gerhard, *Munich*
Wolfram H Gerlich, *Giessen*
Dieter Glebe, *Giessen*
Burkhard Göke, *Munich*
Florian Graepler, *Tuebingen*
Axel M Gressner, *Aachen*
Veit Gülberg, *Munich*
Rainer Haas, *Munich*
Eckhart G Hahn, *Erlangen*
Stephan Hellmig, *Kiel*
Martin Hennenberg, *Bonn*
Johannes Herkel, *Hamburg*
Klaus R Herrlinger, *Stuttgart*
Eva Herrmann, *Homburg/Saar*
Eberhard Hildt, *Berlin*
Joerg C Hoffmann, *Berlin*
Ferdinand Hofstaedter, *Regensburg*
Werner Hohenberger, *Erlangen*
Jörg C Kalff, *Bonn*
Ralf Jakobs, *Ludwigshafen*
Jutta Keller, *Hamburg*
Andrej Khandoga, *Munich*
Sibylle Koletzko, *München*
Stefan Kubicka, *Hannover*
Joachim Labenz, *Siegen*
Frank Lammert, *Bonn*
Thomas Langmann, *Regensburg*
Christian Liedtke, *Aachen*
Matthias Löhr, *Mannheim*
Christian Maaser, *Muenster*
Ahmed Madisch, *Dresden*
Peter Malfertheiner, *Magdeburg*
Michael P Manns, *Hannover*
Helmut Messmann, *Augsburg*
Stephan Miehlke, *Dresden*
Sabine Mihm, *Göttingen*
Silvio Nadalin, *Essen*
Markus F Neurath, *Mainz*
Johann Ockenga, *Berlin*
Florian Obermeier, *Regensburg*
Gustav Paumgartner, *Munich*
Ulrich KS Peitz, *Magdeburg*
Markus Reiser, *Bochum*
Emil C Reisinger, *Rostock*
Steffen Rickes, *Magdeburg*
Tilman Sauerbruch, *Bonn*
Dieter Saur, *Munich*
Hans Scherubl, *Berlin*
Joerg Schirra, *Munich*
Roland M Schmid, *München*
Volker Schmitz, *Bonn*
Andreas G Schreyer, *Regensburg*
Tobias Schroeder, *Essen*
Henning Schulze-Bergkamen, *Mainz*
Hans Seifert, *Oldenburg*
Norbert Senninger, *Muenster*
Manfred V Singer, *Mannheim*
Gisela Sparmann, *Rostock*
Christian J Steib, *München*
Jurgen M Stein, *Frankfurt*
Ulrike S Stein, *Berlin*
Manfred Stolte, *Bayreuth*
Christian P Strassburg, *Hannover*
Wolfgang R Stremmel, *Heidelberg*
Harald F Teutsch, *Ulm*
Robert Thimme, *Freiburg*
Hans L Tillmann, *Leipzig*
Tung-Yu Tsui, *Regensburg*
Axel Ulsenheimer, *Munich*
Patrick Veit-Haibach, *Essen*
Claudia Veltkamp, *Heidelberg*
Siegfried Wagner, *Deggendorf*
Henning Walczak, *Heidelberg*
Heiner Wedemeyer, *Hannover*
Fritz von Weizsacker, *Berlin*
Jens Werner, *Heidelberg*
Bertram Wiedenmann, *Berlin*
Reiner Wiest, *Regensburg*
Stefan Wirth, *Wuppertal*
Stefan JP Zeuzem, *Homburg*

Greece

Alexandra A Alexopoulou, *Athens*
George N Dalekos, *Larissa*
Christos Dervenis, *Athens*
Melanie Maria Deutsch, *Athens*
Tsianos Epameinondas, *Ioannina*
Elias A Kouroumalis, *Heraklion*
George Papatheodoridis, *Athens*
Spiros Sgouros, *Athens*

Hungary

Peter L Lakatos, *Budapest*
Zsuzsa Szondy, *Debrecen*

Iceland

Hallgrimur Gudjonsson, *Reykjavik*

India

Philip Abraham, *Mumbai*
Rakesh Aggarwal, *Lucknow*
Kunissery A Balasubramanian, *Vellore*
Deepak Kumar Bhasin, *Chandigarh*
Sujit K Bhattacharya, *Kolkata*
Yogesh K Chawla, *Chandigarh*
Radha K Dhiman, *Chandigarh*
Sri Prakash Misra, *Allahabad*
Ramesh Roop Rai, *Jaipur*
Nageshwar D Reddy, *Hyderabad*
Rakesh Kumar Tandon, *New Delhi*

Iran

Seyed-Moayed Alavian, *Tehran*
Reza Malekzadeh, *Tehran*
Seyed A Taghavi, *Shiraz*

Ireland

Billy Bourke, *Dublin*
Ronan A Cahill, *Cork*
Anthony P Moran, *Galway*

Israel

Simon Bar-Meir, *Hashomer*
Abraham R Eliakim, *Haifa*
Zvi Fireman, *Hadera*
Yaron Ilan, *Jerusalem*
Avidan U Neumann, *Ramat-Gan*
Yaron Niv, *Pardesia*
Ran Oren, *Tel Aviv*
Ami D Sperber, *Beer-Sheva*

Italy

Giovanni Addolorato, *Roma*
Luigi E Adinolfi, *Naples*
Domenico Alvaro, *Rome*
Mario Angelico, *Rome*
Vito Annese, *San Giovanni Rotond*
Filippo Ansaldi, *Genoa*
Adolfo F Attili, *Roma*
Giovanni Barbara, *Bologna*
Claudio Bassi, *Verona*
Gabrio Bassotti, *Perugia*
Pier M Battezzati, *Milan*
Stefano Bellentani, *Carpi*
Antonio Benedetti, *Ancona*
Mauro Bernardi, *Bologna*
Livia Biancone, *Rome*
Luigi Bonavina, *Milano*
Flavia Bortolotti, *Padova*
Giuseppe Brisinda, *Rome*
Elisabetta Buscarini, *Crema*
Giovanni Cammarota, *Roma*

Antonino Cavallari, *Bologna*
Giuseppe Chiarioni, *Valeggio*
Michele Cicala, *Rome*
Massimo Colombo, *Milan*
Amedeo Columbano, *Cagliari*
Massimo Conio, *Sanremo*
Dario Conte, *Milano*
Gino R Corazza, *Pavia*
Francesco Costa, *Pisa*
Antonio Craxi, *Palermo*
Silvio Danese, *Milan*
Roberto de Franchis, *Milano*
Roberto De Giorgio, *Bologna*
Maria Stella De Mitri, *Bologna*
Giovanni D De Palma, *Naples*
Fabio Farinati, *Padua*
Giammarco Fava, *Ancona*
Francesco Feo, *Sassari*
Fiorucci Stefano, *Perugia*
Andrea Galli, *Firenze*
Valeria Ghisett, *Turin*
Gianluigi Giannelli, *Bari*
Edoardo G Giannini, *Genoa*
Paolo Gionchetti, *Bologna*
Fabio Grizzi, *Milan*
Salvatore Gruttadauria, *Palermo*
Mario Guslandi, *Milano*
Pietro Invernizzi, *Milan*
Ezio Laconi, *Cagliari*
Giacomo Laffi, *Firenze*
Giovanni Maconi, *Milan*
Lucia Malaguarnera, *Catania*
Emanuele D Mangoni, *Napoli*
Paolo Manzoni, *Torino*
Giulio Marchesini, *Bologna*
Fabio Marra, *Florence*
Marco Marzioni, *Ancona*
Giuseppe Mazzella, *Bologna*
Mario U Mondelli, *Pavia*
Giuseppe Montalto, *Palermo*
Giovanni Monteleone, *Rome*
Giovanni Musso, *Torino*
Gerardo Nardone, *Napoli*
Valerio Nobili, *Rome*
Fabio Pace, *Milano*
Luisi Pagliaro, *Palermo*
Francesco Pallone, *Rome*
Fabrizio R Parente, *Milan*
Maurizio Parola, *Torino*
Francesco Perri, *San Giovanni Rotondo*
Raffaele Pezzilli, *Bologna*
Alberto Pilotto, *San Giovanni Rotondo*
Alberto Piperno, *Monza*
Mario Pirisi, *Novara*
Anna C Piscaglia, *Roma*
Paolo Del Poggio, *Treviglio*
Gabriele B Porro, *Milano*
Piero Portincasa, *Bari*
Cosimo Prantera, *Roma*
Bernardino Rampone, *Siena*
Oliviero Riggio, *Rome*
Claudio Romano, *Messina*
Marco Romano, *Napoli*
Gerardo Rosati, *Potenza*
Mario Del Tacca, *Pisa*
Gloria Taliani, *Rome*
Pier A Testoni, *Milan*
Enrico Roda, *Bologna*
Domenico Sansonno, *Bari*
Vincenzo Savarino, *Genova*
Vincenzo Stanghellini, *Bologna*
Giovanni Tarantino, *Naples*
Roberto Testa, *Genoa*
Dino Vaira, *Bologna*
Anna Linda Zignego, *Florence*

Japan

Kyoichi Adachi, *Izumo*
Yasushi Adachi, *Sapporo*
Taiji Akamatsu, *Matsumoto*
Sk Md Fazle Akbar, *Ehime*
Takafumi Ando, *Nagoya*
Akira Andoh, *Otsu*
Taku Aoki, *Tokyo*
Masahiro Arai, *Tokyo*
Tetsuo Arakawa, *Osaka*
Yasuji Arase, *Tokyo*
Masahiro Asaka, *Sapporo*
Hitoshi Asakura, *Tokyo*
Takeshi Azuma, *Fukui*
Yoichi Chida, *Fukuoka*
Takahiro Fujimori, *Tochigi*
Jiro Fujimoto, *Hyogo*
Kazuma Fujimoto, *Saga*
Mitsuhiro Fujishiro, *Tokyo*
Yoshihide Fujiyama, *Otsu*
Hiroyuki Fukui, *Tochigi*
Hiroyuki Hanai, *Hamamatsu*
Naohiko Harada, *Fukuoka*
Makoto Hashizume, *Fukuoka*
Tetsuo Hayakawa, *Nagoya*
Toru Hiyama, *Higashihiroshima*
Kazuhide Higuchi, *Osaka*
Keisuke Hino, *Ube*
Keiji Hirata, *Kitakyushu*
Yuji Iimuro, *Nishinomiya*
Kenji Ikeda, *Tokyo*
Toru Ikegami, *Fukuoka*
Kenichi Ikejima, *Bunkyo-ku*
Fumio Imazeki, *Chiba*
Yutaka Inagaki, *Kanagawa*
Yasuhiro Inokuchi, *Yokohama*
Haruhiro Inoue, *Yokohama*
Masayasu Inoue, *Osaka*
Hiromi Ishibashi, *Nagasaki*
Shunji Ishihara, *Izumo*
Toru Ishikawa, *Niigata*
Kei Ito, *Sendai*
Masayoshi Ito, *Tokyo*
Hiroaki Itoh, *Akita*
Ryuichi Iwakiri, *Saga*
Yoshiaki Iwasaki, *Okayama*
Terumi Kamisawa, *Tokyo*
Hiroshi Kaneko, *Aichi-Gun*
Shuichi Kaneko, *Kanazawa*
Takashi Kanematsu, *Nagasaki*
Mitsuo Katano, *Fukuoka*
Junji Kato, *Sapporo*
Mototsugu Kato, *Sapporo*
Shinzo Kato, *Tokyo*
Norifumi Kawada, *Osaka*
Sunao Kawano, *Osaka*
Mitsuhiro Kida, *Kanagawa*
Yoshikazu Kinoshita, *Izumo*
Tsuneo Kitamura, *Chiba*
Seigo Kitano, *Oita*
Kazuhiko Koike, *Tokyo*
Norihiro Kokudo, *Tokyo*
Satoshi Kondo, *Sapporo*
Shoji Kubo, *Osaka*
Shigeki Kuriyama, *Kagawa*[2]
Katsunori Iijima, *Sendai*
Masato Kusunoki, *Tsu Mie*
Shin Maeda, *Tokyo*
Shigeru Marubashi, *Suita*
Masatoshi Makuuchi, *Tokyo*
Osamu Matsui, *Kanazawa*
Yasuhiro Matsumura, *Chiba*
Yasushi Matsuzaki, *Tsukuba*
Kiyoshi Migita, *Omura*
Kenji Miki, *Tokyo*
Tetsuya Mine, *Kanagawa*
Hiroto Miwa, *Hyogo*
Masashi Mizokami, *Nagoya*
Yoshiaki Mizuguchi, *Tokyo*
Motowo Mizuno, *Hiroshima*
Morito Monden, *Suita*
Hisataka S Moriwaki, *Gifu*
Yasuaki Motomura, *Iizuka*
Yoshiharu Motoo, *Kanazawa*
Naofumi Mukaida, *Kanazawa*
Kazunari Murakami, *Oita*
Kunihiko Murase, *Tusima*
Hiroaki Nagano, *Suita*
Masahito Nagaki, *Gifu*
Masaki Nagaya, *Kawasaki*
Yujl Naito, *Kyoto*
Atsushi Nakajima, *Yokohama*
Hisato Nakajima, *Tokyo*
Hiroki Nakamura, *Yamaguchi*
Shotaro Nakamura, *Fukuoka*
Mikio Nishioka, *Niihama*
Shuji Nomoto, *Nagoya*
Susumu Ohmada, *Maebashi*
Hirohide Ohnishi, *Akita*
Masayuki Ohta, *Oita*
Tetsuo Ohta, *Kanazawa*
Kazuichi Okazaki, *Osaka*
Katsuhisa Omagari, *Nagasaki*
Saburo Onishi, *Nankoku*
Morikazu Onji, *Ehime*
Satoshi Osawa, *Hamamatsu*
Masanobu Oshima, *Kanazawa*
Hiromitsu Saisho, *Chiba*
Hidetsugu Saito, *Tokyo*
Yutaka Saito, *Tokyo*
Isao Sakaida, *Yamaguchi*
Michiie Sakamoto, *Tokyo*
Yasushi Sano, *Chiba*
Hiroki Sasaki, *Tokyo*
Iwao Sasaki, *Sendai*
Motoko Sasaki, *Kanazawa*
Chifumi Sato, *Tokyo*
Shuichi Seki, *Osaka*
Hiroshi Shimada, *Yokohama*
Mitsuo Shimada, *Tokushima*
Tomohiko Shimatan, *Hiroshima*
Hiroaki Shimizu, *Chiba*
Ichiro Shimizu, *Tokushima*
Yukihiro Shimizu, *Kyoto*
Shinji Shimoda, *Fukuoka*
Tooru Shimosegawa, *Sendai*
Tadashi Shimoyama, *Hirosaki*
Ken Shirabe, *Iizuka City*
Yoshio Shirai, *Niigata*
Katsuya Shiraki, *Mie*
Yasushi Shiratori, *Okayama*
Masayuki Sho, *Nara*
Yasuhiko Sugawara, *Tokyo*
Hidekazu Suzuki, *Tokyo*
Minoru Tada, *Tokyo*
Tadatoshi Takayama, *Tokyo*
Tadashi Takeda, *Osaka*
Koji Takeuchi, *Kyoto*
Kiichi Tamada, *Tochigi*
Akira Tanaka, *Kyoto*
Eiji Tanaka, *Matsumoto*
Noriaki Tanaka, *Okayama*
Shinji Tanaka, *Hiroshima*
Hideki Taniguchi, *Yokohama*
Kyuichi Tanikawa, *Kurume*
Akira Terano, *Shimotsugagun*
Hitoshi Togash, *Yamagata*
Shinji Togo, *Yokohama*
Kazunari Tominaga, *Osaka*
Takuji Torimura, *Fukuoka*

Minoru Toyota, *Sapporo*
Akihito Tsubota, *Chiba*
Takato Ueno, *Kurume*
Naomi Uemura, *Tokyo*
Shinichi Wada, *Tochigi*
Hiroyuki Watanabe, *Kanazawa*
Toshio Watanabe, *Osaka*
Yuji Watanabe, *Ehime*
Toshiaki Watanabe, *Tokyo*
Chun-Yang Wen, *Nagasaki*
Satoshi Yamagiwa, *Niigata*
Koji Yamaguchi, *Fukuoka*
Takayuki Yamamoto, *Yokkaichi*
Takashi Yao, *Fukuoka*
Masashi Yoneda, *Tochigi*
Hiroshi Yoshida, *Tokyo*
Masashi Yoshida, *Tokyo*
Norimasa Yoshida, *Kyoto*
Hitoshi Yoshiji, *Nara*
Kentaro Yoshika, *Toyoake*
Yasunobu Yoshikai, *Fukuoka*
Masahide Yoshikawa, *Kashihara*
Katsutoshi Yoshizato, *Higashihiroshima*

Lebanon

Bassam N Abboud, *Beirut*
Ala I Sharara, *Beirut*
Joseph D Boujaoude, *Beirut*

Lithuania

Limas Kupcinskas, *Kaunas*

Macedonia

Vladimir C Serafimoski, *Skopje*

Malaysia

Andrew Seng Boon Chua, *Ipoh*
Khean-Lee Goh, Kuala *Lumpur*
Jayaram Menon, *Sabah*

Mexico

Diego Garcia-Compean, *Monterrey*
Eduardo R Marin-Lopez, *Jesús García*
Nahum Méndez-Sánchez, *Mexico*
Saúl Villa-Treviño, *México*

Monaco

Patrick Rampal, *Monaco*

Morocco

Abdellah Essaid, *Rabat*

The Netherlands

Ulrich Beuers, *Amsterdam*
Gerd Bouma, *Amsterdam*
Lee Bouwman, *Leiden*
J Bart A Crusius, *Amsterdam*
NKH de Boer, *Amsterdam*
Koert P de Jong, *Groningen*
Henrike Hamer, *Maastricht*
Frank Hoentjen, *Haarlem*
Janine K Kruit, *Groningen*
Ernst J Kuipers, *Rotterdam*
CBHW Lamers, *Leiden*
Ton Lisman, *Utrecht*
Yi Liu, *Amsterdam*
Jeroen Maljaars, *Maastricht*
Servaas Morré, *Amsterdam*
Chris JJ Mulder, *Amsterdam*
Michael Müller, *Wageningen*
Amado S Peña, *Amsterdam*
Robert J Porte, *Groningen*
Ingrid B Renes, *Rotterdam*
Andreas Smout, *Utrecht*
Paul E Sijens, *Groningen*
Reinhold W Stockbrugger, *Maastricht*
Luc JW van der Laan, *Rotterdam*
Karel van Erpecum, *Utrecht*
Gerard P VanBerge-Henegouwen,*Utrecht*

New Zealand

Ian D Wallace, *Auckland*

Nigeria

Samuel B Olaleye, *Ibadan*

Norway

Trond Berg, *Oslo*
Tom H Karlsen, *Oslo*
Helge L Waldum, *Trondheim*

Pakistan

Muhammad S Khokhar, *Lahore*
Syed MW Jafri, *Karachi*

Peru

Hector H Garcia, *Lima*

Poland

Tomasz Brzozowski, *Cracow*
Robert Flisiak, *Bialystok*
Hanna Gregorek, *Warsaw*
Dariusz M Lebensztejn, *Bialystok*
Wojciech G Polak, *Wroclaw*
Marek Hartleb, *Katowice*

Portugal

Miguel C De Moura, *Lisbon*

Russia

Vladimir T Ivashkin, *Moscow*
Leonid Lazebnik, *Moscow*
Vasiliy I Reshetnyak, *Moscow*

Saudi Arabia

Ibrahim A Al Mofleh, *Riyadh*
Ahmed Helmy, *Riyadh*

Serbia

Dusan M Jovanovic, *Sremska Kamenica*

Singapore

Bow Ho, *Singapore*
Khek-Yu Ho, *Singapore*
Fock Kwong Ming, *Singapore*
Francis Seow-Choen, *Singapore*

Slovakia

Silvia Pastorekova, *Bratislava*
Anton Vavrecka, *Bratislava*

Slovenia

Sasa Markovic, *Ljubljana*

South Africa

Rosemar Joyce Burnett, *Pretoria*
Michael C Kew, *Parktown*

South Korea

Byung Ihn Choi, *Seoul*
Ho Soon Choi, *Seoul*
Marie Yeo, *Suwon*
Sun Pyo Hong, *Gyeonggi-do*
Jae J Kim, *Seoul*
Jin-Hong Kim, *Suwon*
Myung-Hwan Kim, *Seoul*
Chang Hong Lee, *Seoul*
Jong Kyun Lee, *Seoul*
Eun-Yi Moon, *Seoul*
Jae-Gahb Park, *Seoul*
Dong Wan Seo, *Seoul*
Dong Jin Suh, *Seoul*
Byung Chul Yoo, *Seoul*

Spain

Juan G Abraldes, *Barcelona*
Agustin Albillos, *Madrid*
Raul J Andrade, *Málaga*
Luis Aparisi, *Valencia*
Fernando Azpiroz, *Barcelona*
Ramon Bataller, *Barcelona*
Josep M Bordas, *Barcelona*
Xavier Calvet, *Sabadell*
Jordi Camps, *Catalunya*
Andres Cardenas, *Barcelona*
Vicente Carreño, *Madrid*
Jose Castellote, *Barcelona*
Antoni Castells, *Barcelona*
Vicente Felipo, *Valencia*
Juan C Garcia-Pagán, *Barcelona*
Jaime B Genover, *Barcelona*
Javier P Gisbert, *Madrid*
Jaime Guardia, *Barcelona*
Isabel Fabregat, *Barcelona*
Mercedes Fernandez, *Barcelona*
Angel Lanas, *Zaragoza*
Juan-Ramón Larrubia, *Guadalajara*
Laura Lladóa, *Barcelona*
María IT López, *Jaén*
Juan R Malagelada, *Barcelona*
José M Mato, *Derio*
Juan F Medina, *Pamplona*
Miguel A Muñoz-Navas, *Pamplona*
Julian Panes, *Barcelona*
Miguel M Perez, *Valencia*
Miguel Perez-Mateo, *Alicante*

Josep M Pique, *Barcelona*
Jesús M Prieto, *Pamplona*
Sabino Riestra, *Pola De Siero*
Luis Rodrigo, *Oviedo*
Manuel Romero-Gómez, *Sevilla*
Joan Roselló-Catafau, *Barcelona*

Sweden

Einar S Björnsson, *Gothenburg*
Curt Einarsson, *Huddinge*
Per M Hellström, *Stockholm*
Ulf Hindorf, *Lund*
Elisabeth Hultgren-Hörnquist, *Örebro*
Anders E Lehmann, *Mölndal*
Hanns-Ulrich Marschall, *Stockholm*
Lars C Olbe, *Molndal*
Lars A Pahlman, *Uppsala*
Matti Sallberg, *Stockholm*
Magnus Simrén, *Göteborg*
Xiao-Feng Sun, *Linköping*
Ervin Tóth, *Malmö*
Weimin Ye, *Stockholm*
Christer S von Holstein, *Lund*

Switzerland

Chrish Beglinger, *Basel*
Pierre A Clavien, *Zurich*
Jean-Francois Dufour, *Bern*
Franco Fortunato, *Zürich*
Jean L Frossard, *Geneva*
Gerd A Kullak-Ublick, *Zurich*
Pierre Michetti, *Lausanne*
Francesco Negro, *Genève*
Bruno Stieger, *Zurich*
Radu Tutuian, *Zurich*
Stephan R Vavricka, *Zurich*
Gerhard Rogler, *Zurich*
Arthur Zimmermann, *Berne*

Turkey

Yusuf Bayraktar, *Ankara*
Figen Gurakan, *Ankara*
Aydin Karabacakoglu, *Konya*
Serdar Karakose, *Konya*
Hizir Kurtel, *Istanbul*
Osman C Ozdogan, *Istanbul*
Özlem Yilmaz, *Izmir*
Cihan Yurdaydin, *Ankara*

United Arab Emirates

Sherif M Karam, *Al-Ain*

United Kingdom

David H Adams, *Birmingham*
Simon Afford, *Birmingham*
Navneet K Ahluwalia, *Stockport*
Ahmed Alzaraa, *Manchester*
Lesley A Anderson, *Belfast*
Charalambos G Antoniades, *London*
Anthony TR Axon, *Leeds*
Qasim Aziz, *Manchester*
Nicholas M Barnes, *Birmingham*
Jim D Bell, *London*
Mairi Brittan, *London*
Alastair D Burt, *Newcastle*
Simon S Campbell, *Manchester*
Simon R Carding, *Leeds*
Paul J Ciclitira, *London*
Eithne Costello, *Liverpool*
Tatjana Crnogorac-Jurcevic, *London*
Harry Dalton, *Truro*
Amar P Dhillon, *London*
William Dickey, *Londonderry*
James E East, *London*
Emad M El-Omar, *Aberdeen*
Ahmed M Elsharkawy, *Newcastle Upon Tyne*
Annette Fristscher-Ravens, *London*
Elizabeth Furrie, *Dundee*
Daniel R Gaya, *Edinburgh*
Subrata Ghosh, *London*
William Greenhalf, *Liverpool*
Indra N Guha, *Southampton*
Peter C Hayes, *Edinburgh*
Gwo-Tzer Ho, *Edinburgh*
Anthony R Hobson, *Salford*
Lesley A Houghton, *Manchester*
Stefan G Hübscher, *Birmingham*
Robin Hughes, *London*
Pali Hungin, *Stockton*
David P Hurlstone, *Sheffield*
Rajiv Jalan, *London*
Janusz AZ Jankowski, *Oxford*
Brian T Johnston, *Belfast*
David EJ Jones, *Newcastle*
Roger Jones, *London*
Michael A Kamm, *Harrow*
Peter Karayiannis, *London*
Laurens Kruidenier, *Harlow*
Patricia F Lalor, *Birmingham*
Chee Hooi Lim, *Midlands*
Hong-Xiang Liu, *Cambridge*
Yun Ma, *London*
Kenneth E L McColl, *Glasgow*
Stuart AC McDonald, *London*
Dermot P Mcgovern, *Oxford*
Giorgina Mieli-Vergani, *London*
Nikolai V Naoumov, *London*
John P Neoptolemos, *Liverpool*
James Neuberger, *Birmingham*
Philip Noel Newsome, *Birmingham*
Mark S Pearce, *Newcastle Upon Tyne*
Stephen P Pereira, *London*
D Mark Pritchard, *Liverpool*
Sakhawat Rahman, *London*
Stephen E Roberts, *Swansea*
Marco Senzolo, *Padova*
Soraya Shirazi-Beechey, *Liverpool*
Robert Sutton, *Liverpool*
Simon D Taylor-Robinson, *London*
Paris P Tekkis, *London*
Ulrich Thalheimer, *London*
David G Thompson, *Salford*
Nick P Thompson, *Newcastle*
David Tosh, *Bath*
Frank I Tovey, *London*
Chris Tselepis, *Birmingham*
Diego Vergani, *London*
Geoffrey Warhurst, *Salford*
Alastair John Watson, *Liverpool*
Peter J Whorwell, *Manchester*
Roger Williams, *London*
Karen L Wright, *Bath*
Min Zhao, *Foresterhill*

United States

Manal F Abdelmalek, *Durham*
Gary A Abrams, *Birmingham*
Maria T Abreu, *New York*
Reid B Adams, *Virginia*
Golo Ahlenstiel, *Bethesda*
BS Anand, *Houston*
Frank A Anania, *Atlanta*
M Ananthanarayanan, *New York*
Gavin E Arteel, *Louisville*
Jasmohan S Bajaj, *Milwaukee*
Subhas Banerjee, *Palo Alto*
Peter A Banks, *Boston*
Jamie S Barkin, *Miami Beach*
Kim E Barrett, *San Diego*
Marc D Basson, *Detroit*
Anthony J Bauer, *Pittsburgh*
Wallace F Berman, *Durham*
Timothy R Billiar, *Pittsburgh*
Edmund J Bini, *New York*
David G Binion, *Milwaukee*
Jennifer D Black, *Buffalo*
Herbert L Bonkovsky, *Charlotte*
Carla W Brady, *Durham*
Andrea D Branch, *New York*
Robert S Bresalier, *Houston*
Alan L Buchman, *Chicago*
Ronald W Busuttil, *Los Angeles*
Alan Cahill, *Philadelphia*
John M Carethers, *San Diego*
David L Carr-Locke, *Boston*
Maurice A Cerulli, *New York*
Ravi S Chari, *Nashville*
Jiande Chen, *Galveston*
Xian-Ming Chen, *Omaha*
Xin Chen, *San Francisco*
Ramsey Chi-man Cheung, *Palo Alto*
William D Chey, *Ann Arbor*
John Y Chiang, *Rootstown*
Parimal Chowdhury, *Arkansas*
Raymond T Chung, *Boston*
James M Church, *Cleveland*
Ram Chuttani, *Boston*
Mark G Clemens, *Charlotte*
Ana J Coito, *Los Angeles*
Vincent Coghlan, *Beaverton*
David Cronin II, *New Haven*
John Cuppoletti, *Cincinnati*
Mark J Czaja, *New York*
Peter V Danenberg, *Los Angeles*
Kiron M Das, New *Brunswick*
Conor P Delaney, *Cleveland*
Jose L del Pozo, *Rochester*
Sharon DeMorrow, *Temple*
Deborah L Diamond, *Seattle*
Douglas A Drossman, *Chapel Hill*
Katerina Dvorak, *Tucson*
Bijan Eghtesad, *Cleveland*
Hala El-Zimaity, *Houston*
Michelle Embree-Ku, *Providence*
Sukru Emre, New *Haven*
Douglas G Farmer, *Los Angeles*
Alessio Fasano, *Baltimore*
Mark A Feitelson, *Philadelphia*
Ariel E Feldstein, *Cleveland*
Alessandro Fichera, *Chicago*
Robert L Fine, *New York*
Magali Fontaine, *Stanford*
Chris E Forsmark, *Gainesville*
Glenn T Furuta, *Aurora*
Chandrashekhar R Gandhi, *Pittsburgh*
Susan L Gearhart, *Baltimore*
Xupeng Ge, *Boston*
Xin Geng, *New Brunswick*
M Eric Gershwin, *Suite*
Jean-Francois Geschwind, *Baltimore*
Ignacio Gil-Bazo, *New York*
Shannon S Glaser, *Temple*
Ajay Goel, *Dallas*
Richard M Green, *Chicago*
Julia B Greer, *Pittsburgh*

James H Grendell, *New York*
David R Gretch, *Seattle*
Stefano Guandalini, *Chicago*
Anna S Gukovskaya, *Los Angeles*
Sanjeev Gupta, *Bronx*
David J Hackam, *Pittsburgh*
Stephen B Hanauer, *Chicago*
Gavin Harewood, *Rochester*
Margaret M Heitkemper, *Washington*
Alan W Hemming, *Gainesville*
Samuel B Ho, *San Diego*
Peter R Holt, *New York*
Colin W Howden, *Chicago*
Hongjin Huang, *Alameda*
Jamal A Ibdah, *Columbia*
Atif Iqbal, *Omaha*
Hajime Isomoto, *Rochester*
Hartmut Jaeschke, *Tucson*
Dennis M Jensen, *Los Angeles*
Cheng Ji, *Los Angeles*
Leonard R Johnson, *Memphis*
Michael P Jones, *Chicago*
Peter J Kahrilas, *Chicago*
Anthony N Kalloo, *Baltimore*
Marshall M Kaplan, *Boston*
Neil Kaplowitz, *Los Angeles*
Serhan Karvar, *Los Angeles*
Rashmi Kaul, *Tulsa*
Jonathan D Kaunitz, *Los Angeles*
Ali Keshavarzian, *Chicago*
Miran Kim, *Providence*
Joseph B Kirsner, *Chicago*
Leonidas G Koniaris, *Miami*
Burton I Korelitz, *New York*
Robert J Korst, *New York*
Richard A Kozarek, *Seattle*
Alyssa M Krasinskas, *Pittsburgh*
Michael Kremer, *Chapel Hill*
Shiu-Ming Kuo, *Buffalo*
Paul Y Kwo, *Indianapolis*
Daryl Tan Yeung Lau, *Galvesto*
Stephen J Lanspa, *Omaha*
Joel E Lavine, *San Diego*
Bret Lashner, *Cleveland*
Dirk J van Leeuwen, *Lebanon*
Glen A Lehman, *Indianapolis*
Alex B Lentsch, *Cincinnati*
Andreas Leodolter, *La Jolla*
Gene LeSage, *Houston*
Josh Levitsky, *Chicago*
Cynthia Levy, *Gainesville*
Ming Li, *New Orleans*
Zhiping Li, *Baltimore*
Zhe-Xiong Lian, *Davis*
Lenard M Lichtenberger, *Houston*
Gary R Lichtenstein, *Philadelphia*
Otto Schiueh-Tzang Lin, *Seattle*
Martin Lipkin, *New York*
Chen Liu, *Gainesville*
Edward V Loftus, *Rocheste*
Robin G Lorenz, *Birmingham*
Michael R Lucey, *Madison*
James D Luketich, *Pittsburgh*
Guangbin Luo, *Cheveland*
Henry T Lynch, *Omaha*
Patrick M Lynch, *Houston*
John S Macdonald, *New York*
Bruce V MacFadyen, *Augusta*
Willis C Maddrey, *Dallas*
Ashok Malani, *Los Angeles*
Mercedes Susan Mandell, *Aurora*
Peter J Mannon, *Bethesda*
Charles M Mansbach, *Tennessee*
John F Di Mari, *Texas*
John M Mariadason, *Bronx*
Jorge A Marrero, *Ann Arbor*
Paul Martin, *New York*
Paulo Ney Aguiar Martins, *Boston*
Wendy M Mars, *Pittsburgh*
Laura E Matarese, *Pittsburgh*
Richard W McCallum, *Kansas*
Beth A McCormick, *Charlestown*
Lynne V McFarland, *Washington*
Kevin McGrath, *Pittsburgh*
Harihara Mehendale, *Monroe*
Ali Mencin, *New York*
Fanyin Meng, *Ohio*
Stephan Menne, *New York*
Didier Merlin, *Atlanta*
Howard Mertz, *Nashville*
George W Meyer, *Sacramento*
George Michalopoulos, *Pittsburgh*
James M Millis, *Chicago*
Fabrizio Michelassi, *New York*
Albert D Min, *New York*
Pramod K Mistry, *New Haven*
Emiko Mizoguchi, *Boston*
Smruti R Mohanty, *Chicago*
Satdarshan S Monga, *Pittsburgh*
Timothy H Moran, *Baltimore*
Peter L Moses, *Burlington*
Steven F Moss, *Providence*
Andrew J Muir, *Durham*
Milton G Mutchnick, *Detroit*
Masaki Nagaya, *Boston*
Victor Navarro, *Philadelphia*
Laura E Nagy, *Cleveland*
Hiroshi Nakagawa, *Philadelphia*
Douglas B Nelson, *Minneapolis*
Justin H Nguyen, *Florida*
Patrick G Northup, *Charlottesville*
Christopher O'Brien, *Miami*
Robert D Odze, *Boston*
Brant K Oelschlager, *Washington*
Curtis T Okamoto, *Los Angeles*
Stephen JD O'Keefe, *Pittsburgh*
Dimitry Oleynikov, *Omaha*
Stephen J Pandol, *Los Angeles*
Georgios Papachristou, *Pittsburgh*
Pankaj J Pasricha, *Galveston*
Zhiheng Pei, *New York*
Michael A Pezzone, *Pittsburgh*
CS Pitchumoni, *New Brunswiuc*
Paul J Pockros, *La Jolla*
Jay Pravda, *Gainesville*
Massimo Raimondo, *Jacksonville*
GS Raju, *Galveston*
Raymund R Razonable, *Minnesota*
Murray B Resnick, *Providence*
Adrian Reuben, *Charleston*
Douglas K Rex, *Indianapolis*
Victor E Reyes, *Galveston*
Basil Rigas, *New York*
Yehuda Ringel, *Chapel Hill*
Richard A Rippe, *Chapel Hill*
Maribel Rodriguez-Torres, *Santurce*
Marcos Rojkind, *Washington*
Philip Rosenthal, *San Francisco*
Barry Rosser, *Jacksonville Florida*
Hemant K Roy, *Evanston*
Sammy Saab, *Los Angeles*
Shawn D Safford, *Norfolk*
Dushyant V Sahani, *Boston*
Bruce E Sands, *Boston*
James M Scheiman, *Ann Arbor*
Eugene R Schiff, *Miami*
Nicholas J Shaheen, *Chapel Hill*
Vanessa M Shami, *Charlottesville*
Prateek Sharma, *Kansas City*
Harvey L Sharp, *Minneapolis*
Stuart Sherman, *Indianapolis*
Shivendra Shukla, *Columbia*
Alphonse E Sirica, *Virginia*
Shanthi V Sitaraman, *Atlanta*
Stuart J Spechler, *Dallas*
Shanthi Srinivasan, *Atlanta*
Michael Steer, *Boston*
Peter D Stevens, *New York*
Charmaine A Stewart, *Rochester*
Christian D Stone, *Saint Louis*
Gary D Stoner, *Columbus*
R Todd Stravitz, *Richmond*
Liping Su, *Chicago*
Christina Surawicz, *Seattle*
Robert W Summers, *Iowa City*
Wing-Kin Syn, *Durham*
Gyongyi Szabo, *Worcester*
Yvette Taché, *Los Angeles*
Seng-Lai Tan, *Seattle*
Andrzej S Tarnawski, *Orange*
K-M Tchou-Wong, *New York*
Jonathan P Terdiman, *San Francisco*
Neil D Theise, *New York*
Christopher C Thompson, *Boston*
Swan N Thung, *New York*
Michael Torbenson, *Baltimore*
Natalie J Torok, *Sacramento*
RA Travagli, *Baton Rouge*
George Triadafilopoulos, *Stanford*
Chung-Jyi Tsai, *Lexington*
Janet Elizabeth Tuttle-Newhall, *Durham*
Andrew Ukleja, *Florida*
Michael F Vaezi, *Nashville*
Hugo E Vargas, *Scottsdale*
Arnold Wald, *Wisconsin*
Scott A Waldman, *Philadelphia*
Jian-Ying Wang, *Baltimore*
Timothy C Wang, *New York*
Irving Waxman, *Chicago*
Steven A Weinman, *Galveston*
Steven D Wexner, *Weston*
Keith T Wilson, *Baltimore*
Jacqueline L Wolf, *Boston*
Jackie Wood, *Ohio*
George Y Wu, *Farmington*
Jian Wu, *Sacramento*
Samuel Wyllie, *Houston*
Wen Xie, *Pittsburgh*
Vijay Yajnik, *Boston*
Vincent W Yang, *Atlanta*
Francis Y Yao, *San Francisco*
Hal F Yee, *San Francisco*
Xiao-Ming Yin, *Pittsburgh*
Min You, *Tampa*
Zobair M Younossi, *Virginia*
Liqing Yu, *Winston-Salem*
David Yule, *Rochester*
Ruben Zamora, *Pittsburgh*
Michael E Zenilman, *New York*
Zhi Zhong, *Chapel Hill*
Michael A Zimmerman, *Colorado*
Stephen D Zucker, *Cincinnati*

Uruguay

Henry Cohen, *Montevideo*

[1]Passed away on October 20, 2007
[2]Passed away on June 11, 2007

National Journal Award 2005

World Journal of Gastroenterology®

Weekly Established in October 1995

Volume 14 Number 38
October 14, 2008

Contents

Contents

World Journal of Gastroenterology
Volume 14 **Number** 38 **October** 14, 2008

World Journal of Gastroenterology
Volume 14 **Number** 38 **October** 14, 2008

Contents

NAME OF JOURNAL
World Journal of Gastroenterology

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

EDITING
Editorial Board of *World Journal of Gastroenterology,* Room 903, Ocean International Center, Building D, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

PUBLISHING
The WJG Press and Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Ocean International Center, Building D, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

PRINTING
Beijing Kexin Printing House

OVERSEAS DISTRIBUTOR
Beijing Bureau for Distribution of Newspapers and Journals (Code No. 82-261)
China International Book Trading Corporation PO Box 399, Beijing, China (Code No. M4481)

PUBLICATION DATE
October 14, 2008

EDITOR-IN-CHIEF
Lian-Sheng Ma, *Beijing*

SUBSCRIPTION
RMB 50 Yuan for each issue, RMB 2400 Yuan for one year

CSSN
ISSN 1007-9327
CN 14-1219/R

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5781

World J Gastroenterol 2008 October 14; 14(38): 5781-5788
World Journal of Gastroenterology ISSN 1007-9327

REVIEW

Intrahepatic cholestasis of pregnancy-current achievements and unsolved problems

Jurate Kondrackiene, Limas Kupcinskas

Jurate Kondrackiene, Limas Kupcinskas, Department of Gastroenterology, Kaunas University of Medicine, Kaunas 50009, Lithuania

Author contributions: Kondrackiene J and Kupcinskas L contributed equally to this work; Kondrackiene J and Kupcinskas L wrote the paper based on results of their own experience and recent literature sources (PubMed, ISI Web of Science) on ICP.

Correspondence to: Jurate Kondrackiene, MD, PhD, Department of Gastroenterology, Kaunas University of Medicine, Eiveniu Street 2, Kaunas 50009, Lithuania. jukond@takas.lt

Telephone: +370-37-326896 Fax: +370-37-326508

Received: April 23, 2008 Revised: June 16, 2008
Accepted: June 23, 2008
Published online: October 14, 2008

Abstract

Intrahepatic cholestasis of pregnancy (ICP) is the most common pregnancy-related liver disorder. Maternal effects of ICP are mild; however, there is a clear association between ICP and higher frequency of fetal distress, preterm delivery, and sudden intrauterine fetal death. The cause of ICP remains elusive, but there is evidence that mutations in genes encoding hepatobiliary transport proteins can predispose for the development of ICP. Recent data suggest that ursodeoxycholic acid is currently the most effective pharmacologic treatment, whereas obstetric management is still debated. Clinical trials are required to identify the most suitable monitoring modalities that can specifically predict poor perinatal outcome. This article aims to review current achievements and unsolved problems of ICP.

Key words: Cholestasis of pregnancy; Canalicular ABC transporters; Pruritus; Fetal outcome; Ursodeoxycholic acid

Peer reviewer: Dr. Yogesh K Chawla, Professor, Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

Kondrackiene J, Kupcinskas L. Intrahepatic cholestasis of pregnancy-current achievements and unsolved problems. *World J Gastroenterol* 2008; 14(38): 5781-5788 Available from: URL: http://www.wjgnet.com/1007-9327/14/5781.asp DOI: http://dx.doi.org/10.3748/wjg.14.5781

INTRODUCTION

Intrahepatic cholestasis of pregnancy (ICP) is the most common liver disorder unique to pregnancy. ICP predominantly occurs during the third trimester of pregnancy and characterized by pruritus, and biochemical disturbances in liver tests. While ICP poses little maternal risk, there is an increased risk of preterm delivery and sudden intrauterine fetal death. In spite of the substantial risk, ICP remains widely disregarded as a serious clinical problem[1]. The cause of ICP is not fully understood. Current research on the pathogenesis of ICP focuses on genetic and hormonal factors. The treatment is mainly symptomatic. Ursodeoxycholic acid is currently the most effective pharmacologic treatment, but there is no ideal method for fetal surveillance so far. This review, based on an analysis of the literature and our own experience, attempts to summarize current achievements and unsolved problems of ICP.

EPIDEMIOLOGY

The incidence of ICP varies throughout the world. The highest incidence is considered to be in Chile and Bolivia (5%-15%)[2,3]. In these countries, the incidence of ICP has decreased more recently, whereas it has increased in other parts of Europe, the USA, Asia, Australia and some Latin American countries[3,4]. In Scandinavian and Baltic countries ICP occurs in up to 2% of pregnancies, while in other countries of Europe and North America the incidence is less than 1%[3-5], and in South Asian populations the incidence is 0.8%-1.46%[6]. In Lithuania, a retrospective analysis disclosed a rate of 0.4% of ICP in 16 252 pregnant women over a period of five years[7]. The low incidence of ICP may reflect an underestimation of the problem, and growing awareness of the condition will probably increase the numbers[2].

ETIOLOGY AND PATHOGENESIS

Over the last decade, it has become increasingly apparent that the etiology of ICP is multifactorial, involving genetic and hormonal factors. Hormonal

factors may trigger the transient decompensation of the heterozygous state for genes encoding hepatobiliary transport proteins which fail during pregnancy, leading to ICP[3,8,9].

Estrogens and progesterone

ICP occurs mainly during the third trimester, when serum concentrations of estrogens and progesterone reach their peak. ICP is also more common in twin pregnancies, which are associated with higher levels of hormones than singleton pregnancies[3,10]. All hormones are metabolized by the liver, and an excess of metabolites influences the activity of biliary canalicular transporters. The cholestatic potential of some D-ring estrogens, in particular glucuronides like estradiol-17β-*d*-glucuronide, and mono- or disulfated progesterone metabolites, mainly 3α, 5α-isomers, is supported by experimental and clinical data[11-13]. The formation of large amounts of sulfated progesterone metabolites, possibly related to greater 5-α and 3-α reduction, may result in saturation of the hepatic transport system (s) utilized for biliary excretion of these compounds in some genetically predisposed women[11,14,15]. The function of hepatocellular transporters such as ABCB11 and ABSB4 has been shown to be impaired at the posttranscriptional level *in vitro* by high loads of estrogen glucuronides and progesterone[16-18]. In addition, estrogens impair basolateral as well as canalicular bile acid transporter expression of liver cells *in vitro* by transcriptional mechanisms[19].

Genetic factors

There is increasing evidence that interaction between genetically determined dysfunction in the canalicular ABC transporters, and high levels of sex hormones produced in pregnancy, can predispose for the development of ICP[8,9]. Genetic factors could explain familial cases and the higher incidence in some ethnic groups. Also supporting genetic factors are the high rate of recurrence of ICP in subsequent pregnancies and the susceptibility of affected women to progesterone[3,15,20]. The phospholipid translocator (ABCB4, MDR3) and the bile salt export pump (ABCB11, BSEP) are the main transporters involved in the biliary secretion of cholephilic compounds. The hypothesis that mutations in the canalicular transporters contributes to ICP was first supported by Jacquemin *et al*[21]. Heterozygous mutations in ABCB4 have been found in a large consanguineous family in whom six women had at least one episode of ICP[21,22]. Since then, different studies reported additional mutations in ABCB4 which are associated with the presence of ICP[23-25]. In a recent prospective study on 693 Swedish patients with severe ICP (bile acid levels $\geqslant$ 40 μmol/L), a genetic association with common ABCB4 gene variants was found. These associations were reflected by different frequencies of at-risk alleles of the two tagging polymorphisms [c.711A: Odds ratio (OR) = 2.27, P = 0.04; deletion intron 5: OR = 14.68; P = 0.012][26]. The association between ICP and the SNP c.711A was detected previously in a large UK cohort of 184 ICP patients with bile acid levels > 14 μmol/L[27]. Splicing mutations have been described in ABCB4 with normal gamma-glutamyltranspeptidase (γ-GT) in German women[28], whereas in only a small percentage (7.2%) of Italian women ABCB4 mutations were responsible for the development of ICP[29]. Different genetic background may justify the presence of novel MDR3 gene mutations[30]. It has been suggested that mutations in the ABCB4 are associated with elevated γ-GT levels[25,31], whereas in several recent studies patients with ICP exhibited normal γ-GT activity[28,29]. Floreani and coworkers concluded that γ-GT is not a discriminant for patients carrying ABCB4 mutations[29].

The bile salt export pump (BSEP, ABCB11) and multidrug resistance associated protein 2 (MRP2, ABCC2) have been proposed as alternative candidate proteins involved in the pathogenesis of hormonal cholestasis given their important roles in bile formation and bilirubin secretion[25,32-35]. Meier and coworkers supported a role for the ABCB11 1331T>C polymorphism as a susceptibility factor for the development of estrogen-induced cholestasis[32]. No association was found for ABCC2 in this study[32], whereas Sookoian *et al*[36], found an association between the rs3740066 in exon 28 of the ABCC2 gene and ICP. Also, single British and Finnish patients with ICP carried mutations in the ATP8B1 (or FIC1) gene encoding a potential membrane transporter for phosphatidylserine[37,38].

Other factors

Some characteristics of ICP, such as incomplete recurrence at subsequent pregnancies, the decrease in prevalence and seasonal variations, suggest that environmental factors may contribute to the pathogenesis of this disorder[2,3,39]. Recently Reyes *et al*[40] reported that increased intestinal permeability was detected in ICP patients, and a "leaky gut' may participate in the pathogenesis of this pregnancy disorder by enhancing the absorption of bacterial endotoxin. Could cytokines be the missing link between pregnancy and cholestasis by favoring the absorption of bacterial endotoxin to initiate the liver inflammatory cascade? This hypothesis need to be confirmed in a large group of ICP patients[4]. Future studies may provide a better understanding of the pathogenic mechanisms of ICP.

Fetal pathophysiology

The mechanism underlying poor perinatal outcome is still poorly understood. During ICP there is an increased flux of bile acids from the mother to the fetus[41-43]. The placenta plays a crucial role in protecting the fetus from the adverse effects of potentially toxic endogenous substances including total bile acid (TBA)[44,45]. High levels of maternal TBA affect placental transport, placental hormone production, and chorionic vessel constriction[46]. In animal models,

maternal hypercholanemia may affect the vectorial transfer of bile acids through the creation of inversely directed gradients, as compared with the physiological situation[47], and by impairing the ability of the trophoblast to transport bile acids[48]. Germain *et al*[49] have shown that activation of the oxytocin receptor pathway is possibly caused by a cholic-acid-mediated increase in oxytocin-receptor expression.

CLINICAL FEATURES

ICP is characterized by pruritus starting in the second or third trimester of pregnancy, and disappearing after delivery. It is often generalized but predominates on the palms and the soles of the feet, and is worse at night. Skin lesions are characteristically absent except for excoriations due to scratching[2,3,5]. In approximately 80% of patients pruritus starts in late pregnancy, but there can be unusual forms of ICP[11]: (1) early onset, even in the first weeks; (2) typical pruritus without the usual serum abnormalities; (3) pruritus may fade spontaneously before delivery with or without an improvement in serum liver tests; (4) the disorder exacerbates postpartum with no signs of liver failure, and may last 1-2 mo after delivery, subsiding spontaneously without sequelae. Mild jaundice occurs in 10% to 15% of cases, typically within 4 wk of the onset of itching[50-52]. Subclinical steatorrhea may be seen along with fat malabsorption, which may lead to vitamin K deficiency[53,54]. Abdominal pain is uncommon. Encephalopathy or other features of liver failure are unusual and their presence should initiate a search for other causes of liver disease[39].

The most sensitive laboratory abnormality in ICP is an increase in serum TBA concentrations, which may be the first or only laboratory abnormality[3,5,52]. Serum cholic acid increases more than chenodeoxycholic acid, resulting in a marked elevation of the cholic/chenodeoxycholic acid ratio compared to pregnant women without ICP[55]. Whereas, a study from Argentina has shown that asymptomatic hypercholanemia of pregnancy, defined as TBA > 11 μmol/L in healthy pregnant women, does not necessarily lead to ICP[56]. Serum aminotransferases are elevated, and may reach values greater than 1000 U/L, making distinction from viral hepatitis important. Hyperbilirubinemia, up to 100 μmol/L is observed in 10% to 20% of the cases[3,5,52,53]. During a 3-year period in a prospective study of 84 women with ICP, elevation of aminotransferase activities from 2-fold to 15-fold were noticed in 85% of patients, bilirubin concentration from 2-fold to 4-fold in 14%, fasting serum bile acids from 1.5-fold to 20-fold in 78%, γ-GT was elevated up to 3-fold in 11% and alkaline phosphatase up to 2-3-fold in 60% of patients[51]. Although a sensitive marker of other types of cholestasis, serum γ-GT is usually normal or modestly elevated[3,5,52,53]. Alkaline phosphatase is of poor diagnostic value due to placental and bone production. The prothrombin time is usually normal. When present, prolonged prothrombin times reflect vitamin K deficiency due to cholestasis or to the use of anion exchange resins (such as cholestyramine) rather than liver dysfunction[53,54]. Dann *et al*[57] reported significantly increased levels of low-density lipoprotein cholesterol in ICP, and proposed that this test might be useful to distinguish between ICP and pruritus gravidarum. An upper abdominal ultrasound is considered in patients with biliary colic or other manifestation of gallstone disease. Liver biopsy is rarely necessary for diagnosis. Histology is characterized by cholestasis without inflammation, and bile plugs in hepatocytes and canaliculi predominate in zone 3[58].

DIFFERENTIAL DIAGNOSIS

Pruritus, as the cardinal feature of ICP, helps distinguish it from other types of liver pregnancy-related disease that can share similar laboratory features (such as early HELLP syndrome, acute fatty liver of pregnancy or preeclampsia)[3,7,59]. In patients presenting with jaundice, choledocholithiasis should be excluded, and abdominal ultrasound is indicated in this situation. The differential diagnosis of pruritus without icterus should be focused on skin diseases (eczema, scabies, urticarial papules), allergic reactions, abdominal striae, diabetes and other metabolic diseases. In patients with high transaminases, acute viral hepatitis (A, B, C, E, Epstein-Barr virus, cytomegalovirus, herpes simplex virus), choledocholithiasis, toxic, autoimmune hepatitis might be excluded[3,52,53,59]. In some cases an underlying liver disorder can be diagnosed as ICP[4]. Hormone- and cytokine-induced reduction of expression and function of the principal sinusoidal or canalicular bile acid transporters in late pregnancy could result in the development of symptomatic cholestasis in a previously asymptomatic pregnant woman with hepatitis C or other chronic liver disease[4].

MATERNAL OUTCOME

Maternal prognosis is favourable[5,50,51]. Pruritus usually disappears in the first few days following delivery, accompanied by normalization of serum bile acid concentrations and other liver tests. The patients with ICP generally have no hepatic sequelae. If the pruritus and liver test abnormalities persist after delivery, chronic liver diseases such as primary biliary cirrhosis, primary sclerosing cholangitis or chronic hepatitis must be considered. ICP recurs during subsequent pregnancies in 45% to 70% of cases[5]. Affected women may also be at increased risk for the development of gallstones[60]. Persistent cholestasis frequently leads to malabsorption of fat-soluble vitamins, worsening maternal nutritional status. In addition, ICP is associated with increased incidence of intra- and postpartum hemorrhage. The administration of oral contraceptives to women with a history of ICP rarely results in cholestatic hepatitis. These women should be advised of the risk of cholestasis to use contraceptives with low dose estrogen or progesterone-only products[2,5,61].

FETAL OUTCOME

In contrast to the favourable prognosis for mothers, ICP poses significant risk for the fetus[5,62]. The major complications are premature deliveries in 19 to 60%[5], stillbirths in 0, 4 to 4, 1%[62,63] and fetal distress in 22 to 33% of cases[64,65]. The rate of malformations or abortions is not increased in ICP. Recent data show a marked decrease in fetal complication rates possibly due to greater awareness of the disease, experienced management and treatment[50,51,66]. The mechanism for poor perinatal outcome remains unclear. Because high bile salt levels were found to be associated with more frequent occurrence of fetal distress, this might be of great relevance for fetal prognosis[42]. Autopsies show signs of acute, lethal anoxia with petechial bleeding in pleura, pericardium and adrenal glands, but no signs of chronic anoxia[42,64]. Fetuses of women with ICP have adequate birthweights for gestational age and normal Doppler umbilical artery velocimetry, suggesting that chronic placental insufficiency is not the primary cause of fetal death[67]. Bile acids have been shown to induce vasoconstriction of human placental chorionic veins, and myometrial sensitivity to oxytocin[48,49,68]. In a study from Sweden, a correlation between fetal complications (defined as preterm delivery, asphyxial events, meconium staining of amniotic fluid, placenta, and membranes) and serum bile acid levels ($\geqslant$ 40 μmol/L) was found. The authors reported no increase in fetal risk detected in ICP patients with TBA levels < 40 μmol/L, and proposed that these women can be managed expectantly[50]. Further validation of these results is needed to determine the accuracy of this cutoff value. A recent case of fetal death at 39 wk and 3 d in a patient with ICP, who had low TBA concentrations at the time of diagnosis, has been reported[69]. This raises a crucial question: is the fasting TBA level sufficient to predict fetal outcome? Evaluation of bile acid concentrations may take several days even in major laboratories, making it an impractical tool for immediate risk stratification[70]. Therefore, it is important to evaluate other clinical factors that are possibly associated with prematurity. The risk of prematurity also appears to correlate inversely with onset of pruritus[71]. We recently reported that early onset of pruritus, along with markedly elevation of bile acid levels, may predict premature delivery[72].

MANAGEMENT

Various strategies have been proposed to improve obstetric outcome. Although, there is no ideal method for fetal surveillance in ICP, monitoring of fetal status is recommended in all cases. Nevertheless, in several studies, the investigators have concluded that fetal death in ICP may not be predictable by traditional antepartum surveillance, and that delivery after establishment of fetal lung maturity may reduce fetal mortality rate[62-64]. Obstetric management consists of weighing the risk of premature delivery against the risk of stillbirths. Some obstetricians recommended non stress tests or biophysical assessment[65]. However, no test reliably predicts the risk of fetal demise[62,63]. Intended to recognize a failing placenta, fetal cardiac monitoring cannot forecast an acute event such as an umbilical cord entanglement or a sudden fetal cardiac decompensation or arrhythmia[70]. As the majority of intrauterine fetal deaths in singleton pregnancies complicated by ICP occur after 37 wk, delivery has been recommended no later than 37-38 wk of gestation. Delivery at 36 wk if lung maturity is achieved or as soon thereafter as fetal lung maturity is established[62] or earlier should be considered for severe cases with jaundice and progressive elevation in serum TBA[62,65]. According to UK Guideline for Obstetric Cholestasis (2006), there are insufficient data to support or refute the popular practice of "early" (37 wk of gestation) induction of labour aimed at reducing late stillbirth. The timing and risks of delivery should therefore, be discussed on an individual basis[39]. Because the prognosis remains unpredictable in some cases[69], our current strategy is to begin pharmacological treatment with UDCA after confirmation of diagnosis in all ICP patients[72].

PHARMACOLOGICAL TREATMENT

The goal of pharmacologic treatment in ICP is to reduce maternal symptoms and improve fetal outcome. Antihistamines, benzodiazepines, phenobarbital, dexamethasone, epomediol, S-adenosyl-L-methionine, opioid antagonists, and cholestyramine have been used without clear evidence of efficacy[1-3]. Cholestyramine (8-16 g/d) was a widely used treatment for ICP related pruritus. It binds bile salts and interrupts their enterohepatic circulation. Observational studies suggest that cholestyramine may be associated with improved maternal morbidity without a documented improvement in fetal outcome[73-75]. Cholestyramine may worsen the malabsorption of fat-soluble vitamins, especially vitamin K. A case report of severe fetal intracranial hemorrhage during treatment with cholestyramine for ICP has raised the possibility that severe maternal vitamin K deficiency may lead to fetal vitamin K deficiency and coagulopathy[75].

Currently, the ursodeoxycholic acid (UDCA) is the most promising treatment for ICP. UDCA is hydrophilic bile acid. It is well tolerated by pregnant women, and no adverse effects in mothers or newborns have been observed[50,51,66]. Experimental evidence suggests that UDCA improves impaired hepatocellular secretion by mainly posttranscriptional stimulation of canalicular expression of transporters like the conjugate export pump, MRP2 (ABCC2), or the bile salt export pump, BSEP (ABCB11)[76,77]. In particular, targeting and insertion of these transporter proteins into the canalicular membrane by UDCA conjugates has been demonstrated in experimental models of cholestasis, which led to enhanced elimination of bile acid metabolites and other organic anions as well as steroid mono- and disulfates[76,77]. In addition to effects of UDCA on the maternal liver, UDCA

restores the impaired maternal-placental bile acid transport across the trophoblast. This could be mediated by enhanced expression of plasma membrane transporters involved in the excretory role of the placenta and would prevent structural alterations of the trophoblast induced by maternal cholestasis[78]. Effects of UDCA in ICP resemble the effects observed in other cholestatic diseases, although in ICP the clinical and biochemical effects are obtained faster, and they fade quickly when the drug is discontinued[79]. Following oral administration, approximately 30% to 60% of UDCA is absorbed in the gut[80]. The degree of UDCA enrichment in biliary bile following chronic ingestion correlates with the administered dose. Most clinical trials have used UDCA at a dose of 13-15 mg/kg per day[81,82]. Recently, Mazzella *et al*[83] reported positive results and no adverse reactions of high dose UDCA (1.5-2 g/d). In an open, randomized, parallel group study from Lithuania, 84 patients with ICP were randomized to either UDCA, 8-10 mg/kg body weight per day, or cholestyramine, 8 g per day, for 14 d. The onset of pruritus was approximately 31 wk to 32 wk of gestation while treatment was started at approximately 34 wk of gestation. A significant improvement of pruritus severity, aminotransferase activities, serum TBA concentrations and a more favorable outcome of pregnancy and absence of adverse events after treatment with UDCA was demonstrated. In contrast, cholestyramine alleviated pruritus only mildly and caused side effects[51,84]. In another double-blind, placebo-controlled trial, comparing UDCA (1 g/d for 3 wk) and dexamethasone (12 mg per day for one week) in 130 women with ICP, relief of pruritus was significantly more pronounced in the UDCA subgroup with bile acid levels > 40 μmol/L, and significant improvement of serum alanine aminotransferase and bilirubin levels irrespective of disease severity was observed in the UDCA group only. Dexamethasone produced no alleviation of pruritus or reduction of ALT activity and was less effective than UDCA in reducing bile acids and bilirubin levels[85]. In a retrospective, non-randomized analysis over a 12-year observation period, 32 patients had received UDCA (15 mg/kg per day) for at least 3 wk before delivery. Patients were compared with 16 historic controls who did not receive UDCA. UDCA treatment was associated with improvement in pruritus, serum bilirubin, aminotransferase and TBA levels, higher birth weight, and a greater proportion of deliveries at term. Twenty-six children whose mothers received UDCA were reexamined after 1 year to 12 years of follow-up all of whom were healthy[66].

The efficacy of the glutathione precursor S-adenosyl-methionine (SAMe) is controversial[86,87]. A controlled trial (46 patients) comparing SAMe with UDCA found that UDCA was significantly more effective at improving the concentration of serum bile acids and other liver biochemical tests, whereas both drugs equally reduced pruritus[88]. Another randomized placebo-controlled trial involving 32 patients showed the superiority of the combination of UDCA with SAMe over either drug alone for alleviating pruritus and improving liver tests[89]. A recent randomized prospective comparative study of UDCA (750 mg per day) and S-adenosyl-L-methionine (1000 mg per day intravenously) in 78 patients with ICP suggested that both regimens improved pruritus, but the combined therapy had no additive effect on pruritus as compared to UDCA monotherapy[90].

CONCLUSION

Intrahepatic cholestasis of pregnancy should be considered a high-risk condition, and careful fetal assessment and appropriate medical intervention might improve perinatal outcome. Although genetic research has not yet led to better prediction of the disease course, remarkable progress has been made in our understanding of the pathogenesis of ICP. Genetic risk profiling might help to stratify cases at the time of diagnosis, even before bile acid concentrations exceed levels that indicate an increased fetal risk. Additional studies in ICP populations from different countries are needed to further characterize the genetic background in these patients. UDCA is currently considered as first line therapy for ICP. Future prospective controlled studies may provide a better understanding of the underlying pathophysiological mechanisms of fetal risk, identify the most suitable monitoring modalities, and clarify the obstetrical management near term.

REFERENCES

1 **Riely CA**, Bacq Y. Intrahepatic cholestasis of pregnancy. *Clin Liver Dis* 2004; **8**: 167-176
2 **Reyes H**. Review: intrahepatic cholestasis. A puzzling disorder of pregnancy. *J Gastroenterol Hepatol* 1997; **12**: 211-216
3 **Lammert F**, Marschall HU, Glantz A, Matern S. Intrahepatic cholestasis of pregnancy: molecular pathogenesis, diagnosis and management. *J Hepatol* 2000; **33**: 1012-1021
4 **Beuers U**, Pusl T. Intrahepatic cholestasis of pregnancy--a heterogeneous group of pregnancy-related disorders? *Hepatology* 2006; **43**: 647-649
5 **Bacq Y**, Sapey T, Brechot MC, Pierre F, Fignon A, Dubois F. Intrahepatic cholestasis of pregnancy: a French prospective study. *Hepatology* 1997; **26**: 358-364
6 **Abedin P**, Weaver JB, Egginton E. Intrahepatic cholestasis of pregnancy: prevalence and ethnic distribution. *Ethn Health* 1999; **4**: 35-37
7 **Kondrackiene J**, Kupcinskas L. Liver diseases unique to pregnancy. *Medicina* (Kaunas) 2008; **44**: 337-345
8 **Pauli-Magnus C**, Meier PJ. Pharmacogenetics of hepatocellular transporters. *Pharmacogenetics* 2003; **13**: 189-198
9 **Savander M**, Ropponen A, Avela K, Weerasekera N, Cormand B, Hirvioja ML, Riikonen S, Ylikorkala O, Lehesjoki AE, Williamson C, Aittomaki K. Genetic evidence of heterogeneity in intrahepatic cholestasis of pregnancy. *Gut* 2003; **52**: 1025-1029
10 **Gonzalez MC**, Reyes H, Arrese M, Figueroa D, Lorca B, Andresen M, Segovia N, Molina C, Arce S. Intrahepatic cholestasis of pregnancy in twin pregnancies. *J Hepatol* 1989; **9**: 84-90
11 **Reyes H**, Sjovall J. Bile acids and progesterone metabolites in intrahepatic cholestasis of pregnancy. *Ann Med* 2000; **32**: 94-106
12 **Reyes H**, Simon FR. Intrahepatic cholestasis of pregnancy:

an estrogen-related disease. *Semin Liver Dis* 1993; **13**: 289-301
13 **Kreek MJ**. Female sex steroids and cholestasis. *Semin Liver Dis* 1987; **7**: 8-23
14 **Meng LJ**, Reyes H, Axelson M, Palma J, Hernandez I, Ribalta J, Sjovall J. Progesterone metabolites and bile acids in serum of patients with intrahepatic cholestasis of pregnancy: effect of ursodeoxycholic acid therapy. *Hepatology* 1997; **26**: 1573-1579
15 **Meng LJ**, Reyes H, Palma J, Hernandez I, Ribalta J, Sjovall J. Effects of ursodeoxycholic acid on conjugated bile acids and progesterone metabolites in serum and urine of patients with intrahepatic cholestasis of pregnancy. *J Hepatol* 1997; **27**: 1029-1040
16 **Stieger B**, Fattinger K, Madon J, Kullak-Ublick GA, Meier PJ. Drug- and estrogen-induced cholestasis through inhibition of the hepatocellular bile salt export pump (Bsep) of rat liver. *Gastroenterology* 2000; **118**: 422-430
17 **Huang L**, Smit JW, Meijer DK, Vore M. Mrp2 is essential for estradiol-17beta(beta-D-glucuronide)-induced cholestasis in rats. *Hepatology* 2000; **32**: 66-72
18 **Debry P**, Nash EA, Neklason DW, Metherall JE. Role of multidrug resistance P-glycoproteins in cholesterol esterification. *J Biol Chem* 1997; **272**: 1026-1031
19 **Simon FR**, Fortune J, Iwahashi M, Gartung C, Wolkoff A, Sutherland E. Ethinyl estradiol cholestasis involves alterations in expression of liver sinusoidal transporters. *Am J Physiol* 1996; **271**: G1043-G1052
20 **Leevy CB**, Koneru B, Klein KM. Recurrent familial prolonged intrahepatic cholestasis of pregnancy associated with chronic liver disease. *Gastroenterology* 1997; **113**: 966-972
21 **Jacquemin E**, Cresteil D, Manouvrier S, Boute O, Hadchouel M. Heterozygous non-sense mutation of the MDR3 gene in familial intrahepatic cholestasis of pregnancy. *Lancet* 1999; **353**: 210-211
22 **de Vree JM**, Jacquemin E, Sturm E, Cresteil D, Bosma PJ, Aten J, Deleuze JF, Desrochers M, Burdelski M, Bernard O, Oude Elferink RP, Hadchouel M. Mutations in the MDR3 gene cause progressive familial intrahepatic cholestasis. *Proc Natl Acad Sci USA* 1998; **95**: 282-287
23 **Jacquemin E**. Role of multidrug resistance 3 deficiency in pediatric and adult liver disease: one gene for three diseases. *Semin Liver Dis* 2001; **21**: 551-562
24 **Jacquemin E**, De Vree JM, Cresteil D, Sokal EM, Sturm E, Dumont M, Scheffer GL, Paul M, Burdelski M, Bosma PJ, Bernard O, Hadchouel M, Elferink RP. The wide spectrum of multidrug resistance 3 deficiency: from neonatal cholestasis to cirrhosis of adulthood. *Gastroenterology* 2001; **120**: 1448-1458
25 **Pauli-Magnus C**, Lang T, Meier Y, Zodan-Marin T, Jung D, Breymann C, Zimmermann R, Kenngott S, Beuers U, Reichel C, Kerb R, Penger A, Meier PJ, Kullak-Ublick GA. Sequence analysis of bile salt export pump (ABCB11) and multidrug resistance p-glycoprotein 3 (ABCB4, MDR3) in patients with intrahepatic cholestasis of pregnancy. *Pharmacogenetics* 2004; **14**: 91-102
26 **Wasmuth HE**, Glantz A, Keppeler H, Simon E, Bartz C, Rath W, Mattsson LA, Marschall HU, Lammert F. Intrahepatic cholestasis of pregnancy: the severe form is associated with common variants of the hepatobiliary phospholipid transporter ABCB4 gene. *Gut* 2007; **56**: 265-270
27 **Mullenbach R**, Linton KJ, Wiltshire S, Weerasekera N, Chambers J, Elias E, Higgins CF, Johnston DG, McCarthy MI, Williamson C. ABCB4 gene sequence variation in women with intrahepatic cholestasis of pregnancy. *J Med Genet* 2003; **40**: e70
28 **Schneider G**, Paus TC, Kullak-Ublick GA, Meier PJ, Wienker TF, Lang T, van de Vondel P, Sauerbruch T, Reichel C. Linkage between a new splicing site mutation in the MDR3 alias ABCB4 gene and intrahepatic cholestasis of pregnancy. *Hepatology* 2007; **45**: 150-158
29 **Floreani A**, Carderi I, Paternoster D, Soardo G, Azzaroli F, Esposito W, Montagnani M, Marchesoni D, Variola A, Rosa Rizzotto E, Braghin C, Mazzella G. Hepatobiliary phospholipid transporter ABCB4, MDR3 gene variants in a large cohort of Italian women with intrahepatic cholestasis of pregnancy. *Dig Liver Dis* 2008; **40**: 366-370
30 **Floreani A**, Carderi I, Paternoster D, Soardo G, Azzaroli F, Esposito W, Variola A, Tommasi AM, Marchesoni D, Braghin C, Mazzella G. Intrahepatic cholestasis of pregnancy: three novel MDR3 gene mutations. *Aliment Pharmacol Ther* 2006; **23**: 1649-1653
31 **Milkiewicz P**, Gallagher R, Chambers J, Eggington E, Weaver J, Elias E. Obstetric cholestasis with elevated gamma glutamyl transpeptidase: incidence, presentation and treatment. *J Gastroenterol Hepatol* 2003; **18**: 1283-1286
32 **Meier Y**, Zodan T, Lang C, Zimmermann R, Kullak-Ublick GA, Meier PJ, Stieger B, Pauli-Magnus C. Increased susceptibility for intrahepatic cholestasis of pregnancy and contraceptive-induced cholestasis in carriers of the 1331T>C polymorphism in the bile salt export pump. *World J Gastroenterol* 2008; **14**: 38-45
33 **Byrne JA**, Strautnieks SS, Mieli-Vergani G, Higgins CF, Linton KJ, Thompson RJ. The human bile salt export pump: characterization of substrate specificity and identification of inhibitors. *Gastroenterology* 2002; **123**: 1649-1658
34 **Doppelfeld IS**, Parnham MJ. Experimental conditions determine effects of ascorbic acid on reperfusion injury: comparison of tissue damage with hemodynamic parameters in rat isolated hearts. *Methods Find Exp Clin Pharmacol* 1992; **14**: 419-430
35 **Nies AT**, Keppler D. The apical conjugate efflux pump ABCC2 (MRP2). *Pflugers Arch* 2007; **453**: 643-659
36 **Sookoian S**, Castano G, Burgueno A, Gianotti TF, Pirola CJ. Association of the multidrug-resistance-associated protein gene (ABCC2) variants with intrahepatic cholestasis of pregnancy. *J Hepatol* 2008; **48**: 125-132
37 **Mullenbach R**, Bennett A, Tetlow N, Patel N, Hamilton G, Cheng F, Chambers J, Howard R, Taylor-Robinson SD, Williamson C. ATP8B1 mutations in British cases with intrahepatic cholestasis of pregnancy. *Gut* 2005; **54**: 829-834
38 **Painter JN**, Savander M, Ropponen A, Nupponen N, Riikonen S, Ylikorkala O, Lehesjoki AE, Aittomaki K. Sequence variation in the ATP8B1 gene and intrahepatic cholestasis of pregnancy. *Eur J Hum Genet* 2005; **13**: 435-439
39 **Pusl T**, Beuers U. Intrahepatic cholestasis of pregnancy. *Orphanet J Rare Dis* 2007; **2**: 26
40 **Reyes H**, Zapata R, Hernandez I, Gotteland M, Sandoval L, Jiron MI, Palma J, Almuna R, Silva JJ. Is a leaky gut involved in the pathogenesis of intrahepatic cholestasis of pregnancy? *Hepatology* 2006; **43**: 715-722
41 **Heikkinen J**, Maentausta O, Tuimala R, Ylostalo P, Janne O. Amniotic fluid bile acids in normal and pathologic pregnancy. *Obstet Gynecol* 1980; **56**: 60-64
42 **Laatikainen T**, Tulenheimo A. Maternal serum bile acid levels and fetal distress in cholestasis of pregnancy. *Int J Gynaecol Obstet* 1984; **22**: 91-94
43 **Rodrigues CM**, Marin JJ, Brites D. Bile acid patterns in meconium are influenced by cholestasis of pregnancy and not altered by ursodeoxycholic acid treatment. *Gut* 1999; **45**: 446-452
44 **Marin JJ**, Macias RI, Serrano MA. The hepatobiliary-like excretory function of the placenta. A review. *Placenta* 2003; **24**: 431-438
45 **Macias RI**, Pascual MJ, Bravo A, Alcalde MP, Larena MG, St-Pierre MV, Serrano MA, Marin JJ. Effect of maternal cholestasis on bile acid transfer across the rat placenta-maternal liver tandem. *Hepatology* 2000; **31**: 975-983
46 **Meng LJ**, Reyes H, Palma J, Hernandez J, Ribalta J, Sjovall J. Progesterone metabolism in normal human pregnancy and in patients with intrahepatic cholestasis of pregnancy. In: Reyes HB, Leuschner U, Arias IM. editors. Pregnancy,

sex hormones and the liver. Dordrecht: Kluver Academic Publishers, 1996: 91-100
47 **Monte MJ**, Rodriguez-Bravo T, Macias RI, Bravo P, el-Mir MY, Serrano MA, Lopez-Salva A, Marin JJ. Relationship between bile acid transplacental gradients and transport across the fetal-facing plasma membrane of the human trophoblast. *Pediatr Res* 1995; **38**: 156-163
48 **Sepulveda WH**, Gonzalez C, Cruz MA, Rudolph MI. Vasoconstrictive effect of bile acids on isolated human placental chorionic veins. *Eur J Obstet Gynecol Reprod Biol* 1991; **42**: 211-215
49 **Germain AM**, Kato S, Carvajal JA, Valenzuela GJ, Valdes GL, Glasinovic JC. Bile acids increase response and expression of human myometrial oxytocin receptor. *Am J Obstet Gynecol* 2003; **189**: 577-582
50 **Glantz A**, Marschall HU, Mattsson LA. Intrahepatic cholestasis of pregnancy: Relationships between bile acid levels and fetal complication rates. *Hepatology* 2004; **40**: 467-474
51 **Kondrackiene J**, Beuers U, Kupcinskas L. Efficacy and safety of ursodeoxycholic acid versus cholestyramine in intrahepatic cholestasis of pregnancy. *Gastroenterology* 2005; **129**: 894-901
52 **Nichols AA**. Cholestasis of pregnancy: a review of the evidence. *J Perinat Neonatal Nurs* 2005; **19**: 217-225
53 **Mullally BA**, Hansen WF. Intrahepatic cholestasis of pregnancy: review of the literature. *Obstet Gynecol Surv* 2002; **57**: 47-52
54 **Reyes H**, Radrigan ME, Gonzalez MC, Latorre R, Ribalta J, Segovia N, Alvarez C, Andresen M, Figueroa D, Lorca B. Steatorrhea in patients with intrahepatic cholestasis of pregnancy. *Gastroenterology* 1987; **93**: 584-590
55 **Brites D**, Rodrigues CM, Oliveira N, Cardoso M, Graca LM. Correction of maternal serum bile acid profile during ursodeoxycholic acid therapy in cholestasis of pregnancy. *J Hepatol* 1998; **28**: 91-98
56 **Castano G**, Lucangioli S, Sookoian S, Mesquida M, Lemberg A, Di Scala M, Franchi P, Carducci C, Tripodi V. Bile acid profiles by capillary electrophoresis in intrahepatic cholestasis of pregnancy. *Clin Sci* (Lond) 2006; **110**: 459-465
57 **Dann AT**, Kenyon AP, Wierzbicki AS, Seed PT, Shennan AH, Tribe RM. Plasma lipid profiles of women with intrahepatic cholestasis of pregnancy. *Obstet Gynecol* 2006; **107**: 106-114
58 **Rolfes DB**, Ishak KG. Liver disease in pregnancy. *Histopathology* 1986; **10**: 555-570
59 **Ropponen A**, Sund R, Riikonen S, Ylikorkala O, Aittomaki K. Intrahepatic cholestasis of pregnancy as an indicator of liver and biliary diseases: a population-based study. *Hepatology* 2006; **43**: 723-728
60 **Hirvioja ML**, Kivinen S. Inheritance of intrahepatic cholestasis of pregnancy in one kindred. *Clin Genet* 1993; **43**: 315-317
61 **Saleh MM**, Abdo KR. Intrahepatic cholestasis of pregnancy: review of the literature and evaluation of current evidence. *J Womens Health* (Larchmt) 2007; **16**: 833-841
62 **Rioseco AJ**, Ivankovic MB, Manzur A, Hamed F, Kato SR, Parer JT, Germain AM. Intrahepatic cholestasis of pregnancy: a retrospective case-control study of perinatal outcome. *Am J Obstet Gynecol* 1994; **170**: 890-895
63 **Alsulyman OM**, Ouzounian JG, Ames-Castro M, Goodwin TM. Intrahepatic cholestasis of pregnancy: perinatal outcome associated with expectant management. *Am J Obstet Gynecol* 1996; **175**: 957-960
64 **Fisk NM**, Bye WB, Storey GN. Maternal features of obstetric cholestasis: 20 years experience at King George V Hospital. *Aust N Z J Obstet Gynaecol* 1988; **28**: 172-176
65 **Heinonen S**, Kirkinen P. Pregnancy outcome with intrahepatic cholestasis. *Obstet Gynecol* 1999; **94**: 189-193
66 **Zapata R**, Sandoval L, Palma J, Hernandez I, Ribalta J, Reyes H, Sedano M, Toha D, Silva JJ. Ursodeoxycholic acid in the treatment of intrahepatic cholestasis of pregnancy. A 12-year experience. *Liver Int* 2005; **25**: 548-554
67 **Zimmermann P**, Koskinen J, Vaalamo P, Ranta T. Doppler umbilical artery velocimetry in pregnancies complicated by intrahepatic cholestasis. *J Perinat Med* 1991; **19**: 351-355
68 **Simpson LL**. Maternal medical disease: risk of antepartum fetal death. *Semin Perinatol* 2002; **26**: 42-50
69 **Sentilhes L**, Verspyck E, Pia P, Marpeau L. Fetal death in a patient with intrahepatic cholestasis of pregnancy. *Obstet Gynecol* 2006; **107**: 458-460
70 **Egerman RS**, Riely CA. Predicting fetal outcome in intrahepatic cholestasis of pregnancy: is the bile acid level sufficient? *Hepatology* 2004; **40**: 287-288
71 **Williamson C**, Hems LM, Goulis DG, Walker I, Chambers J, Donaldson O, Swiet M, Johnston DG. Clinical outcome in a series of cases of obstetric cholestasis identified via a patient support group. *BJOG* 2004; **111**: 676-681
72 **Kondrackiene J**, Beuers U, Zalinkevicius R, Tauschel HD, Gintautas V, Kupcinskas L. Predictors of premature delivery in patients with intrahepatic cholestasis of pregnancy. *World J Gastroenterol* 2007; **13**: 6226-6230
73 **Jenkins JK**, Boothby LA. Treatment of itching associated with intrahepatic cholestasis of pregnancy. *Ann Pharmacother* 2002; **36**: 1462-1465
74 **Burrows RF**, Clavisi O, Burrows E. Interventions for treating cholestasis in pregnancy. *Cochrane Database Syst Rev* 2001; CD000493
75 **Sadler LC**, Lane M, North R. Severe fetal intracranial haemorrhage during treatment with cholestyramine for intrahepatic cholestasis of pregnancy. *Br J Obstet Gynaecol* 1995; **102**: 169-170
76 **Paumgartner G**, Beuers U. Ursodeoxycholic acid in cholestatic liver disease: mechanisms of action and therapeutic use revisited. *Hepatology* 2002; **36**: 525-531
77 **Beuers U**. Drug insight: Mechanisms and sites of action of ursodeoxycholic acid in cholestasis. *Nat Clin Pract Gastroenterol Hepatol* 2006; **3**: 318-328
78 **Serrano MA**, Brites D, Larena MG, Monte MJ, Bravo MP, Oliveira N, Marin JJ. Beneficial effect of ursodeoxycholic acid on alterations induced by cholestasis of pregnancy in bile acid transport across the human placenta. *J Hepatol* 1998; **28**: 829-839
79 **Palma J**, Reyes H, Ribalta J, Hernandez I, Sandoval L, Almuna R, Liepins J, Lira F, Sedano M, Silva O, Toha D, Silva JJ. Ursodeoxycholic acid in the treatment of cholestasis of pregnancy: a randomized, double-blind study controlled with placebo. *J Hepatol* 1997; **27**: 1022-1028
80 **Rubin RA**, Kowalski TE, Khandelwal M, Malet PF. Ursodiol for hepatobiliary disorders. *Ann Intern Med* 1994; **121**: 207-218
81 **Berkane N**, Cocheton JJ, Brehier D, Merviel P, Wolf C, Lefevre G, Uzan S. Ursodeoxycholic acid in intrahepatic cholestasis of pregnancy. A retrospective study of 19 cases. *Acta Obstet Gynecol Scand* 2000; **79**: 941-946
82 **Angulo P**. Use of ursodeoxycholic acid in patients with liver disease. *Curr Gastroenterol Rep* 2002; **4**: 37-44
83 **Mazzella G**, Rizzo N, Azzaroli F, Simoni P, Bovicelli L, Miracolo A, Simonazzi G, Colecchia A, Nigro G, Mwangemi C, Festi D, Roda E. Ursodeoxycholic acid administration in patients with cholestasis of pregnancy: effects on primary bile acids in babies and mothers. *Hepatology* 2001; **33**: 504-508
84 **Kondrackiene J**, Kupcinskas L. Liver disease in pregnancy: diagnosis and treatment. In: Diehl AM, Hayashi N, Manns MP, Sauerbruch T, editors. Chronic hepatitis: metabolic, cholestatic, viral and autoimmune. Dordrecht: Springer, 2007: 43-53
85 **Glantz A**, Marschall HU, Lammert F, Mattsson LA. Intrahepatic cholestasis of pregnancy: a randomized controlled trial comparing dexamethasone and ursodeoxycholic acid. *Hepatology* 2005; **42**: 1399-1405

86 **Ribalta J**, Reyes H, Gonzalez MC, Iglesias J, Arrese M, Poniachik J, Molina C, Segovia N. S-adenosyl-L-methionine in the treatment of patients with intrahepatic cholestasis of pregnancy: a randomized, double-blind, placebo-controlled study with negative results. *Hepatology* 1991; **13**: 1084-1089

87 **Floreani A**, Paternoster D, Melis A, Grella PV. S-adenosylmethionine versus ursodeoxycholic acid in the treatment of intrahepatic cholestasis of pregnancy: preliminary results of a controlled trial. *Eur J Obstet Gynecol Reprod Biol* 1996; **67**: 109-113

88 **Roncaglia N**, Locatelli A, Arreghini A, Assi F, Cameroni I, Pezzullo JC, Ghidini A. A randomised controlled trial of ursodeoxycholic acid and S-adenosyl-l-methionine in the treatment of gestational cholestasis. *BJOG* 2004; **111**: 17-21

89 **Nicastri PL**, Diaferia A, Tartagni M, Loizzi P, Fanelli M. A randomised placebo-controlled trial of ursodeoxycholic acid and S-adenosylmethionine in the treatment of intrahepatic cholestasis of pregnancy. *Br J Obstet Gynaecol* 1998; **105**: 1205-1207

90 **Binder T**, Salaj P, Zima T, Vitek L. Randomized prospective comparative study of ursodeoxycholic acid and S-adenosyl-L-methionine in the treatment of intrahepatic cholestasis of pregnancy. *J Perinat Med* 2006; **34**: 383-391

S- Editor Zhong XY **L- Editor** Lalor PF **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5789

World J Gastroenterol 2008 October 14; 14(38): 5789-5796
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Dr. Kalpesh Jani, *Series Editor*

Pancreatic fistula after pancreatectomy: Evolving definitions, preventive strategies and modern management

Shailesh V Shrikhande, Melroy A D'Souza

Shailesh V Shrikhande, Melroy A D'Souza, Department of GI and HPB Surgical Oncology, Tata Memorial Hospital, Mumbai 400-012, India
Author contributions: Shrikhande SV conceptualised the paper, provided important intellectual contributions and modified the manuscript; D'Souza MA wrote the paper and provided intellectual contributions.
Correspondence to: Shailesh V Shrikhande, MS, MD, Associate Professor and Consultant Surgeon, Department of GI and HPB Surgical Oncology, Ernest Borges Marg, Parel, Mumbai 400-012, India. shailushrikhande@hotmail.com
Telephone: + 91-22-24144489 Fax: + 91-22-24148114
Received: June 11, 2008 Revised: July 28, 2008
Accepted: August 4, 2008
Published online: October 14, 2008

Abstract

Pancreatic resection is the treatment of choice for pancreatic malignancy and certain benign pancreatic disorders. However, pancreatic resection is technically a demanding procedure and whereas mortality after a pancreaticoduodenectomy is currently < 3%-5% in experienced high-volume centers, post-operative morbidity is considerable, about 30%-50%. At present, the single most significant cause of morbidity and mortality after pancreatectomy is the development of pancreatic leakage and fistula (PF). The occurrence of a PF increases the length of hospital stay and the cost of treatment, requires additional investigations and procedures, and can result in life-threatening complications. There is no universally accepted definition of PF that would allow standardized reporting and proper comparison of outcomes between different centers. However, early recognition of a PF and prompt institution of appropriate treatment is critical to the prevention of potentially devastating consequences. The present article, reviews the evolution of post resection pancreatic fistula as a concept, and discusses evolving definitions, the current preventive strategies and the management of this problem.

Key words: Pancreatic fistula; Pancreaticoduodenectomy; Pancreatic anastomosis; Pancreatic anastomotic failure; Complications

Peer reviewer: Laura Llado, PhD, Liver Transplant Unit, Dept of Surgery, Hospital U Bellvitge, Feixa Llarga S/N, Hospitalet LL (Barcelona) 08907, Spain

Shrikhande SV, D'Souza MA. Pancreatic fistula after pancreatectomy: Evolving definitions, preventive strategies and modern management. *World J Gastroenterol* 2008; 14(38): 5789-5796 Available from: URL: http://www.wjgnet.com/1007-9327/14/5789.asp DOI: http://dx.doi.org/10.3748/wjg.14.5789

INTRODUCTION

Pancreatic resection is the standard treatment for pancreatic malignancy and certain benign pancreatic disorders. However, pancreatic resection is technically a demanding procedure. At diagnosis, more than 85% of pancreatic tumors are at an advanced stage. Thus, potentially curative resections are possible only in 10%-15% patients[1,2]. The standard surgical procedure for a lesion in the pancreatic head is pancreaticoduodenectomy (PD), while distal pancreatectomy (DP) with or without splenectomy, is performed for tumors in the body and tail.

At experienced high-volume centers, mortality after PD is currently 3%-5%. However, there is considerable post-operative morbidity, around 30%-50%[3,4]. At present, the single most significant cause of morbidity and mortality after PD is the development of pancreatic leak and fistula (PF), and rates of up to 20% are reported from centers specializing in pancreatic surgery[4-6]. The development of PF increases the length of hospital stay and the cost of treatment, necessitates the use of additional investigations and procedures, and can cause life-threatening complications.

Various strategies have been employed to decrease the incidence of PF, including pharmacologic manipulation, and modifications and refinements in the surgical technique. These have resulted in varying degrees of success. However, it is clear that early recognition of PF and prompt institution of appropriate treatment is the cornerstone in the prevention of potentially devastating consequences. The present article reviews the evolution of post-resection pancreatic fistula, and discusses the evolving definitions, and current preventive strategies and management approaches.

Table 1 The different components of previously used definitions of pancreatic fistula and the new grading system used by the International Study Group for Pancreatic Fistula (ISGPF)

Commonly used definitions used prior to the ISGPF recommendations

Output > 10 mL/d of amylase rich fluid on post-operative day 5 or for > 5 d.
Output > 10 mL/d of amylase rich fluid on post-operative day 8 or for > 8 d.
Output between 25 mL/d and 100 mL/d of amylase rich fluid after post-operative day 8 or for > 8 d.
Output > 50 mL/d of amylase rich fluid after post-operative day 11 or for > 11 d.

ISGPF Definition: "Output *via* an operatively placed drain (or a subsequently placed percutaneous drain) of any measurable volume of drain fluid on or after postoperative d 3, with an amylase content greater than 3 times the upper normal serum value"

Grade A	"Transient fistula"	No clinical impact	No peri-pancreatic collections on CT scan; little/no change in management	Clinically well; no sepsis; no prolongation of hospital stay; slow removal of operatively placed drains
Grade B		Clinical impact	Peri-pancreatic drains in place or repositioned to drain collections; Change in management is required	Clinically fairly well; degree of infection requiring specific treatment; prolongation of hospital stay; patients often discharged with drains in situ and observed in outpatient setting
Grade C		Severe clinical impact	Worrisome peri-pancreatic collections that require percutaneous drains; major change in management usually in ICU setting; possible re-surgery to salvage a difficult situation (completion pancreatectomy *etc*)	Clinically unwell; associated sepsis requiring aggressive antibiotics, octreotide and other intensive care support; major prolongation of hospital stay; associated complications and possibility of mortality

DEFINITIONS

There is no universally accepted definition of PF. While some workers have emphasized on the volume (and colour) of the drain output, and its duration, others have stressed more on the amylase content of the drainage fluid[7-9]. In a study published in 2004, Bassi *et al*[10] summarized 4 definitions of PF (Table 1) and applied each definition to 242 patients who had undergone pancreatic resection. The results revealed wide variations in the incidence of PF, from 10% to 29% depending upon the definition used. Therefore, it is essential to standardize the reporting of post-PD complications, especially PF. This led to the unified definition, now known as the International Study Group on Pancreatic Fistula (ISGPF) definition[11]. The definition was intended to standardize the reporting of postoperative PF. The essential component of an anastomotic leak was the high amylase content (> 3 times the upper normal serum value), of the drain fluid (of any measurable volume), at any time on or after the 3rd postoperative day. The ISGPF definition also graded PF (Grades A, B and C) according to the clinical impact on the patient's hospital course and eventual outcome. The various components of the previously used definitions and the new grading of the ISGPF are shown in Table 1. More recently, Pratt *et al*[12] sought to validate the ISGPF classification in 176 patients who underwent PD and concluded that with increasing fistula grades, there was a negative clinical and economic impact on patients and their healthcare resources.

However the applicability and utility of the ISGPF definition in allowing uniform comparisons of fistula rates has been questioned by some workers. In a recent publication, Strasberg *et al*[13] proposed that intra-abdominal collections along with hemorrhage and peritonitis are also the result of a pancreatic anastomotic failure. These workers sought to redefine pancreatic fistulae as "pancreatic anastomotic failures (PAF)" which includes the entire spectrum of clinically relevant problems associated with the loss of integrity of a pancreatico-enterostomy. They also sought to categorize fistulae that occur after DP or segmental resection and enucleation, situations which do not involve pancreatico-enterostomy, as an entity that is distinct from fistulae occurring after PD. These fistulae were termed as pancreatic occlusion failure (POF). POF commonly runs a more benign course (compared to PAF), since enzyme activation does not occur in the absence of a pancreaticoenteric anastomosis. Strasberg *et al* also noted that the definition of PAF should only include patients in whom there is a change in the management, whereas asymptomatic fistulae with merely high drain amylase and no change in the clinical course should not be considered as an operative complication, as proposed by the ISGPF definition. They thus defined PAF and graded its severity into 7 categories in 5 grades (Table 2). They proposed the adoption of the definition of PF used by the Johns Hopkins group[14]. These workers considered any intra-abdominal fluid collection after pancreatic surgery as PAF, if it was not found to be caused by the failure of other anastomoses performed during a PD. In addition, any hemorrhage was considered as PAF unless the pancreatico-enterostomy was shown to be intact. With respect to intra-abdominal collections, a recent study concluded that post-operative intra-abdominal collections after PD were PF. In this study, it was observed that when the initial drain amylase levels were normal, repeat estimation of the amylase level helped uncover previously undiagnosed PF or newly developed PF, thus providing a more precise estimate of postoperative PF rate[15]. From the preceding discussion it is evident that the definition of what constitutes a pancreatic fistula or anastomotic failure is still a matter of considerable debate. Clearly, more studies are needed before a common unifying definition of PF can be adopted.

PREVENTIVE STRATEGIES FOR PF

Considering the dismal outcome of a PF, much effort

Table 2 Pancreatic anastomotic failure (PAF) as described by Strasberg *et al*

Definition: "Drainage of greater than 50 mL amylase-rich fluid (> 3-fold elevation above upper limit of normal in serum) per day through the drains on or after postoperative d 10, or pancreatic anastomotic disruption shown radiographically"	
Grade 1	Deviation from normal postoperative course without pharmacologic, endoscopic, surgical or radiologic intervention (certain drugs allowed)
Grade 2	Pharmacologic treatment needed. Includes blood transfusions and total parenteral nutrition
Grade 3 (a/b)	Surgical, endoscopic or interventional radiologic treatment needed a: Not under general anesthesia b: Under general anesthesia
Grade 4 (a/b)	Life threatening complications and organ dysfunction a: Single organ b: Multi-organ
Grade 5	Death due to PAF

has gone into preventing its occurrence. These measures primarily include technical modifications of constructing a pancreatic anastomosis. The correct management of a pancreatic remnant after a PD is a matter of much debate and this is reflected in the variety of techniques that have evolved over the years for the construction of a safe pancreatic anastomosis. Furthermore, the outcome of a pancreatic anastomosis depends, among other factors, on the consistency of the pancreatic parenchyma and the diameter of the main pancreatic duct. Clearly, the outcome is better when the pancreas is hard and the duct is dilated (*e.g.* in chronic pancreatitis) as compared to a soft pancreas with a non dilated duct (*e.g.* in low common bile duct cancer).

Other innovations include the use of biological adhesives designed to seal the anastomosis, ligation or occlusion of the main pancreatic duct, optimization of the blood supply to the pancreatic remnant, use of somatostatin and its analogues to inhibit pancreatic secretion, and even total pancreatectomy[16].

Pancreatic duct occlusion

Occlusion of the pancreatic duct can be achieved by simple suture ligation of the duct or injection of the duct with non-reabsorbable or reabsorbable glues. Simple duct ligation, advocated in the past, has been largely abandoned due to high PF rates, nearing 50%[17,18]. However in a recent prospective randomized controlled trial (RCT) by Tran *et al*[19] which compared pancreaticojejunostomy with duct occlusion alone, there were no significant differences in the morbidity or mortality, but the incidence of diabetes mellitus was higher in patients with duct occlusion.

In a study on 51 patients, Di Carlo *et al*[20] used a nonreabsorbable glue (neoprene) to occlude the pancreatic duct after PD. The authors concluded that intra-ductal injection of Neoprene after pancreaticoduodenectomy was a safer procedure compared to pancreaticojejunal anastomosis and was not associated with post surgical diabetes. In a prospective, multi-center RCT of 182 patients undergoing either PD or DP, 102 patients received pancreatic ductal occlusion with fibrin glue. Analysis showed that duct occlusion had no effect on the rate or severity of intra-abdominal complications after pancreatic resection[21].

Type of pancreatic anastomosis: Pancreaticojeju-nostomy (PJ) versus pancreaticogastrostomy (PG)

The safe reconstruction of pancreaticoenteric continuity is the key to preventing a PF. The risk of fistula formation depends on the consistency of the remnant pancreas, caliber of the main pancreatic duct, pancreatic vascularity and the technique of construction of the pancreatic anastomosis[22,23]. PJ and PG are the two most commonly employed techniques for the reconstruction of pancreaticoenteric continuity. PJ can be performed by the dunking method or the duct-to-mucosal anastomosis. Surgical techniques such as PG and PJ which are employed for the management of the pancreatic remnant after PD have been evaluated in only a few randomized controlled studies[14,24,25]. Earlier uncontrolled studies were in favor of PG[26,27]. Due to the close proximity of the stomach, a PG was believed to be easier to perform and less prone to ischemia as a result of the rich blood supply of the stomach. However, all the RCTs have failed to show any difference in the overall post-operative complication rate or incidence of PF. Two recent meta-analyses have shown that while non randomized observational studies showed a superiority of PG over PJ, RCTs failed to show superiority of any one technique, thereby concluding that both PJ and PG provided equivalent results[28,29]. Thus, it can be concluded that as long as a tension-free anastomosis between well perfused tissues is performed, employing fine sutures and using the same technique, any type of pancreatic anastomosis should result in a good outcome[3,30].

Stenting of the main pancreatic duct

Stenting of the pancreatic duct during pancreaticoenteric anastomosis facilitates the precise placement of mucosal sutures, diverts pancreatic juice away from the anastomotic site and decreases the risk of inadvertent pancreatic duct occlusion. In doing so, it is believed that the anastomotic integrity improves, thereby reducing the PF rate. The results of this strategy have been encouraging[31,32]. In a prospective but non-randomized trial in 85 patients, Roder *et al*[31] demonstrated that stenting the pancreatic duct reduced the PF rate from 68% to 29.3%, and the median hospital stay from 29 d to 13 d. Poon *et al*[33] reported that external drainage of the pancreatic duct with a stent reduced the leakage of PJ after PD. On

the other hand, some well designed studies have shown no benefit of internal stenting in preventing PF[34,35]. Thus, the available evidence is conflicting and the use of stents depends on personal choice and experience of the pancreatic surgeon.

Role of magnification in pancreatic anastomosis

Since a duct to mucosa anastomosis is crucial for good outcome, a meticulous approximation assumes great importance. Operating loupes have been used by many experts to allow precise construction of a pancreatic anastomosis. Wada *et al*[36] in a retrospective analysis highlighted the role of the operating microscope in constructing a pancreatic anastomosis. Technical errors which may occur during anastomosis include crossing of the sutures, including both sides of the pancreatic duct while passing the suture, taking unequal and inadequate amounts of pancreatic duct and jejunal mucosa, and incorrect knot placement resulting in air knots. All these events can be avoided by using magnification. The study by Traverso, reported a markedly reduced incidence of PF with the operating microscope compared to operating loupes.

OTHER SURGICAL TECHNICAL MODIFICATIONS/APPROACHES

Blood supply based technique of PJ

One of the few modifications which have demonstrated a substantial reduction in the rate of PF after PD was proposed by Strasberg *et al*[37]. These workers put forward the concept of vascular watershed in the pancreatic neck and its role in ischemia of the cut surface of the pancreatic remnant. In their technique, the blood supply at the cut surface of the pancreas was evaluated, and if necessary, the pancreas was cut back 1.5 cm to 2.0 cm to improve the blood supply (n = 47; 38% patients). Thereafter, the anastomosis was performed meticulously under magnification. The authors concluded that a combination of optimization of blood supply to the pancreatic remnant, and a meticulous technique resulted in reduced PF rate, from previous reports of 10% to 1.6% in their series of 123 resections.

Duct-to-mucosa versus invagination anastomosis

A number of studies have demonstrated a lower rate of PF using the duct-to-mucosa technique for pancreatic anastomosis[38-40]. However a prospective RCT by Bassi *et al*[41] revealed no significant difference in the morbidity and PF rate between duct-to-mucosa anastomosis and single layer end-to-side pancreatico-jejunostomy.

Total pancreatectomy

The rationale for total pancreatectomy is that it allows a more extensive lymphadenectomy, obviates the risk of leak from the pancreatic anastomosis and decreases the chances of a positive resection margin. However, total pancreatectomy is associated with obligatory diabetes mellitus, decreased immunity because of splenectomy, and loss of pancreatic exocrine function. Most studies have reported either worse survival or no survival difference between total pancreatectomy and standard PD[42-44]. Total pancreatectomy should not be performed in most cases of carcinoma of the pancreatic head, unless serial positive resection margins are obtained on frozen section examination, or the pancreas is deemed to be very soft with a very high risk of pancreatic leak, and in patients with documented family history of multi-centric disease[45].

Stapled or hand-sewn closure of the pancreatic remnant after DP

PF remains a major cause of morbidity after DP. A number of techniques have been used to reduce the incidence of PF after DP, including hand-sewn closure, staple closure, combined staple and suture closure, fibrin glue application, serosal jejunal patch and prolamine injection[46]. While hand-sewn closure has stood the test of time, the use of staplers is gaining increasing acceptance, especially with the advent of laparoscopic DP. Knaebel *et al*[47] performed a meta-analysis that included six studies comparing stapler versus hand-sutured closure, which showed a non-significant combined odds ratio for pancreatic fistula of 0.66 (95% confidence interval 0.35 to 1.26, P = 0.21) in favor of staple closure. However, a large retrospective study of 302 DP's, showed that stapler closure was associated with a higher rate of pancreatic fistula[48]. Thus, the jury is still out and surgeons must follow their own individual experience when dealing with pancreatic remnant after DP. In hand sewn closure, the guiding principle is to make every effort to identify the pancreatic duct, close it with fine sutures and then close the entire stump with sutures.

Role of Octreotide and Somatostatin analogues in decreasing the rate of PF after pancreatic surgery

Octreotide is a synthetic analogue of somatostatin, and like somatostatin inhibits pancreatic exocrine secretion. Several prospective RCTs conducted in Europe evaluated the use of subcutaneous octreotide/somatostatin in patients undergoing elective pancreatic resection for different indications[8,49-52]. The results show that octreotide reduced the development of pancreatic fistula and other complications. However RCTs by Sarr *et al*[9], Yeo *et al*[53] and Lowy *et al*[54], failed to show a similar benefit in the peri-operative use of somatostatin analogues in patients undergoing pancreatic resection. Meta-analysis and systematic reviews of octreotide use have also yielded conflicting results[55,56]. These findings notwithstanding, many surgeons continue to use octreotide in patients undergoing pancreatic surgery. Selective administration of octreotide in patients considered to have high risk pancreas (soft texture, small duct size, and presence of ampullary, duodenal, cystic or islet cell pathology) may be associated with a decreased incidence of PF[57].

MANAGEMENT OF PF (FIGURE 1)

Despite numerous novel strategies designed to prevent the development of postoperative PF, it is clear that in order to minimize the potentially devastating effect of PF,

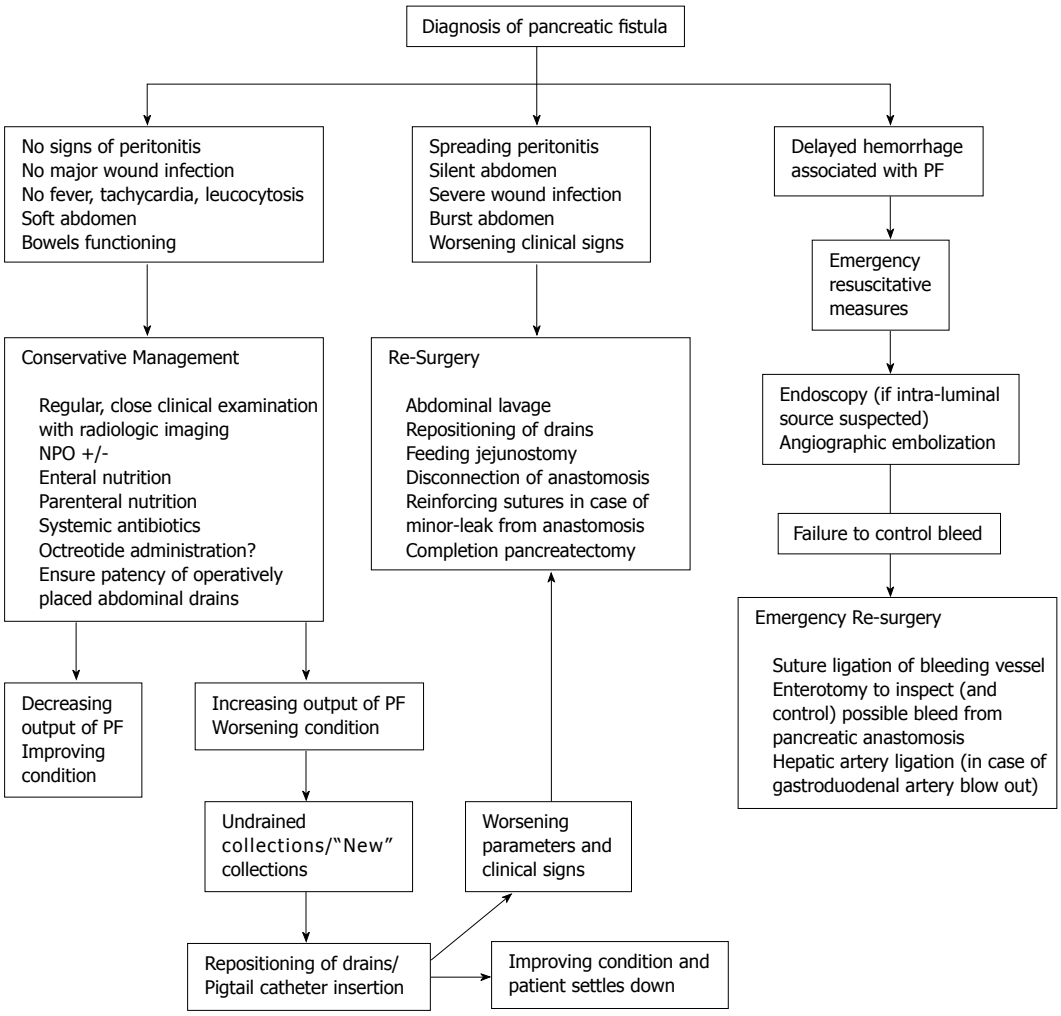


Figure 1 Approach to management of post pancreatectomy PF. Other procedures to manage complex PF may have to be added.

it is essential that this complication is recognized as soon as it develops, and appropriate treatment measures are instituted promptly. The suspicion of PF begins whenever there is a deviation in the normal clinical course of a patient who has just undergone a major pancreatic surgery. This may mean a patient who develops unexpected upper abdominal discomfort (often associated with fever), leucocytosis, increasing tachycardia, or just feels unwell after an apparently "normal" initial post-operative recovery. Furthermore, there may be high amylase content of a drain, a persistently high drain output, altered drain colour and quality, and other complications such as severe wound infection and hemorrhage. Routine radiologic evaluation is neither necessary nor recommended for establishing a diagnosis of PF[57]. What constitutes a PF is a matter of which definition is used, and varies from center to center. Regardless, once a diagnosis of PF is established, aggressive and appropriate conservative management is the key to successful outcome.

The management in the majority of patients is based on conservative measures. However, interventional radiological assistance is sometimes required, but repeat surgery is rarely indicated[16,58].

CONSERVATIVE MANAGEMENT

A conservative approach to the management of PF is successful in over 90% patients[59,60]. This involves clinical evaluation of the patient at short intervals. If the patient does not have any fever, tachycardia, leucocytosis, severe wound infection, and the abdomen is soft (with functioning bowel), and no signs of peritonitis, it is safe to continue with conservative measures. These measures include maintenance of enteral nutrition (through an operatively placed nasojejunal tube or a feeding jejunostomy), nasogastric suction (in the presence of delayed gastric emptying secondary to PF), and appropriate antibiotic coverage. In situations where the abdomen has not "really settled", the option of total parenteral nutrition should be considered. All along, the abdominal drains and the main wound require close attention. The effectiveness of octreotide in aiding the closure of a PF has not provided encouraging results[61]. The interventional radiologist may play a crucial role by image-guided repositioning of operatively placed drains and insertion of percutaneous catheters to drain collections seen on CT scan[60,62]. Delayed hemorrhage following PF is perhaps best managed by angiography and embolization of the bleeding vessel. This treatment is successful in stopping the bleeding in 80% patients[63]. The prognosis of patients with post-pancreatectomy hemorrhage depends on whether or not PF is present. The decision-making should be guided by factors such as the time of onset of the bleeding, presence of PF, vascular pathology, and the

underlying disease process[63]. The failure to successfully control hemorrhage by conservative measures like angiographic embolization may necessitate repeat surgery[63-66]. Obviously, the management of complications associated with PF requires a multidisciplinary approach, involving the pancreatic surgeon, intensive care team, and interventional radiologists. Kazanjian *et al*[59] evaluated 437 patients who underwent PD. A total of 55 (12.6%) developed PF; 52 patients (94.5%) had successful conservative management with prolonged tube drainage, 4 required percutaneous drainage and only 3 patients (5.5%) had repeat surgery.

OPERATIVE MANAGEMENT

Pancreatic resection is now considered a safe procedure when performed in high volume centers. PF can be successfully managed by conservative measures, as described earlier. The indications for surgical intervention in PF include worsening clinical parameters, signs of spreading peritonitis, severe wound infection, wound dehiscence, and delayed hemorrhage. When a decision is made to reoperate a patient with PF, the following measures should be considered: abdominal lavage with repositioning of drains, control of hemorrhage, use of sutures to control a small dehiscence, disconnection of the pancreatic anastomosis, a feeding jejunostomy (if not already in place) and occasionally completion pancreatectomy[61,67]. In patients with delayed hemorrhage who require repeat surgery, a thorough exploration of the resection site is required and if necessary, ligation of the arterial stumps (including occasionally the common hepatic artery) and inspection of the anastomosis by enterotomy[64]. It is worth noting that with improvements in the results of pancreatic surgery and the success of interventional radiology in managing complications, completion pancreatectomy is seldom required, and it has even been suggested that it should no longer be considered in patients with a PF[58].

The approach to the management of a patient with PF is summarized in Figure 1.

REFERENCES

1 **Wagner M**, Redaelli C, Lietz M, Seiler CA, Friess H, Buchler MW. Curative resection is the single most important factor determining outcome in patients with pancreatic adenocarcinoma. *Br J Surg* 2004; **91**: 586-594

2 **D'souza MA**, Shrikhande SV. Pancreatic resectional surgery: an evidence-based perspective. *J Cancer Res Ther* 2008; **4**: 77-83

3 **Shrikhande SV**, Barreto G, Shukla PJ. Pancreatic fistula after pancreaticoduodenectomy: the impact of a standardized technique of pancreaticojejunostomy. *Langenbecks Arch Surg* 2008; **393**: 87-91

4 **Schmidt CM**, Powell ES, Yiannoutsos CT, Howard TJ, Wiebke EA, Wiesenauer CA, Baumgardner JA, Cummings OW, Jacobson LE, Broadie TA, Canal DF, Goulet RJ Jr, Curie EA, Cardenes H, Watkins JM, Loehrer PJ, Lillemoe KD, Madura JA. Pancreaticoduodenectomy: a 20-year experience in 516 patients. *Arch Surg* 2004; **139**: 718-725; discussion 725-727

5 **Bassi C**, Falconi M, Salvia R, Mascetta G, Molinari E, Pederzoli P. Management of complications after pancreaticoduodenectomy in a high volume centre: results on 150 consecutive patients. *Dig Surg* 2001; **18**: 453-457; discussion 458

6 **Balcom JH 4th**, Rattner DW, Warshaw AL, Chang Y, Fernandez-del Castillo C. Ten-year experience with 733 pancreatic resections: changing indications, older patients, and decreasing length of hospitalization. *Arch Surg* 2001; **136**: 391-398

7 **Buchler MW**, Friess H, Wagner M, Kulli C, Wagener V, Z'Graggen K. Pancreatic fistula after pancreatic head resection. *Br J Surg* 2000; **87**: 883-889

8 **Pederzoli P**, Bassi C, Falconi M, Camboni MG. Efficacy of octreotide in the prevention of complications of elective pancreatic surgery. Italian Study Group. *Br J Surg* 1994; **81**: 265-269

9 **Sarr MG**. The potent somatostatin analogue vapreotide does not decrease pancreas-specific complications after elective pancreatectomy: a prospective, multicenter, double-blinded, randomized, placebo-controlled trial. *J Am Coll Surg* 2003; **196**: 556-564; discussion 564-565; author reply 565

10 **Bassi C**, Butturini G, Molinari E, Mascetta G, Salvia R, Falconi M, Gumbs A, Pederzoli P. Pancreatic fistula rate after pancreatic resection. The importance of definitions. *Dig Surg* 2004; **21**: 54-59

11 **Bassi C**, Dervenis C, Butturini G, Fingerhut A, Yeo C, Izbicki J, Neoptolemos J, Sarr M, Traverso W, Buchler M. Postoperative pancreatic fistula: an international study group (ISGPF) definition. *Surgery* 2005; **138**: 8-13

12 **Pratt WB**, Maithel SK, Vanounou T, Huang ZS, Callery MP, Vollmer CM Jr. Clinical and economic validation of the International Study Group of Pancreatic Fistula (ISGPF) classification scheme. *Ann Surg* 2007; **245**: 443-451

13 **Strasberg SM**, Linehan DC, Clavien PA, Barkun JS. Proposal for definition and severity grading of pancreatic anastomosis failure and pancreatic occlusion failure. *Surgery* 2007; **141**: 420-426

14 **Yeo CJ**, Cameron JL, Maher MM, Sauter PK, Zahurak ML, Talamini MA, Lillemoe KD, Pitt HA. A prospective randomized trial of pancreaticogastrostomy versus pancreaticojejunostomy after pancreaticoduodenectomy. *Ann Surg* 1995; **222**: 580-588; discussion 588-592

15 **Barreto SG**, Shukla PJ, Shrikhande SV. The gray zone between postpancreaticoduodenectomy collections and pancreatic fistula. *Pancreas* 2008; **37:** 422-425

16 **Aranha GV**, Aaron JM, Shoup M, Pickleman J. Current management of pancreatic fistula after pancreaticoduodenectomy. *Surgery* 2006; **140**: 561-568; discussion 568-569

17 **Goldsmith HS**, Ghosh BC, Huvos AG. Ligation versus implantation of the pancreatic duct after pancreaticoduodenectomy. *Surg Gynecol Obstet* 1971; **132**: 87-92

18 **Papachristou DN**, Fortner JG. Pancreatic fistula complicating pancreatectomy for malignant disease. *Br J Surg* 1981; **68**: 238-240

19 **Tran K**, Van Eijck C, Di Carlo V, Hop WC, Zerbi A, Balzano G, Jeekel H. Occlusion of the pancreatic duct versus pancreaticojejunostomy: a prospective randomized trial. *Ann Surg* 2002; **236**: 422-428; discussion 428

20 **Di Carlo V**, Chiesa R, Pontiroli AE, Carlucci M, Staudacher C, Zerbi A, Cristallo M, Braga M, Pozza G. Pancreatoduodenectomy with occlusion of the residual stump by Neoprene injection. *World J Surg* 1989; **13**: 105-110; discussion 110-111

21 **Suc B**, Msika S, Fingerhut A, Fourtanier G, Hay JM, Holmieres F, Sastre B, Fagniez PL. Temporary fibrin glue occlusion of the main pancreatic duct in the prevention of intra-abdominal complications after pancreatic resection: prospective randomized trial. *Ann Surg* 2003; **237**: 57-65

22 **Sakorafas GH**, Friess H, Balsiger BM, Buchler MW, Sarr MG. Problems of reconstruction during pancreatoduodenectomy. *Dig Surg* 2001; **18**: 363-369

23 **Hamanaka Y**, Nishihara K, Hamasaki T, Kawabata A,

Yamamoto S, Tsurumi M, Ueno T, Suzuki T. Pancreatic juice output after pancreatoduodenectomy in relation to pancreatic consistency, duct size, and leakage. *Surgery* 1996; **119**: 281-287
24 **Bassi C**, Falconi M, Molinari E, Salvia R, Butturini G, Sartori N, Mantovani W, Pederzoli P. Reconstruction by pancreaticojejunostomy versus pancreaticogastrostomy following pancreatectomy: results of a comparative study. *Ann Surg* 2005; **242**: 767-771, discussion 771-773
25 **Duffas JP**, Suc B, Msika S, Fourtanier G, Muscari F, Hay JM, Fingerhut A, Millat B, Radovanowic A, Fagniez PL. A controlled randomized multicenter trial of pancreatogastrostomy or pancreatojejunostomy after pancreatoduodenectomy. *Am J Surg* 2005; **189**: 720-729
26 **Takano S**, Ito Y, Watanabe Y, Yokoyama T, Kubota N, Iwai S. Pancreaticojejunostomy versus pancreaticogastrostomy in reconstruction following pancreaticoduodenectomy. *Br J Surg* 2000; **87**: 423-427
27 **Oussoultzoglou E**, Bachellier P, Bigourdan JM, Weber JC, Nakano H, Jaeck D. Pancreaticogastrostomy decreased relaparotomy caused by pancreatic fistula after pancreaticoduodenectomy compared with pancreaticojejunostomy. *Arch Surg* 2004; **139**: 327-335
28 **McKay A**, Mackenzie S, Sutherland FR, Bathe OF, Doig C, Dort J, Vollmer CM Jr, Dixon E. Meta-analysis of pancreaticojejunostomy versus pancreaticogastrostomy reconstruction after pancreaticoduodenectomy. *Br J Surg* 2006; **93**: 929-936
29 **Wente MN**, Shrikhande SV, Muller MW, Diener MK, Seiler CM, Friess H, Buchler MW. Pancreaticojejunostomy versus pancreaticogastrostomy: systematic review and meta-analysis. *Am J Surg* 2007; **193**: 171-183
30 **Shrikhande SV**, Qureshi SS, Rajneesh N, Shukla PJ. Pancreatic anastomoses after pancreaticoduodenectomy: do we need further studies? *World J Surg* 2005; **29**: 1642-1649
31 **Roder JD**, Stein HJ, Bottcher KA, Busch R, Heidecke CD, Siewert JR. Stented versus nonstented pancreaticojejunostomy after pancreatoduodenectomy: a prospective study. *Ann Surg* 1999; **229**: 41-48
32 **Yoshimi F**, Ono H, Asato Y, Ohta T, Koizumi S, Amemiya R, Hasegawa H. Internal stenting of the hepaticojejunostomy and pancreaticojejunostomy in patients undergoing pancreatoduodenectomy to promote earlier discharge from hospital. *Surg Today* 1996; **26**: 665-667
33 **Poon RT**, Fan ST, Lo CM, Ng KK, Yuen WK, Yeung C, Wong J. External drainage of pancreatic duct with a stent to reduce leakage rate of pancreaticojejunostomy after pancreaticoduodenectomy: a prospective randomized trial. *Ann Surg* 2007; **246**: 425-433; discussion 433-435
34 **Imaizumi T**, Harada N, Hatori T, Fukuda A, Takasaki K. Stenting is unnecessary in duct-to-mucosa pancreaticojejunostomy even in the normal pancreas. *Pancreatology* 2002; **2**: 116-121
35 **Winter JM**, Cameron JL, Campbell KA, Chang DC, Riall TS, Schulick RD, Choti MA, Coleman J, Hodgin MB, Sauter PK, Sonnenday CJ, Wolfgang CL, Marohn MR, Yeo CJ. Does pancreatic duct stenting decrease the rate of pancreatic fistula following pancreaticoduodenectomy? Results of a prospective randomized trial. *J Gastrointest Surg* 2006; **10**: 1280-1290; discussion 1290
36 **Wada K**, Traverso LW. Pancreatic anastomotic leak after the Whipple procedure is reduced using the surgical microscope. *Surgery* 2006; **139**: 735-742
37 **Strasberg SM**, Drebin JA, Mokadam NA, Green DW, Jones KL, Ehlers JP, Linehan D. Prospective trial of a blood supply-based technique of pancreaticojejunostomy: effect on anastomotic failure in the Whipple procedure. *J Am Coll Surg* 2002; **194**: 746-758; discussion 759-760
38 **Shrikhande SV**, Kleeff J, Buchler MW, Friess H. Pancreatic anastomosis after pancreaticoduodenectomy: How we do it. *Ind J Surg* 2007; **6**: 224-229
39 **Hosotani R**, Doi R, Imamura M. Duct-to-mucosa pancreaticojejunostomy reduces the risk of pancreatic leakage after pancreatoduodenectomy. *World J Surg* 2002; **26**: 99-104
40 **Murakami Y**, Uemura K, Hayashidani Y, Sudo T, Hashimoto Y, Nakagawa N, Ohge H, Sueda T. No mortality after 150 consecutive pancreatoduodenctomies with duct-to-mucosa pancreaticogastrostomy. *J Surg Oncol* 2008; **97**: 205-209
41 **Bassi C**, Falconi M, Molinari E, Mantovani W, Butturini G, Gumbs AA, Salvia R, Pederzoli P. Duct-to-mucosa versus end-to-side pancreaticojejunostomy reconstruction after pancreaticoduodenectomy: results of a prospective randomized trial. *Surgery* 2003; **134**: 766-771
42 **Sarr MG**, Behrns KE, van Heerden JA. Total pancreatectomy. An objective analysis of its use in pancreatic cancer. *Hepatogastroenterology* 1993; **40**: 418-421
43 **Karpoff HM**, Klimstra DS, Brennan MF, Conlon KC. Results of total pancreatectomy for adenocarcinoma of the pancreas. *Arch Surg* 2001; **136**: 44-47; discussion 48
44 **Ihse I**, Anderson H, Andren-Sandberg. Total pancreatectomy for cancer of the pancreas: is it appropriate? *World J Surg* 1996; **20**: 288-293; discussion 294
45 **Reddy SK**, Tyler DS, Pappas TN, Clary BM. Extended resection for pancreatic adenocarcinoma. *Oncologist* 2007; **12**: 654-663
46 **Ridolfini MP**, Alfieri S, Gourgiotis S, Di Miceli D, Rotondi F, Quero G, Manghi R, Doglietto GB. Risk factors associated with pancreatic fistula after distal pancreatectomy, which technique of pancreatic stump closure is more beneficial? *World J Gastroenterol* 2007; **13**: 5096-5100
47 **Knaebel HP**, Diener MK, Wente MN, Buchler MW, Seiler CM. Systematic review and meta-analysis of technique for closure of the pancreatic remnant after distal pancreatectomy. *Br J Surg* 2005; **92**: 539-546
48 **Kleeff J**, Diener MK, Z'graggen K, Hinz U, Wagner M, Bachmann J, Zehetner J, Muller MW, Friess H, Buchler MW. Distal pancreatectomy: risk factors for surgical failure in 302 consecutive cases. *Ann Surg* 2007; **245**: 573-582
49 **Buchler M**, Friess H, Klempa I, Hermanek P, Sulkowski U, Becker H, Schafmayer A, Baca I, Lorenz D, Meister R. Role of octreotide in the prevention of postoperative complications following pancreatic resection. *Am J Surg* 1992; **163**: 125-130; discussion 130-131
50 **Montorsi M**, Zago M, Mosca F, Capussotti L, Zotti E, Ribotta G, Fegiz G, Fissi S, Roviaro G, Peracchia A. Efficacy of octreotide in the prevention of pancreatic fistula after elective pancreatic resections: a prospective, controlled, randomized clinical trial. *Surgery* 1995; **117**: 26-31
51 **Friess H**, Beger HG, Sulkowski U, Becker H, Hofbauer B, Dennler HJ, Buchler MW. Randomized controlled multicentre study of the prevention of complications by octreotide in patients undergoing surgery for chronic pancreatitis. *Br J Surg* 1995; **82**: 1270-1273
52 **Gouillat C**, Chipponi J, Baulieux J, Partensky C, Saric J, Gayet B. Randomized controlled multicentre trial of somatostatin infusion after pancreaticoduodenectomy. *Br J Surg* 2001; **88**: 1456-1462
53 **Yeo CJ**, Cameron JL, Lillemoe KD, Sauter PK, Coleman J, Sohn TA, Campbell KA, Choti MA. Does prophylactic octreotide decrease the rates of pancreatic fistula and other complications after pancreaticoduodenectomy? Results of a prospective randomized placebo-controlled trial. *Ann Surg* 2000; **232**: 419-429
54 **Lowy AM**, Lee JE, Pisters PW, Davidson BS, Fenoglio CJ, Stanford P, Jinnah R, Evans DB. Prospective, randomized trial of octreotide to prevent pancreatic fistula after pancreaticoduodenectomy for malignant disease. *Ann Surg* 1997; **226**: 632-641
55 **Poon RT**, Lo SH, Fong D, Fan ST, Wong J. Prevention of pancreatic anastomotic leakage after pancreaticoduodenectomy. *Am J Surg* 2002; **183**: 42-52
56 **Stojadinovic A**, Brooks A, Hoos A, Jaques DP, Conlon KC, Brennan MF. An evidence-based approach to the surgical management of resectable pancreatic adenocarcinoma. *J Am*

Coll Surg 2003; **196**: 954-964
57 **Vanounou T**, Pratt WB, Callery MP, Vollmer CM Jr. Selective administration of prophylactic octreotide during pancreaticoduodenectomy: a clinical and cost-benefit analysis in low- and high-risk glands. *J Am Coll Surg* 2007; **205**: 546-557
58 **Buchler MW**, Wagner M, Schmied BM, Uhl W, Friess H, Z'graggen K. Changes in morbidity after pancreatic resection: toward the end of completion pancreatectomy. *Arch Surg* 2003; **138**: 1310-1314; discussion 1315
59 **Kazanjian KK**, Hines OJ, Eibl G, Reber HA. Management of pancreatic fistulas after pancreaticoduodenectomy: results in 437 consecutive patients. *Arch Surg* 2005; **140**: 849-854; discussion 854-856
60 **Munoz-Bongrand N**, Sauvanet A, Denys A, Sibert A, Vilgrain V, Belghiti J. Conservative management of pancreatic fistula after pancreaticoduodenectomy with pancreaticogastrostomy. *J Am Coll Surg* 2004; **199**: 198-203
61 **Cullen JJ**, Sarr MG, Ilstrup DM. Pancreatic anastomotic leak after pancreaticoduodenectomy: incidence, significance, and management. *Am J Surg* 1994; **168**: 295-298
62 **Halloran CM**, Ghaneh P, Bosonnet L, Hartley MN, Sutton R, Neoptolemos JP. Complications of pancreatic cancer resection. *Dig Surg* 2002; **19**: 138-146
63 **Yekebas EF**, Wolfram L, Cataldegirmen G, Habermann CR, Bogoevski D, Koenig AM, Kaifi J, Schurr PG, Bubenheim M, Nolte-Ernsting C, Adam G, Izbicki JR. Postpancreatectomy hemorrhage: diagnosis and treatment: an analysis in 1669 consecutive pancreatic resections. *Ann Surg* 2007; **246**: 269-280
64 **van Berge Henegouwen MI**, Allema JH, van Gulik TM, Verbeek PC, Obertop H, Gouma DJ. Delayed massive haemorrhage after pancreatic and biliary surgery. *Br J Surg* 1995; **82**: 1527-1531
65 **Makowiec F**, Riediger H, Euringer W, Uhl M, Hopt UT, Adam U. Management of delayed visceral arterial bleeding after pancreatic head resection. *J Gastrointest Surg* 2005; **9**: 1293-1299
66 **de Castro SM**, Kuhlmann KF, Busch OR, van Delden OM, Lameris JS, van Gulik TM, Obertop H, Gouma DJ. Delayed massive hemorrhage after pancreatic and biliary surgery: embolization or surgery? *Ann Surg* 2005; **241**: 85-91
67 **van Berge Henegouwen MI**, De Wit LT, Van Gulik TM, Obertop H, Gouma DJ. Incidence, risk factors, and treatment of pancreatic leakage after pancreaticoduodenectomy: drainage versus resection of the pancreatic remnant. *J Am Coll Surg* 1997; **185**: 18-24

S- Editor Zhong XY **L- Editor** Anand BS **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5797

World J Gastroenterol 2008 October 14; 14(38): 5797-5809
World Journal of Gastroenterology ISSN 1007-9327

Depletion of $CD4^+CD25^+$ regulatory T cells can promote local immunity to suppress tumor growth in benzo[a]pyrene-induced forestomach carcinoma

Yi-Ling Chen, Jung-Hua Fang, Ming-Derg Lai, Yan-Shen Shan

Yi-Ling Chen, Department of Childhood Education and Nursery, Chia-Nan University of Pharmacy and Science, Tainan 70428, Taiwan, China
Jung-Hua Fang, Yan-Shen Shan, Department of Surgery, National Cheng Kung University Hospital, National Cheng Kung University, Tainan 70428, Taiwan, China
Ming-Derg Lai, Department of Biochemistry and Molecular Biology, College of Medicine, National Cheng Kung University, Tainan 70428, Taiwan, China
Author contributions: Chen YL performed research and wrote the manuscript, Shan YS designed the research and revised the manuscript, Fang JH helped to perform the research, Lai MD helped to modify the design of research.
Supported by Grant NSC93-2320-B41-010 and NSC93-2314-B-006-112 from the National Science Council, Taiwan, China
Correspondence to: Yan-Shen Shan, MD, PhD, Assistant Professor, Division of General Surgery, Department of Surgery, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, 138 Sheng-Li Road, Tainan 70428, Taiwan, China. ysshan@mail.ncku.edu.tw
Telephone: +886-6-2353535 Fax: +886-6-2766676
Received: March 8, 2008 Revised: September 23, 2008
Accepted: September 30, 2008
Published online: October 14, 2008

Abstract

AIM: To elucidate the distribution of $CD4^+CD25^+$ regulatory T cells (Tregs) in different lymphoid tissues and its local enhancement on tumor growth before and after depletion of $CD4^+CD25^+$ Tregs.

METHODS: Female ICR mice were gavaged with benzo[a]pyrene (BaP) to induce forestomach carcinoma. $CD4^+CD25^+$ Tregs were intraperitoneally depleted with monoclonal antibody PC61. These mice were divided into BaP-only, BaP + IgG, BaP + PC61, and control groups. The forestomach of mice was dissected for histological analysis, and tunnel test was performed for apoptosis of tumor cells. $CD4^+CD25^+$ Tregs were sorted from different lymphoid tissues and expression of Foxp3, IL-10, and chemokine receptors was analyzed by flow cytometry, semi-quantitative and real-time polymerase chain reaction.

RESULTS: The mice gavaged with only BaP showed increased forestomach papilloma and carcinoma at wk 16 and 32. The proportion of $CD4^+CD25^+$ Tregs was significantly higher in peri-stomach regional lymph nodes than in other lymphoid tissues. These $CD4^+CD25^+$ Tregs in regional lymph nodes expressed higher levels of Foxp3 and IL-10, enriched in the CD62L-subset, and CCR1 and CCR5 chemokine receptors. In mice gavaged with BaP + PC61, the number of tumor nodules and tumor volume decreased significantly with massive infiltrating cells and apoptosis of tumor cells. In the draining regional lymph nodes, the number of $CD4^+CD25^+$ Tregs also decreased significantly.

CONCLUSION: Inducible and activated $CD4^+CD25^+$ Tregs in the draining regional lymph nodes suppress host local immunity during tumor growth. Depletion of $CD4^+CD25^+$ Tregs can promote host local immunity to suppress tumor growth.

Key words: $CD4^+CD25^+$ regulatory T cells; Forestomach tumor; Foxp3

Peer reviewer: Ana J Coito, Associate Professor, Department of Surgery, The Dumont UCLA Transplant Center, 77-120 CHS BOX 90095-7054, Los Angeles 90095, United States

Chen YL, Fang JH, Lai MD, Shan YS. Depletion of $CD4^+CD25^+$ regulatory T cells can promote local immunity to suppress tumor growth in benzo[a]pyrene-induced forestomach carcinoma. *World J Gastroenterol* 2008; 14(38): 5797-5809 Available from: URL: http://www.wjgnet.com/1007-9327/14/5797.asp DOI: http://dx.doi.org/10.3748/wjg.14.5797

INTRODUCTION

$CD4^+CD25^+$ regulatory T cells (Tregs) constitute 5%-10% of peripheral $CD4^+$ T cells in humans[1]. The immune regulatory function of these $CD4^+CD25^+$ Tregs has been attributed to their ability to secrete immuno-suppressive cytokines, including IL-10 and TGF-β1[2]. Although beneficial to protecting the host from autoimmune disease, $CD4^+CD25^+$ Tregs may also dampen anti-tumor response. Evidence from various cancers demonstrates that the proportion of $CD4^+CD25^+$ Tregs increases in tumor-infiltrating lymphocytes (TILs) and peripheral circulation of gastric cancers[3-5], and in the peripheral blood of patients with

ovarian and lung cancers, hepatocellular carcinoma, and pancreas/breast adenocarcinoma[6-10]. Local accumulation of $CD4^+CD25^+$ Tregs in tumors is associated with a high death rate and reduced survival of ovarian carcinoma patients[9]. These reports suggest that $CD4^+CD25^+$ Tregs participate in cancer immunopathogenesis and are responsible for impairment of host immune surveillance to cancer. Viguier *et al*[11] reported that accumulation of $CD4^+CD25^+$ Tregs is higher in metastatic melanoma lymph nodes of humans and further demonstrated that these cells inhibit proliferation and cytokine production of infiltrating $CD4^+CD25^-$ and $CD8^+$ T cells *in vitro*. In contrast, Curiel *et al*[9] reported that $CD4^+CD25^+$ Tregs accumulate in ovarian tumors and malignant ascites but not in draining lymph nodes in later cancer stages. Thus, it is necessary to elucidate the potentially differential distributions and effects of $CD4^+CD25^+$ Tregs in local lymphoid tissues during cancer progression.

Preserved, smoked, and salted foods are highly associated with the development of gastric cancer. Polycyclic aromatic hydrocarbons (PAHs) are carcinogens that have been detected in cigarette smoke and in broiled and smoked foods[12]. Among PAHs, benzo[a]pyrene (BaP) is the most extensively studied compound and induces many carcinogen-specific effects[13]. Gavage-delivered BaP induces forestomach tumor in mice, providing a commonly used model for studying the chemopreventive effect of substances on gastric cancer[14-17]. Depletion of $CD4^+CD25^+$ Tregs has been shown to induce immune responses to a variety of different tumors in mice, including myeloma, leukemia, melanoma, and fibrosarcoma[18-20]. Lymphoid metastasis is also the determinative for gastric cancer. In this study, to understand the immunosurveillance effect of $CD4^+CD25^+$ Tregs in local lymphoid tissues during cancer progression, we used the BaP-induced forestomach tumor model to evaluate the differential effect of $CD4^+CD25^+$ Tregs by intervention at the initiation, promotion, or progression stages of multistage carcinogenesis. We first used tumor progression of BaP-induced autochthonous forestomach tumors in female mice to analyze the distribution and characteristics of $CD4^+CD25^+$ Tregs in different lymphoid tissues over time. Second, we used a $CD4^+CD25^+$ Treg-specific antibody to determine whether $CD4^+CD25^+$ Tregs are required for controlling tumor formation.

MATERIALS AND METHODS

Reagents and antibodies

Benzo[a]pyrene and glycerol gelatin were purchased from Sigma-Aldrich (St. Louis, MO). An aminoethyl carbazole substrate kit was purchased from Zymed (San Francisco, CA). The following antibodies (Abs) were purchased from BD PharMingen (San Diego, CA) and used in this study: mouse anti-CD4 PE (H129.19), anti-CD8a PE (53-6.7), anti-CD3e PE (145-2C11), anti-CD25 FITC (7D4), anti-CD62L FITC (MEL-14), and anti-CD62L PE (MEL-14). FITC-anti-Foxp3 (FJK-16s) mAb and FITC rat IgG2a isotype control Ab were purchased from eBioscience.

BaP-induced forestomach tumorigenesis

BaP-induced forestomach tumors were generated in female mice, as previously reported[21]. Eight-week-old ICR mice were purchased from the Laboratory Animal Center of National Cheng Kung University. All animal experiments were carried out with the approval of the ethical committee of our institution. BaP was dissolved in corn oil. Corn oil control mice (n = 14) and BaP-only treated mice (n = 9) received 200 μL of corn oil with or without 3 mg of BaP, respectively, by *po* gavage twice weekly for 4 wk. The mice were then sacrificed at week 7 (n = 3), 16 (n = 3), and 32 (n = 3) after the first administration of BaP.

Buffered formalin-phosphate (10%) was immediately injected into the stomach by oral intubation to distend and fix the stomach. Each stomach was removed and placed on a plastic sheet. The tumor incidence, number, and size in the forestomach were measured under a dissection microscope. Tumor volume was calculated using the following formula: Volume = $4\pi R^3/3$[22]. Stomach samples were excised and immersed in a buffered 10% formalin solution for paraffin block preparation. Four-micron sections were dehydrated, embedded, and stained with hematoxylin and eosin.

In vivo depletion of $CD4^+CD25^+$ Tregs

$CD25^+$ T cell-specific antibody, hybridoma PC61, was a gift from the Laboratory of Dr. Lai, MD. To deplete the $CD25^+$ T cells, BaP-treated mice (n = 19) were injected intraperitoneally with 200 μL PC61 mAb fluid at a concentration of 3 mg protein per mL[23], twice a week beginning 5 wk after the initial BaP treatment until the time of sacrifice. The mice in the BaP + PC61 group were then sacrificed at weeks 7 (n = 3), 16 (n = 7), 24 (n = 5), and 32 (n = 4) respectively, after the first administration of BaP. BaP-treated mice were injected with purified rat IgG antibody as a control (ICN, Pharmaceuticals Inc. Cappel, OH, USA). The mice in the BaP + IgG group were then sacrificed at weeks 7 (n = 4), 16 (n = 6), 24 (n = 5), and 32 (n = 4) respectively, after the first administration of BaP.

To confirm the effectiveness of the depletion, splenocytes, thymocytes, or lymphocytes from the peripheral lymph nodes were harvested on the third and seventh days every week after BaP treatment and incubated with anti-CD4 PE, anti-CD8 PE, and anti-CD25-FITC respectively, at 4℃ for 40 min in the dark. The mixture was washed twice with ice-cold HBSS, and then re-suspended in HBSS containing 2% FCS/0.1% NaN_3. Stained splenocytes, thymocytes, or lymphocytes were analyzed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA).

Cell preparations, sorting, and flow cytometry analysis

Lymph nodes were collected from the mice at weeks 7 (n = 10), 16 (n = 12), and 32 (n = 11) respectively, after the initial BaP administration. Lymph nodes around the stomach and in the mesentery were collected and defined as regional lymph nodes (RLNs). Lymph nodes in the axillary, inguinal, brachial, and popliteal areas were collected and defined as PLNs. Cell suspensions

from the lymph nodes, spleen, or thymus were isolated by flow sorting after sterile dissociation, filtration, and washing. Cells were stained using PE-conjugated anti-CD4 and FITC-conjugated anti-CD25 and then purified on a FACSAria-sorting flow cytometer (BD Biosciences, San Jose, CA). The purity of these $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells was > 90%. To determine the expression of cell surface markers in T cells, the cells were incubated with antibodies appropriately diluted for staining and further stained with anti-Foxp3 (Foxp3 regulates Treg development and function) or isotype control Ab according to the manufacturer's protocol. Stained lymphocytes were analyzed by flow cytometry.

Detection of apoptotic cells

Cytotoxic T-cells are able to directly induce apoptosis in cells, which is the one of the host immune surveillances. To identify a host anti-tumor response after depletion of $CD4^+CD25^+$ Tregs, apoptotic cells in tumor nodules were detected by TUNEL labeling detection of free 3'-OH groups in fragmented DNA *in situ* (ApopTag® peroxidase *in situ* apoptosis detection kit, Chemicon). Paraffin-embedded, slide-mounted tissue sections were deparaffinized and treated with proteinase K for 15 min followed by 3% H_2O_2 for 5 min at room temperature. After nick-end labeling with digoxigenin-deoxyuridine triphosphate by terminal deoxynucleotidyl transferase, immunostaining was performed using peroxidase-conjugated anti-digoxigenin Ab. Apoptotic cells were visualized with diaminobenzidine substrate, becoming a dark-brown color. Specimens were then counterstained with hematoxylin.

Semi-quantitative and real-time PCR

We used PCR to assess expression levels of several $CD4^+CD25^+$ Treg-related genes. Total RNA was extracted from 5×10^5 sorted cells and purified using the RNeasy kit according to the manufacturer's instructions (Qiagen, Valencia, CA) and converted to cDNA by moloney murine leukemia virus (M-MLV) reverse transcriptase with oligo(dT) primer in the presence of RNAsin (Promega, Madison, WI). Semi-quantitative PCR for Foxp3, TGF-β_1, IL-10, CCR1, CCR5, CCR6, CCR7, and β-actin was performed as described previously[24-28]. The cDNA generated was subjected to 30-35 cycles of PCR amplification on a DNA thermal cycler (Perkin Elmer, GeneAmp PCR System). PCR was performed with specific primers for CCR4 and CCR8: CCR4 (forward, AGAGGCTCAAGTCCATGACG; reverse, AGTGTTGCAGAGTCCTAATG), and CCR8 (forward, CGGCATGCTACAATGTTTCC; reverse, TCTCTCCTATGAACGCGTAG). The β-actin served as a quantitative control for PCR. PCR products were fractionated by agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light.

Real-time PCR was performed on a LightCycler detection system (Roche Applied Science). Analyses were performed using primers, an internal fluorescent TaqMan probe specific to Foxp3 or HPRT, and the LightCycler TaqMan Master kit (Roche Applied Science, Penzberg, Germany). The primer and TaqMan probe sequences used as previously described[26] are as follows: *Foxp3* primers (5'-CCCAGGAAAGACAGCAACCTT-3' and 5'-TTCTCACAACCAGGCCACTTG-3'), Foxp3 probe (5'-FAM-ATCCTACCCACTGCTGGCAAATGG AGTC-3'), HPRT primers (5'-TGAAGAGCTACTGTA ATGATCAGTCAAC-3' and 5'-AGCAAGCTTGCA ACCTTAACCA-3'), HPRT probe (5'-FAM-TGCTTT CCCTGGTTAAGCAGTACAGCCC-3'). Standard curves of cDNAs from ICR mice $CD4^+CD25^+$ T cells were used as previously described[27]. The normalized values for Foxp3 mRNA were calculated as the relative quantity of Foxp3 mRNA levels divided by the relative quantity of HPRT mRNA levels. All samples were run in triplicate.

Statistical analysis

Results are expressed as mean ± SE. Because of time constraints involving the large number of antibodies and RNA analyses in this study, values were compared using the Mann-Whitney test for independent experiments. $P \leqslant 0.05$ was considered statistically significant.

RESULTS

Depletion of $CD4^+CD25^+$ Tregs reduced growth of tumor nodules in BaP-treated mice

A total 65 mice used in this study were divided into corn oil control (n = 14), BaP-only (n = 9), BaP + IgG (n = 19), and BaP + PC61 (n = 19) groups. To prove the effect of a short course treatment with PC61, 4 mice in the BaP + PC61 only group received 5 wk of PC61 injection before sacrifice. The tumor incidence was 100% in the forestomach of BaP-treated mice at weeks 16, 24, and 32, respectively. There was no difference in tumor incidence, growth and pathological alternation between the BaP-only and BaP + IgG-treated mice. Formation of some small rash-like tumor nodules and more significant tumor nodules was observed in the forestomach at weeks 16 and 32, respectively (Figure 1B and E), but no tumor growth was detected in the forestomachs of mice in the corn oil control group (Figure 1A and D). Typical pathological alternations in tumor nodules, including papilloma and squamous cell carcinomas, were observed, but few lymphocytes and granulocytes accumulated in and around the tumor nodes (Figure 2B and E). The number of tumor nodules, both papilloma and squamous cell carcinoma, significantly increased in this period (Table 1).

The constituted number of $CD4^+CD25^+$ T cells in PLNs, spleen, and thymus of naïve mice after depletion by PC61 was maintained at less than 1% (Figure 3A and C). These naïve mice treated with PC61 mAb did not develop histologically evident autoimmunity. After a short-course treatment with PC61 for 5 wk, the tumor volume decreased significantly by as much as 60% in individual mice of BaP + PC61 group at week 16 (Table 1). The effect of a long-course treatment, until sacrifice, was more significant. The forestomach tumor growth was more

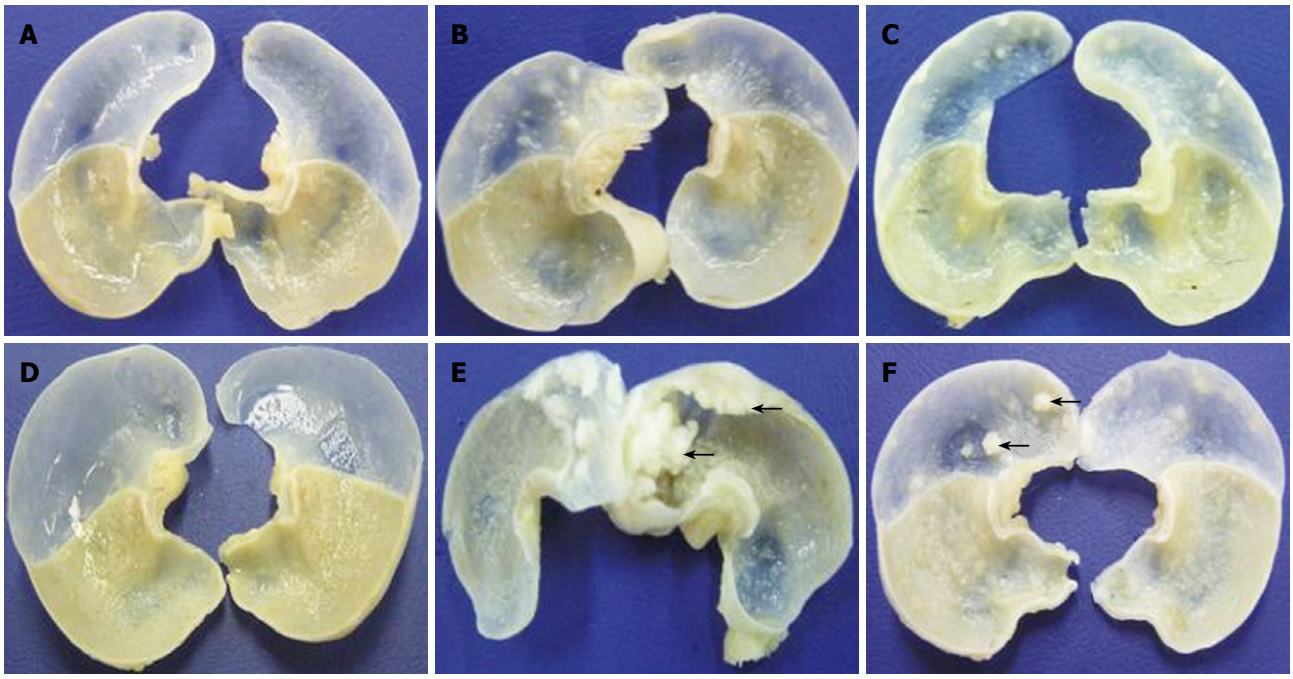


Figure 1 Forestomach tumors (arrow) in BaP-treated mice with/without depletion of Tregs. Mice were sacrificed at wk 16 (**A**-**C**) and wk 32 (**D**-**F**) after the first administration of benzo[a]pyrene (BaP) in control mice (**A** and **D**), BaP + IgG-treated mice (**B** and **E**), and BaP + PC61-treated mice (**C** and **F**). After depletion of Tregs by PC61, the size and volume of forestomach carcinoma reduced significantly.

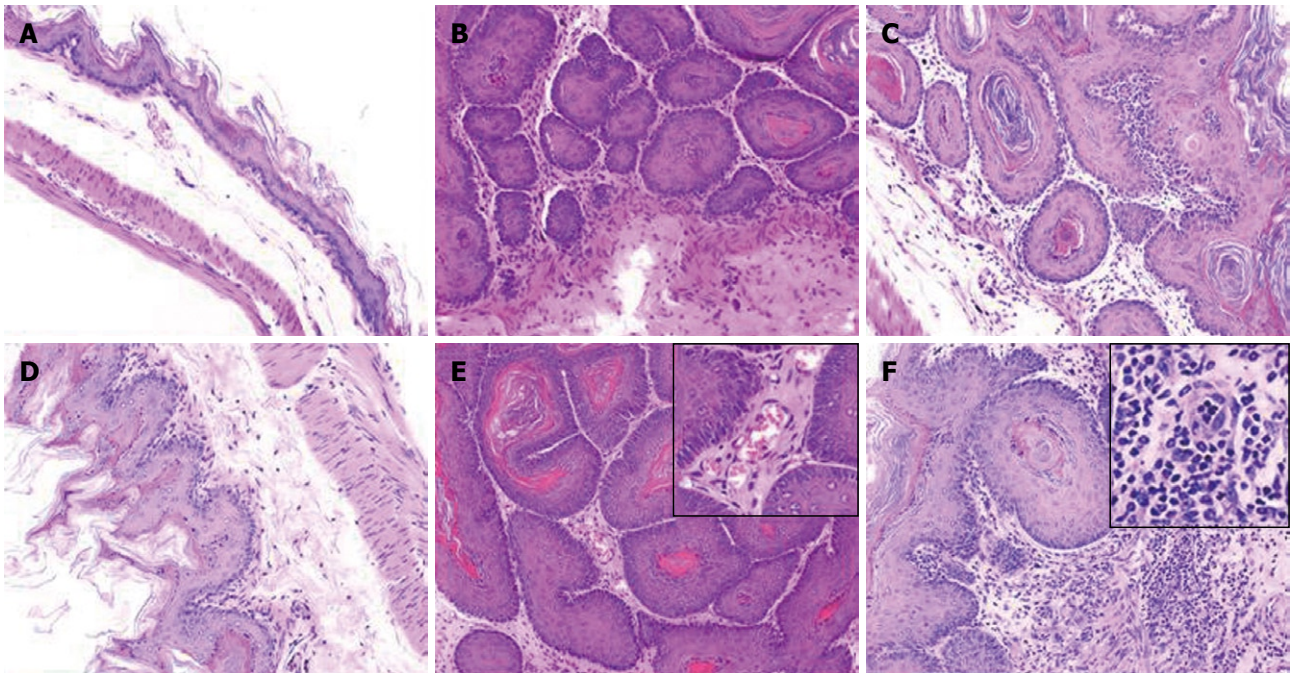


Figure 2 Histology of forestomach carcinoma in mice. Mice were sacrificed at wk 16 (**A**-**C**) and wk 32 (**D**-**F**) with their stomachs excised. The number of squamous cell carcinomas was increased in BaP + IgG-treated (**B**, **E**) and BaP + PC61-treated mice (**C**, **F**). However, there was a significant infiltration of lymphocytes and granulocytes into tumors of BaP + PC61-treated mice (**F**, inset). **A** and **D**: control mice; **B** and **E**: BaP + IgG-treated mice; **C** and **F**: BaP + PC61-treated mice.

progressively reduced (Figure 1C and E), and the tumor volume decreased by as much as 80% per mouse in comparison to the mice in the BaP + IgG group at weeks 16 and 32, respectively (Table 1). A significant increase in infiltration of lymphocytes and granulocytes at the tumor sites in mice of the BaP + PC61 group was also observed upon histological examination (Figure 2C and F).

Depletion of CD4$^+$ CD25$^+$ Tregs enhanced host anti-tumor immunity

We further investigated whether depletion of $CD4^+CD25^+$ Tregs increases host anti-tumor immunity, using apoptosis as a marker. After depletion using PC61, the portion of $CD4^+CD25^+$ Tregs in $CD4^+$ and $CD8^+CD25^+$ in $CD8^+$ was significantly decreased, the tumor infiltrating lymphocytes (TILs) were significantly

Table 1 Depletion of CD25+ regulatory cells suppresses forestomach tumor growth

Group	Body wt. (g)/mouse	Stomach wt. (g)/mouse	% of mice with tumors	Tumors/mouse	Tumor vol. (mm^3)/tumor	Total tumor vol. (mm^3)/mouse
16 wk						
Control	34.7 ± 3.1	0.30 ± 0.02	0 (0/3)	0	0	0
BaP-only	33.5 ± 2.7	0.34 ± 0.02	100 (3/3)	5.3 ± 1.2	2.8 ± 0.5	15.0 ± 5.0
BaP + IgG	35.0 ± 2.9	0.32 ± 0.04	100 (6/6)	6.2 ± 1.3	2.5 ± 0.8	15.2 ± 4.2
BaP + PC61	33.5 ± 2.7	0.30 ± 0.03	100 (3/3)	3.7 ± 1.5[a]	0.8 ± 0.5[b]	3.1 ± 2.4[b]
BaP + PC61/5 wk[1]	32.3 ± 8.2	0.31 ± 0.05	100 (4/4)	4.3 ± 1.5	1.0 ± 0.5[b]	4.6 ± 2.8[b]
24 wk						
Control	36.0 ± 3.3	0.33 ± 0.01	0 (0/5)	0	0	0
BaP + IgG	35.6 ± 2.9	0.44 ± 0.08	100 (5/5)	ND	ND	ND
BaP + PC61	36.4 ± 4.1	0.32 ± 0.02[b]	100 (5/5)	ND	ND	ND
32 wk						
Control	36.0 ± 4.5	0.35 ± 0.03	0 (0/3)	0	0	0
BaP-only	36.7 ± 2.1	ND	100 (3/3)	ND	ND	ND
BaP + IgG	36.0 ± 2.5	0.53 ± 0.12	100 (4/4)	15.5 ± 4.2	21.1 ± 16.4	276.8 ± 134.5
BaP + PC61	39.7 ± 8.5	0.44 ± 0.03	100 (4/4)	8.3 ± 2.2[a]	6.8 ± 3.2	57.1 ± 33.6[a]

[1]Mice were treated with anti-CD25 mAb twice a wk beginning 5 wk after benzo[a]pyrene treatment. [a]$P < 0.05$, [b]$P < 0.01$ *vs* BaP/IgG group; ND: Not determined.

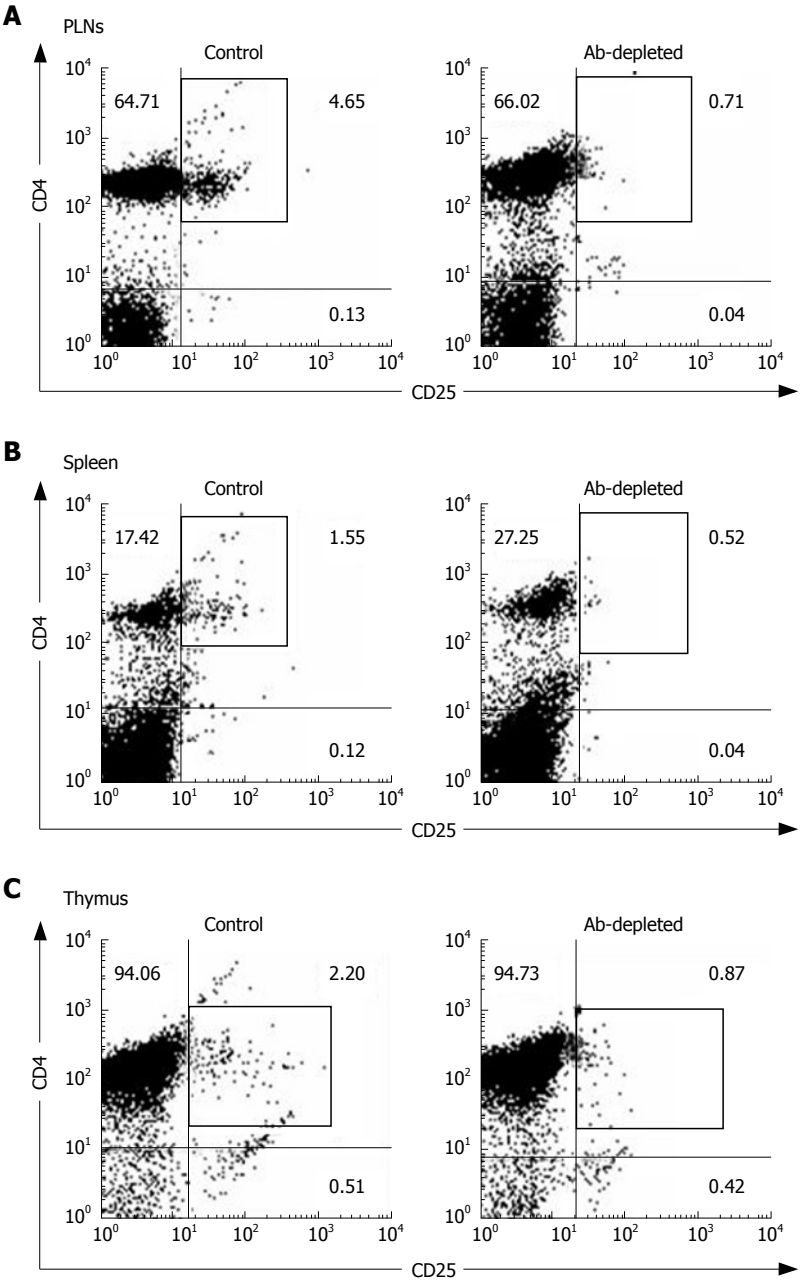


Figure 3 Flow cytometry showing reduced CD4+CD25+ T cells in PLNs (**A**), spleen (**B**), and thymus (**C**) of antibody-treated mice. Cells were double-stained with anti-CD4 and anti-CD25 Ab.

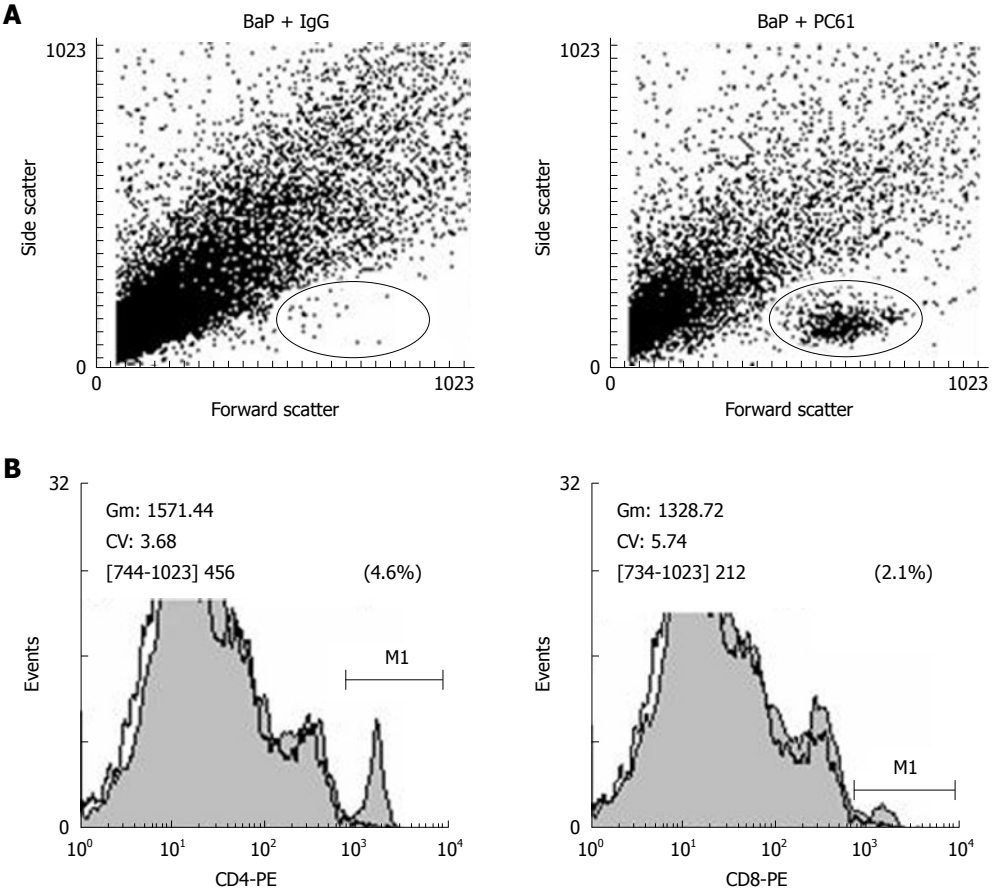


Figure 4 Flow cytometry showing lymphocyte infiltration of the stomach in Treg-undepleted/-depleted mice (**A**) and cells stained with PE-conjugated anti-CD4, CD8 mAb (solid peak) and isotype control (hollow peak), and the infiltration of $CD4^+$ and $CD8^+$ cells of the stomach quantified in Treg-depleted mice (**B**).

Table 2 Effects of depleted Tregs on tumor formation

	Time	BaP + IgG	BaP + PC61
Infiltrating cells	16 wk	-/+	+
	32 wk	-/+	+++
Apoptotic cells	16 wk	-	+
	32 wk	+	+++
Apoptosis occurred in tumor nodules	16 wk	-	+
	32 wk	-	+++

+++: Severe; ++: Moderate; +: Mild; -/+: Few; -: Not found.

increased in the nodules (Figure 4A), and the fraction of $CD4^+$ (3.90% ± 0.99%) and $CD8^+$ (2.25% ± 0.21%) cells in TILs was also observed in mice of the BaP + PC61 group at week 32 (Figure 4B). The tumor size and volume were decreased, and massive apoptotic cells were observed in the forestomach tumors of mice in the BaP + PC61 group (Figure 5C and F), but rare apoptotic cells were found in the tumor mass of mice in the BaP + IgG group at weeks 16 and 32 (Figure 5B and E). The effect of depletion of $CD4^+$ $CD25^+$ Tregs is summarized in Table 2.

$CD4^+CD25^+$ Tregs increased in RLNs responsible for local immune surveillance

To elucidate whether $CD4^+CD25^+$ Tregs are responsible for local immune surveillance during tumor growth, the population of $CD4^+CD25^+$ Tregs in different lymphoid organs was analyzed. The RLNs in mice of the BaP-only group had a significantly higher proportion of $CD4^+CD25^+$ Tregs at weeks 7, 16, and 32, respectively (9.26% ± 1.56%, 9.61% ± 0.52%, 5.09% ± 2.03%) than in mice of the control group (3.72% ± 0.83%, 3.07% ± 1.52%, 2.45% ± 0.70%). The population of total $CD4^+$ Tregs at weeks 7, 16 and 32 (13.51% ± 2.21%, 17.63% ± 2.62%, 9.36% ± 4.75%) was also significantly higher than that in mice of the control group (5.57% ± 1.79%, 5.73% ± 2.95%, 4.51% ± 1.15%) (Figure 6A). The proportion of total $CD4^+$ Tregs (6.42% ± 0.59% and 10.96% ± 0.28%, respectively) in the PLNs of mice of the BaP-only group was significantly higher than that in mice of the control group (3.50% ± 1.11%; 5.52% ± 1.78%, respectively) at week 16 (Figure 6B). In spleen, the proportion of Tregs in total $CD4^+$ T cells was significantly higher at weeks 7 and 32 (Figure 6C). However, a difference in Treg distribution was not observed in thymus (Figure 6D).

The population of $CD8^+CD4^+$ Tregs in different lymphoid organs was also analyzed. The RLNs in mice of the BaP-only group had a significantly higher proportion of $CD8^+CD25^+$ Tregs at weeks 7, 16 and 32, respectively (1.68% ± 0.28%, 5.24% ± 1.90%, 1.14% ± 0.29%) than those in mice of the control group (1.93% ± 0.34%, 0.65% ± 0.36%, 0.46% ± 0.20%). The population of $CD8^+CD25^+$ in total $CD8^+$ T cells at weeks 7, 16, and 32 (9.36% ± 0.52%, 18.54% ± 4.48%,

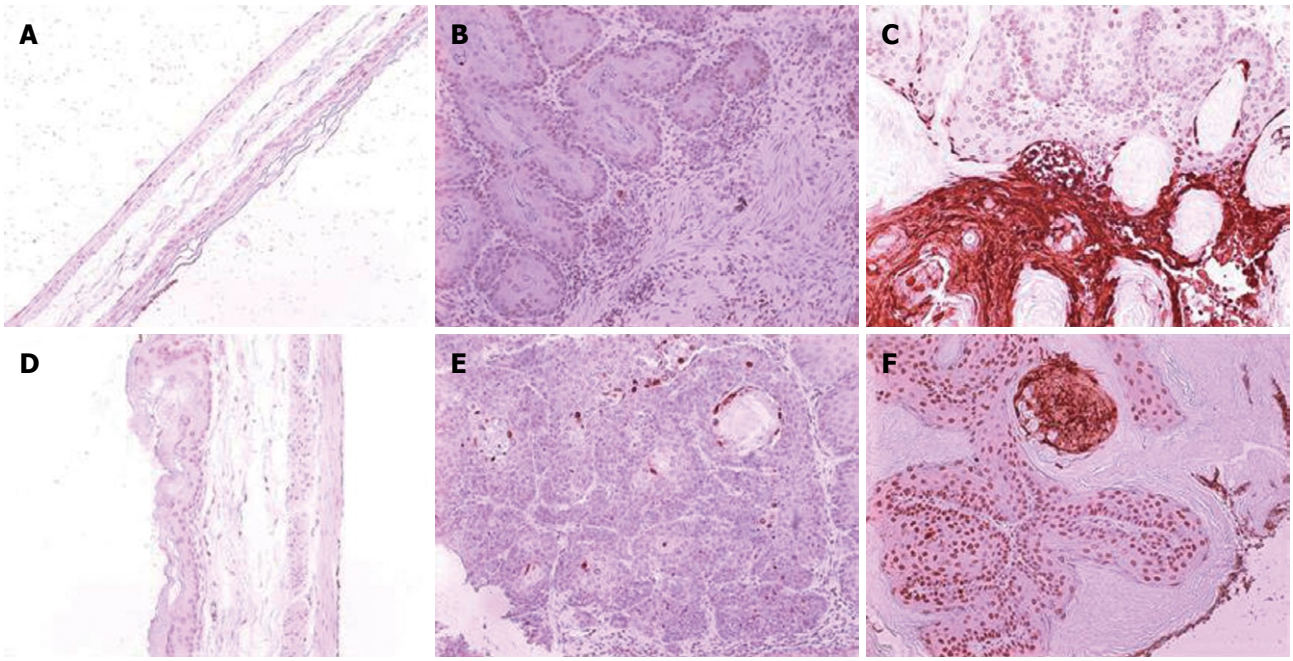


Figure 5 Apoptotic cells in forestomach tumors. Apoptotic cells were detected by TUNEL-staining as described in Materials and Methods. Apoptotic cells were revealed by a brown color. Stomach tumor nodules of mice were surgically obtained at wk 16 (**A**-**C**) and wk 32 (**D**-**F**) after the first administration of BaP. Significant apoptotic cells were found at wk 32 in BaP + PC61-treated mice. **A** and **D**: Control mice; **B** and **E**: BaP+IgG-treated mice; **C** and **F**: BaP + PC61-treated mice.

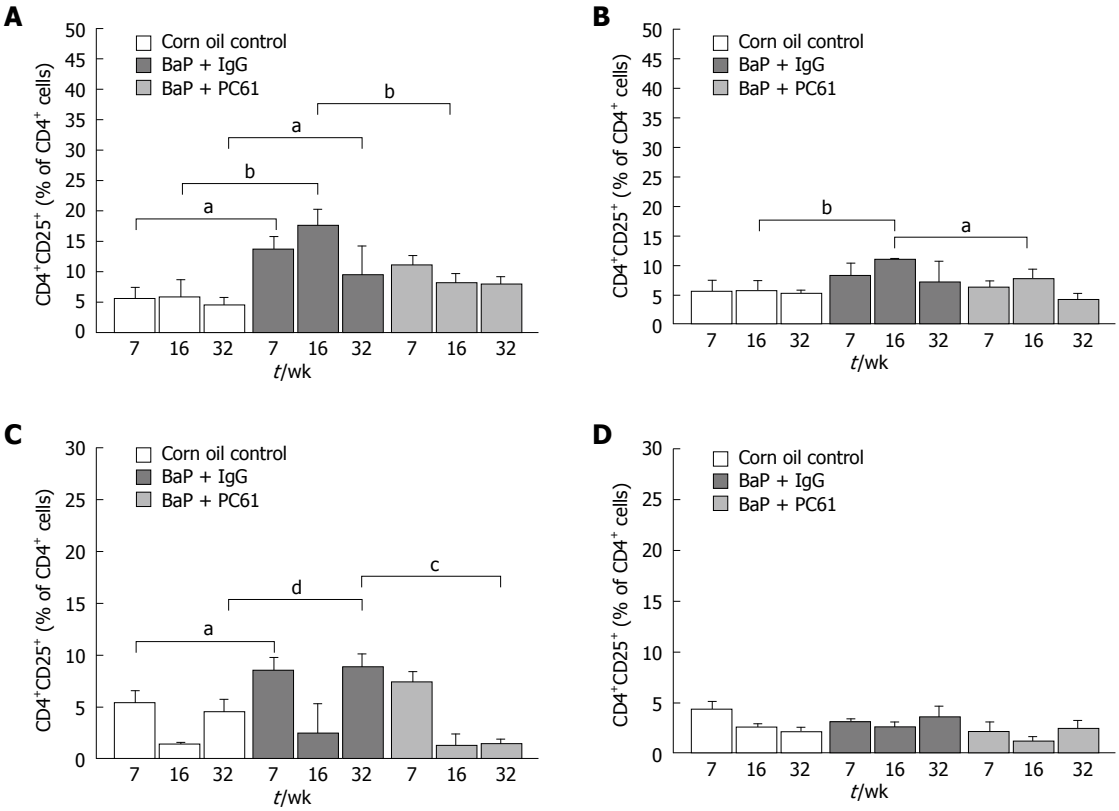


Figure 6 $CD4^+CD25^+$ T cells in distinct microenvironments. Lymphocytes in RLNs (**A**), PLNs (**B**), spleen (**C**), and thymus (**D**) were double-stained with FITC-anti-CD25 and PE-anti-CD4 mAbs and quantified by flow cytometry in the control, BaP + IgG-treated, and BaP + PC61-treated mice at wk 7, wk 16, and wk 32. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.005$, $^dP < 0.001$ *vs* the control mice.

6.74% ± 2.24%) was also significantly higher in mice of the BaP-only group than in mice of the control group (11.98%± 0.13%, 4.49% ± 1.91%, 2.82% ± 1.32%). The proportion of $CD8^+CD25^+$ and $CD8^+CD25^+$ in total $CD8^+$ T cells in the PLNs of mice of the BaP-only group at weeks 7, 16, and 32, respectively, was significantly higher in mice of the BaP-only group than in mice of the control group (data not shown). In

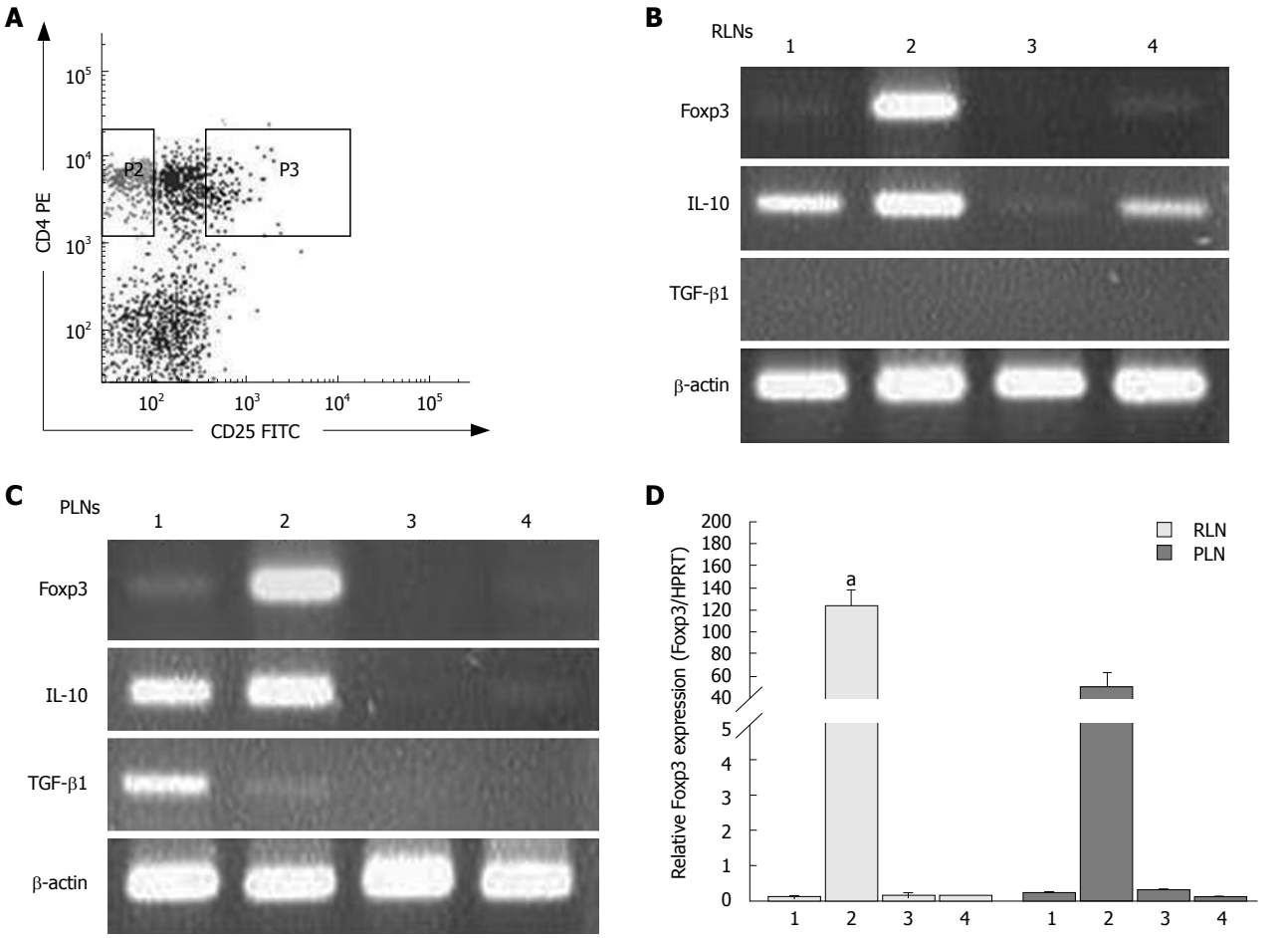


Figure 7 $CD4^+CD25^+$ T cells express high levels of Foxp3 transcripts in RLNs and PLNs of $CD4^+CD25^-$ (P2) and $CD4^+CD25^+$ (P3) T cells (**A**) which were sorted and stained with FITC-anti-CD25 and PE-anti-CD4 mAbs, and of Foxp3, IL-10, TGF-β1, and β-actin mRNA in $CD4^+CD25^+$ and $CD4^+CD25^-$ cells of RLNs (**B**) and PLNs (**C**) in BaP-only-treated and control mice at wk 32 using RT-PCR (Lanes 1 and 2: BaP-only-treated mice; Lanes 3 and 4: control mice; Lanes 1 and 3: $CD4^+CD25^-$ cells; Lanes 2 and 4: $CD4^+CD25^+$ cells), as well as of relative Foxp3 from $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells using real-time quantitative PCR after normalization to HPRT expression (**D**). $^aP < 0.05$ *vs* control mice.

spleen, the proportion of $CD8^+CD25^+$ and $CD8^+CD25^+$ in total $CD8^+$ T cells was significantly higher in mice of the BaP-only group than in mice of the control group at wk 32 (0.75%± 0.14% and 12.99% ± 1.87% *versus* 0.20% ± 0.07% and 3.74% ± 1.40%). However, no difference in $CD8^+CD25^+$ distribution was observed in thymus.

The population of $CD4^+$T cells and the pattern of surface expression for CD3 in RLNs, PLNs, spleen, and thymus was consistent among the corn oil control, BaP + IgG, and BaP + PC61 groups at weeks 7, 16 and 32 (data not shown), revealing that the increased proportion of Tregs and $CD8^+CD25^+$ in the RLNs of BaP-treated mice may be responsible for local immune surveillance during progression of forestomach tumors.

Inducible $CD4^+CD25^+$ Tregs in RLNs suppressed local immunity via secretion of IL-10

Foxp3 is a crucial transcription factor and the specific marker for inducible $CD4^+CD25^+$ Tregs. After enumeration of isolated $CD4^+CD25^+$ (combined $CD25^{int}$ and $CD25^{high}$-expressing cells) and $CD4^+CD25^-$ T cells within RLNs and PLNs (Figure 7A), the expression of Foxp3 in $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ t cells in the RLNs and PLNs of mice in the BaP-only and corn oil control groups was examined. As expected, $CD4^+CD25^+$ Tregs in the RLNs and PLNs expressed high levels of Foxp3, while $CD4^+CD25^-$ T cells expressed very low levels in mice of the BaP group (Figure 7B and C). Furthermore, the Foxp3 transcripts of $CD4^+CD25^+$ Tregs in RLNs of mice of the BaP group were significantly higher than those in PLNs of mice of the BaP group at wk 32 (Figure 7D).

We further analyzed whether the $CD4^+CD25^+$ Tregs or $CD4^+CD25^-$ T cells in the RLNs and PLNs can produce immunosuppressive cytokines. The two types of T cells in the RLNs and PLNs of the BaP-only treated mice enhanced the expression of IL-10 at transcription levels at wk 32 (Figure 7B and C), but only the $CD4^+CD25^+$ Tregs in the RLNs of mice in the control group expressed low-level IL-10 transcripts at wk 32 . The Tregs in the RLNs and PLNs of mice in the BaP-only and control groups produced few or no IL-10 transcripts at weeks 7 and 16 (data not shown). Regarding the TGF-β1 transcripts, the RLNs and PLNs in mice of the BaP-only and control groups were negative at weeks 7, 16, and 32, and only the $CD4^+CD25^-$ T cells from the PLNs in week of the BaP-only group produced low levels of TGF-β1 transcripts

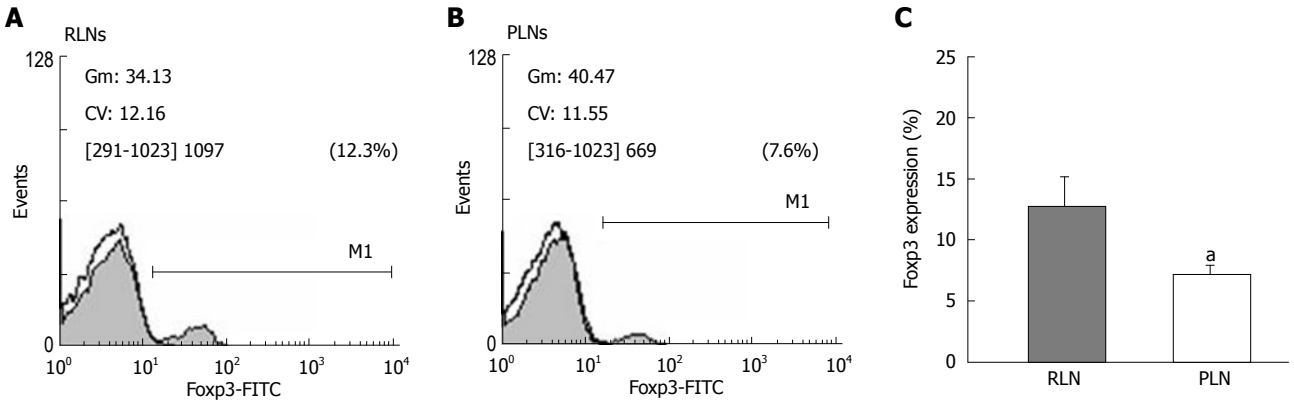


Figure 8 Expression of Foxp3 protein of RLNs (**A**) and PLNs (**B**) in BaP-only-treated mice at wk 32 and quantified by flow cytometry (**C**). Foxp3 expression was determined as described in Materials and Methods. The histogram demonstrates FITC-conjugated Foxp3 staining (solid peak) and isotype control (hollow peak). $^{a}P < 0.05$ *vs* control mice.

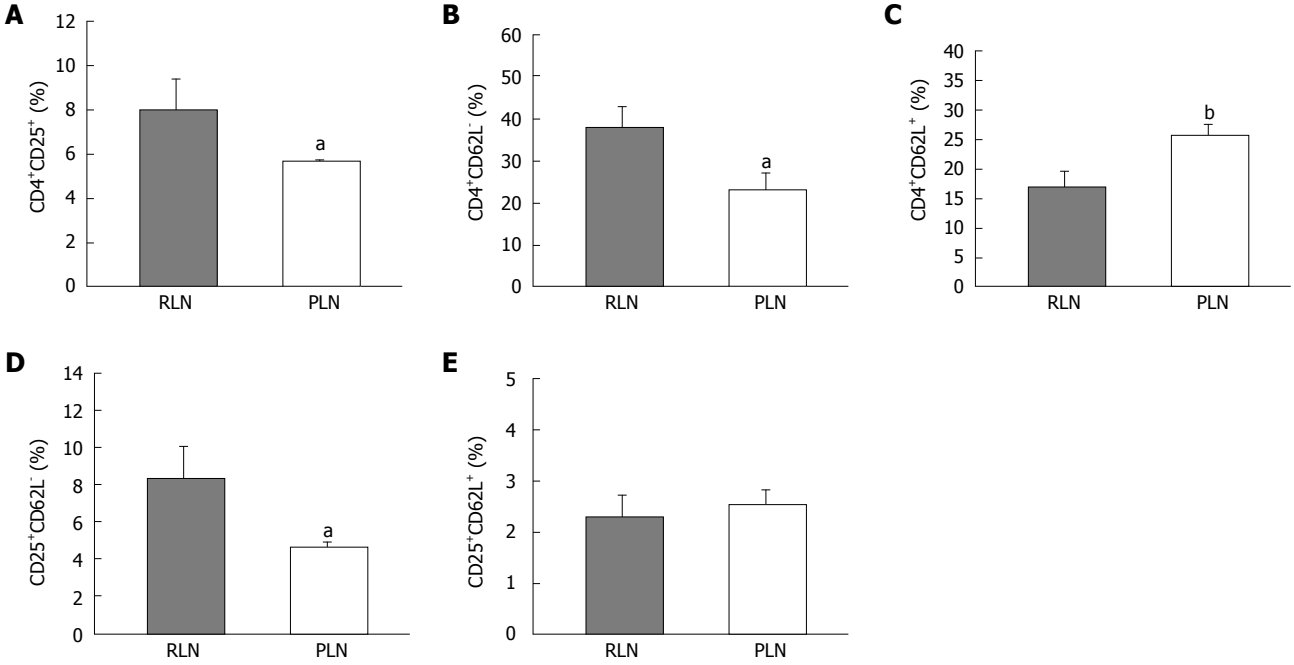


Figure 9 Surface expression of $CD4^{+}CD25^{+}$ (**A**), $CD4^{+}CD62L^{-}$ (**B**), $CD4^{+}CD62L^{+}$ (**C**), $CD25^{+}CD62L^{-}$(**D**), and $CD25^{+}CD62L^{+}$ (**E**) in RLNs and PLNs of BaP-only-treated mice at wk 32. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control mice.

at week 32 (Figure 7B and C).

To confirm the difference in the proportion of Tregs between RLN and PLN, we analyzed the expression of Foxp3 by flow cytometry (Figure 8A and B). The expression of Foxp3 in lymphocytes of RLNs was significantly higher than that in lymphocytes of PLNs of BaP-only-treated mice at week 32 (Figure 8C), suggesting that activation of inducible $CD4^{+}CD25^{+}$ Tregs is associated with tumor formation and that $CD4^{+}CD25^{+}$ Tregs secrete IL-10 but not TGF-β1, as a mediator for local inhibition of host immune functions during tumor progression.

Loss of expression of CD62L protein on Tregs of RLNs

The down-regulation of expression of L-selectin (CD62L) on the cell surface depends on cell activation. Loss of CD62L expression is a useful indicator for T cell maturation or terminal differentiation. The RLNs had a significantly higher proportion of Tregs than the PLNs (Figure 9A). The percentage of $CD4^{+}CD62L^{-}$ cells in RLNs was higher than that in PLNs of mice in the BaP-only group at week 32 (Figure 9B), and the percentage of $CD4^{+}$ $CD62L^{+}$ cells in PLNs, belonging to the naïve pool, was higher than that in RLNs (Figure 9C). Furthermore, the percentage of $CD25^{+}CD62L^{-}$ cells in RLNs was significantly higher than that in PLNs, but there was no difference in $CD25^{+}CD62L^{+}$ between RLNs and PLNs (Figure 9D and E), indicating that more Tregs in the RLNs are at the activation stage.

Expression of CCR1 and CCR5 transcripts in Tregs of RLNs

Because chemokine receptors are important for T cell migration to lymph nodes, the expression of these

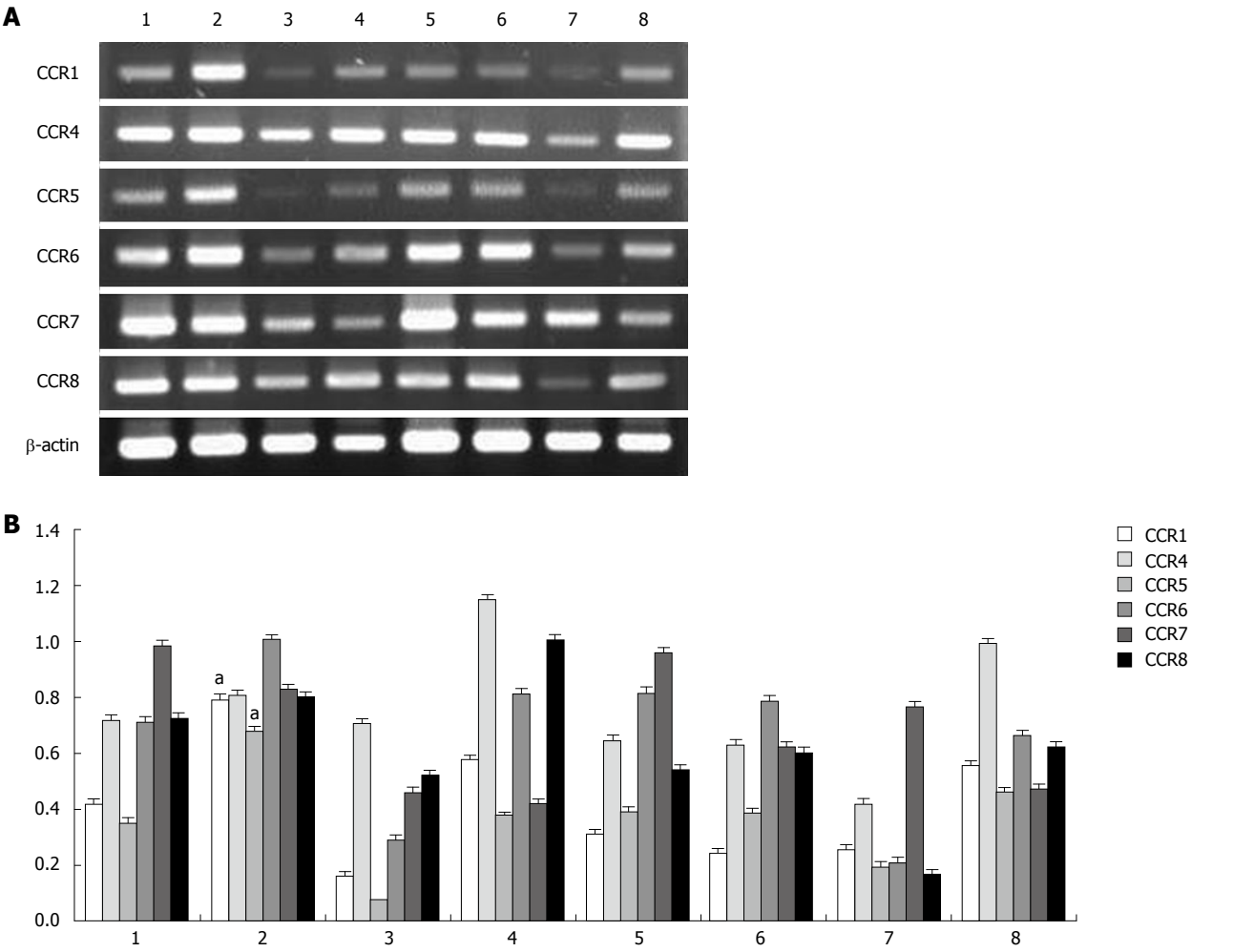


Figure 10 PCR analysis of chemokine receptor transcripts in $CD4^+CD25^-$ and $CD4^+CD25^+$ cells (**A**). CCR1, CCR4, CCR5, CCR6, CCR7, CCR8, and β-actin mRNA expression of $CD4^+CD25^-$ cells (lanes 1, 3, 5, 7) and $CD4^+CD25^+$ (lanes 2, 4, 6, 8) in RLNs (lanes 1-4) and PLNs (lanes 5-8) in BaP-only (lanes 1, 2, 5, 6) and control (lanes 3, 4, 7, 8) mice at 32 wk using RT-PCR. Relative expression of chemokine mRNA was compared (**B**). $CD4^+CD25^+$ cells expressed significantly higher levels of CCR1 (a) and CCR5 (a) mRNA. a: $P < 0.05$

receptors might distinguish the characteristics of $CD4^+$ cell subsets of RLNs and PLNs. To distinguish which factor attracts Tregs migration to RLNs, the expression of CCR1, CCR4, CCR5, CCR6, CCR7, and CCR8 transcripts in purified $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells of RLNs and PLNs was analyzed. According to the results of RT-PCR, $CD4^+CD25^+$ T cells of RLNs specifically expressed a higher level of CCR1 and CCR5 mRNA in mice of the BaP-only group at week 32 (Figure 10). Both $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells of RLNs and PLNs in mice of the BaP-only group exhibited a high expression of CCR4, CCR6, CCR7, and CCR8 mRNA. These results suggest that Tregs of RLNs in the forestomach tumors might possess a unique chemotactic profile for migration of Tregs, which is responsible for local immune suppression during tumor progression.

DISCUSSION

Gastric cancer is one of the most common cancers in the world, and lymph node metastasis is the strongest determinant of patient survival. There are many submucosa-associated lymphoid tissues responsible for immunosurveillance in the gastrointestinal tract. An increase in Tregs has been correlated with immunosuppression and tumor progression in patients with gastrointestinal cancers[4]. So far, there is no evidence that addresses changes in Tregs of lymph nodes at the initial stage of gastric cancer development in human beings or animals. In this study, we clearly demonstrated that the proportion of Tregs increased in the RLNs during tumor progression in BaP-induced mouse forestomach carcinoma. These Tregs significantly displayed enhanced expression levels of Foxp3 mRNA and protein and down-regulation of CD62L, and exhibited a unique chemotactic receptor profile, including high CCR1 and CCR5 mRNA levels, for migration to inflammation sites. These inducible Tregs also expressed high levels of IL-10 for inhibition of host immunity. After depletion of Tregs, the tumor volume decreased, and more infiltrating and apoptotic cells in the tumors were found. Our results indicate that Tregs in RLNs may suppress local host immune response during tumor progression.

Tregs are known to be immunoregulatory, and play an important role in immunological tolerance to self-

antigens and in inhibition of T-cell proliferation[2,29-32]. These observations demonstrate that Tregs are involved in immune dysfunction of cancer patients. Sutmuller *et al*[20] demonstrated that Treg depletion enhances T-cell reactivity to a known tumor-associated antigen. However, depletion of Tregs alone is not sufficient to treat any established cancer. In most of the relevant studies, anti-CD25 mAb was administered to deplete Tregs several days prior to tumor implantation or only 1 day afterwards[19,20,23,33-36]. In this study, although the $CD25^+$ cells in lymph nodes were not completely depleted, forestomach tumor growth was still remarkably reduced, suggesting that increased Tregs in tumor-draining lymph nodes inhibit host local immune response during tumorigenesis. A consecutive course of depleted-antibody injections, for mice without histologically proven autoimmune disease, is sufficient to cause regression of the tumors. It was reported that depletion of $CD25^+$ cells in tumor models could reduce tumor growth as I in our study[37].

Previous studies have shown that Tregs could suppress T cell priming in lymph nodes[30,38,39]. Depletion of Tregs could enhance T cell sensitization in tumor-draining lymph nodes and increase generation of specific immune T cells, and depletion of $CD25^+$ T cells could facilitate infiltration of $CD8^+$ T cells at tumor sites[19,23]. The Tregs are probably the main subset of $CD4^+$ T cells responsible for the observed enhancement of tumor growth[40]. These studies suggest that removal of Tregs can generate tumor-specific CD8 T cells and tumor-nonspecific $CD4^-CD8^-$ effector cells *in vivo*. In this study, Tregs were more abundant in RLNs around the forestomach carcinoma. After depletion, the number of Tregs in the RLNs decreased significantly, more granulocytes, $CD4^+$, and $CD8^+$ cells infiltrated into the tumors, and massive apoptotic cells were found in the tumor mass, proving that Tregs in the RLNs are strongly associated with tumor growth, and that depletion of Tregs can promote effective local tumor-specific immune responses.

Adhesion molecules (such as CD62L) and chemokine receptors are involved in lymphocyte trafficking. Tregs can be subdivided into different functional subsets based on the expression of CD62L. Loss of CD62L expression in Tregs could result from selective migration to forestomach cancer and accumulation at the tumor nodules or in draining lymph nodes after stimulation and activation. Iellem *et al*[40] reported that Tregs specifically express the chemokine receptors CCR4 and CCR8. Bystry *et al*[41] reported that Tregs express CCR5 and respond to the CCL4 ligand, and CCL4 is the most potent chemokine for Tregs. Chemokine receptor CCR1 and its two ligands, CCL5 and CCL7, or CCR5 and its ligand CCL4 may be involved in regulating the trafficking of Tregs. In this study, the expression profile of adhesion molecule and chemokine receptors differed from that of the RLN and PLN Tregs. There were more activated (CD62L negative) Tregs in RLNs, and these Tregs highly expressed the chemokine receptors CCR1 and CCR5, suggesting that a higher expression of CCR1 and CCR5 in activated Tregs may allow their migration toward RLNs or the tumor area to suppress local host immunity.

The above results highly suggest that, during tumor progression, tumor cells may recruit Tregs via a specific subset of chemokines and their receptors to control local host anti-tumor immunity. It is conceivable that it may be advantageous to use antibodies or fusion proteins to reduce the action of Tregs at the local sites in combination with chemoprevention drugs to gain the maximal benefit during treatment of gastric cancer, even though we did not perform functional inhibition studies of Treg cells on host cytotoxic cells. Chen *et al*[42] have induced apoptosis of Tregs in a lymphoma animal model using intratumorally injecting FasL protein to deplete Tregs in the tumor and tumor-draining lymph nodes. Viehl *et al*[43] have also depleted Tregs by intraperitoneal injection with PC61 three times a week, in combination with tumor vaccine. After depletion, the tumor-specific immune response was significantly increased in tumor-draining lymph nodes. Their results are consistent with our studies.

In conclusion, increased Tregs in draining regional lymph nodes suppress host local immunity during tumor growth and depletion of Tregs can promote host local immunity to prevent tumor progression, implying the significance of lymph node dissection in surgery for gastric cancer.

COMMENTS

Background

$CD4^+CD25^+$ regulatory T cells (Tregs) are correlated with the prognosis of malignant tumors and can compromise host immunity surveillance to cancer. Accumulation of Tregs in the drained lymph nodes may reduce host local immunity against gastric cancer. The role of Tregs in the drained lymph nodes was elucidated during cancer development was elucidated in this study.

Research frontiers

Lymph node metastasis is the key factor for prognosis of gastric cancer patients. Lymph node dissection is the radical gastrectomy for gastric cancer, but it is a highly technique-demanding surgery. Intraperitoneal treatment with monoclonal antibody to deplete Tregs in the drained lymph nodes might assist in increasing the curative effect on gastric cancer.

Innovations and breakthroughs

The high incidence of lymph node metastasis is still the milestone of the prognosis of gastric cancer. Decreased host local immunity surveillance might be the determinative for lymph node metastasis. The study established mouse for stomach carcinoma by garage with benzo[a]pyrene (BaP). Then, the mice received anti-CD25 treatment. The results clearly demonstrate that depletion of Tregs with anti-CD25 antibody could decrease Tregs distributed in drained lymph nodes, increase local immune cell infiltration in tumor and reduce tumor growth. This is the first successful treatment of solid tumor with monoclonal antibody to deplete Tregs in mice, thus laying an experimental foundation of development of drugs for treatment of cancer.

Applications

Intraperitoneal injection of anti-CD25 monoclonal antibody is a promising therapeutic approach to forestomach carcinoma. The results implicate that anti-CD25 therapy could be used as a chemoprevention modality for malignan tumors and a local immunity therapy for gastric cancer.

Peer review

The authors of this paper showed that depletion of Tregs can decrease growth of tumor in an animal model of forestomach carcinoma. The study was well designed and its findings are interesting and informative.

REFERENCES

1 **Wolf AM**, Wolf D, Steurer M, Gastl G, Gunsilius E, Grubeck-Loebenstein B. Increase of regulatory T cells in the peripheral blood of cancer patients. *Clin Cancer Res* 2003; **9**: 606-612

2 **Piccirillo CA**, Thornton AM. Cornerstone of peripheral tolerance: naturally occurring CD4+CD25+ regulatory T cells. *Trends Immunol* 2004; **25**: 374-380

3 **Ichihara F**, Kono K, Takahashi A, Kawaida H, Sugai H, Fujii H. Increased populations of regulatory T cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. *Clin Cancer Res* 2003; **9**: 4404-4408

4 **Sasada T**, Kimura M, Yoshida Y, Kanai M, Takabayashi A. CD4+CD25+ regulatory T cells in patients with gastrointestinal malignancies: possible involvement of regulatory T cells in disease progression. *Cancer* 2003; **98**: 1089-1099

5 **Kawaida H**, Kono K, Takahashi A, Sugai H, Mimura K, Miyagawa N, Omata H, Ooi A, Fujii H. Distribution of CD4+CD25high regulatory T-cells in tumor-draining lymph nodes in patients with gastric cancer. *J Surg Res* 2005; **124**: 151-157

6 **Woo EY**, Chu CS, Goletz TJ, Schlienger K, Yeh H, Coukos G, Rubin SC, Kaiser LR, June CH. Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res* 2001; **61**: 4766-4772

7 **Liyanage UK**, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G, Drebin JA, Strasberg SM, Eberlein TJ, Goedegebuure PS, Linehan DC. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J Immunol* 2002; **169**: 2756-2761

8 **Woo EY**, Yeh H, Chu CS, Schlienger K, Carroll RG, Riley JL, Kaiser LR, June CH. Cutting edge: Regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. *J Immunol* 2002; **168**: 4272-4276

9 **Curiel TJ**, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, Zou W. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004; **10**: 942-949

10 **Ormandy LA**, Hillemann T, Wedemeyer H, Manns MP, Greten TF, Korangy F. Increased populations of regulatory T cells in peripheral blood of patients with hepatocellular carcinoma. *Cancer Res* 2005; **65**: 2457-2464

11 **Viguier M**, Lemaitre F, Verola O, Cho MS, Gorochov G, Dubertret L, Bachelez H, Kourilsky P, Ferradini L. Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. *J Immunol* 2004; **173**: 1444-1453

12 **Phillips DH**. Polycyclic aromatic hydrocarbons in the diet. *Mutat Res* 1999; **443**: 139-147

13 **Halliwell B**, Gutteridge JM. Free radicals, ageing and disease. In: Halliwell B, Gutteridge JM. Free radicals in biology and medicine, 2nd edition. New York: Oxford University, Claredon Press, 1989: 446-493

14 **Deshpande SS**, Ingle AD, Maru GB. Inhibitory effects of curcumin-free aqueous turmeric extract on benzo[a]pyrene-induced forestomach papillomas in mice. *Cancer Lett* 1997; **118**: 79-85

15 **Agha AM**, El-Fattah AA, Al-Zuhair HH, Al-Rikabi AC. Chemopreventive effect of Ginkgo biloba extract against benzo(a)pyrene-induced forestomach carcinogenesis in mice: amelioration of doxorubicin cardiotoxicity. *J Exp Clin Cancer Res* 2001; **20**: 39-50

16 **Fahey JW**, Haristoy X, Dolan PM, Kensler TW, Scholtus I, Stephenson KK, Talalay P, Lozniewski A. Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of Helicobacter pylori and prevents benzo[a]pyrene-induced stomach tumors. *Proc Natl Acad Sci USA* 2002; **99**: 7610-7615

17 **Goswami UC**, Sharma N. Efficiency of a few retinoids and carotenoids in vivo in controlling benzo[a]pyrene-induced forestomach tumour in female Swiss mice. *Br J Nutr* 2005; **94**: 540-543

18 **Onizuka S**, Tawara I, Shimizu J, Sakaguchi S, Fujita T, Nakayama E. Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. *Cancer Res* 1999; **59**: 3128-3133

19 **Shimizu J**, Yamazaki S, Sakaguchi S. Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. *J Immunol* 1999; **163**: 5211-5218

20 **Sutmuller RP**, van Duivenvoorde LM, van Elsas A, Schumacher TN, Wildenberg ME, Allison JP, Toes RE, Offringa R, Melief CJ. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *J Exp Med* 2001; **194**: 823-832

21 **Wattenberg LW**, Lam LK, Fladmoe AV. Inhibition of chemical carcinogen-induced neoplasia by coumarins and alpha-angelicalactone. *Cancer Res* 1979; **39**: 1651-1654

22 **Tanaka H**, Tanaka J, Kjaergaard J, Shu S. Depletion of CD4+ CD25+ regulatory cells augments the generation of specific immune T cells in tumor-draining lymph nodes. *J Immunother* 2002; **25**: 207-217

23 **Shull MM**, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G, Calvin D. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 1992; **359**: 693-699

24 **Chen YL**, Wang JY, Chen SH, Yang BC. Granulocytes mediates the Fas-L-associated apoptosis during lung metastasis of melanoma that determines the metastatic behaviour. *Br J Cancer* 2002; **87**: 359-365

25 **Hori S**, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; **299**: 1057-1061

26 **Gough M**, Crittenden M, Thanarajasingam U, Sanchez-Perez L, Thompson J, Jevremovic D, Vile R. Gene therapy to manipulate effector T cell trafficking to tumors for immunotherapy. *J Immunol* 2005; **174**: 5766-5773

27 **Matsuura M**, Okazaki K, Nishio A, Nakase H, Tamaki H, Uchida K, Nishi T, Asada M, Kawasaki K, Fukui T, Yoshizawa H, Ohashi S, Inoue S, Kawanami C, Hiai H, Tabata Y, Chiba T. Therapeutic effects of rectal administration of basic fibroblast growth factor on experimental murine colitis. *Gastroenterology* 2005; **128**: 975-986

28 **Maloy KJ**, Powrie F. Regulatory T cells in the control of immune pathology. *Nat Immunol* 2001; **2**: 816-822

29 **Sakaguchi S**, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M, Kuniyasu Y, Nomura T, Toda M, Takahashi T. Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev* 2001; **182**: 18-32

30 **Gavin M**, Rudensky A. Control of immune homeostasis by naturally arising regulatory CD4+ T cells. *Curr Opin Immunol* 2003; **15**: 690-696

31 **Piccirillo CA**, Shevach EM. Naturally-occurring CD4+CD25+ immunoregulatory T cells: central players in the arena of peripheral tolerance. *Semin Immunol* 2004; **16**: 81-88

32 **Jones E**, Dahm-Vicker M, Simon AK, Green A, Powrie F, Cerundolo V, Gallimore A. Depletion of CD25+ regulatory cells results in suppression of melanoma growth and

induction of autoreactivity in mice. *Cancer Immun* 2002; **2**: 1
33 **Tawara I**, Take Y, Uenaka A, Noguchi Y, Nakayama E. Sequential involvement of two distinct CD4+ regulatory T cells during the course of transplantable tumor growth and protection from 3-methylcholanthrene-induced tumorigenesis by CD25-depletion. *Jpn J Cancer Res* 2002; **93**: 911-916
34 **Casares N**, Arribillaga L, Sarobe P, Dotor J, Lopez-Diaz de Cerio A, Melero I, Prieto J, Borras-Cuesta F, Lasarte JJ. CD4+/CD25+ regulatory cells inhibit activation of tumor-primed CD4+ T cells with IFN-gamma-dependent antiangiogenic activity, as well as long-lasting tumor immunity elicited by peptide vaccination. *J Immunol* 2003; **171**: 5931-5939
35 **Li J**, Hu P, Khawli LA, Epstein AL. Complete regression of experimental solid tumors by combination LEC/chTNT-3 immunotherapy and CD25(+) T-cell depletion. *Cancer Res* 2003; **63**: 8384-8392
36 **Shevach EM**. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol* 2002; **2**: 389-400
37 **Zou W**. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* 2006; **6**: 295-307
38 **von Herrath MG**, Harrison LC. Antigen-induced regulatory T cells in autoimmunity. *Nat Rev Immunol* 2003; **3**: 223-232
39 **Lin CC**, Chou CW, Shiau AL, Tu CF, Ko TM, Chen YL, Yang BC, Tao MH, Lai MD. Therapeutic HER2/Neu DNA vaccine inhibits mouse tumor naturally overexpressing endogenous neu. *Mol Ther* 2004; **10**: 290-301
40 **Iellem A**, Mariani M, Lang R, Recalde H, Panina-Bordignon P, Sinigaglia F, D'Ambrosio D. Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells. *J Exp Med* 2001; **194**: 847-853
41 **Bystry RS**, Aluvihare V, Welch KA, Kallikourdis M, Betz AG. B cells and professional APCs recruit regulatory T cells via CCL4. *Nat Immunol* 2001; **2**: 1126-1132
42 **Chen A**, Liu S, Park D, Kang Y, Zheng G. Depleting intratumoral CD4+CD25+ regulatory T cells via FasL protein transfer enhances the therapeutic efficacy of adoptive T cell transfer. *Cancer Res* 2007; **67**: 1291-1298
43 **Viehl CT**, Moore TT, Liyanage UK, Frey DM, Ehlers JP, Eberlein TJ, Goedegebuure PS, Linehan DC. Depletion of CD4+CD25+ regulatory T cells promotes a tumor-specific immune response in pancreas cancer-bearing mice. *Ann Surg Oncol* 2006; **13**: 1252-1258

S- Editor Zhong XY **L- Editor** Wang XL **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5810

World J Gastroenterol 2008 October 14; 14(38): 5810-5815
World Journal of Gastroenterology ISSN 1007-9327

GASTRIC CANCER

Suppression of gastric cancer growth by baculovirus vector-mediated transfer of normal epithelial cell specific-1 gene

Wei Huang, Xiang-Long Tian, Yun-Lin Wu, Jie Zhong, Li-Fen Yu, Sheng-Ping Hu, Biao Li

Wei Huang, Sheng-Ping Hu, Biao Li, Department of Nuclear Medicine, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025, China
Wei Huang, Xiang-Long Tian, Yun-Lin Wu, Jie Zhong, Li-Fen Yu, Department of Gastroenterology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025, China
Author contributions: Li B, Wu YL, Zhong J and Yu LF designed the research; Huang W, Tian XL and Hu SP performed the research; Huang W and Tian XL analyzed the data; Huang W and Li B wrote the paper.
Supported by The Doctoral Fund from the Ministry of Education of China, No. BXJ0710
Correspondence to: Biao Li, Department of Nuclear Medicine, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025, China. lb10363@rjh.com.cn
Telephone: +86-21-64370045-665558 Fax: +86-21-64333548
Received: April 9, 2008 Revised: September 2, 2008
Accepted: September 9, 2008
Published online: October 14, 2008

Abstract

AIM: To study the inhibitory effect of baculovirus-mediated normal epithelial cell specific-1 (*NES1*) gene therapy on gastric cancer (GC) *in vitro* and *in vivo*.

METHODS: We first constructed recombinant baculovirus vectors and then transfected them into gastric cancer cells (SGC-7901). Efficiency of the baculovirus for gene transfer into SGC-7901 cells and cell growth curves were detected by fluorescence microscopy, Western blot and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay *in vitro*, respectively. The therapeutic effect of this gene therapy on GC was confirmed in xenografted nude mice. Tumor growth was determined by tumor volume, and expression of NES1 in tumor was analyzed by immunohistochemistry.

RESULTS: Baculovirus vectors were successfully transfected into SGC-7901 cells. SGC-7901 cells transfected with the *NES1* gene inhibited cell growth. In the Bac-NES1 treated group, tumor growth was significantly reduced with a high level of NES1 expression

CONCLUSION: Baculovirus-mediated *NES1* gene can be used in gene therapy for GC.

Key words: Gastric cancer; Normal epithelial cell specific-1 gene; Baculovirus; Gene therapy

Peer reviewer: Yaron Niv, Professor, Department of Gastroenterology, Rabin Medical Center, Beilinson Campus, Tel Aviv University, 2 Hadekel St., Pardesia 42815, Israel

Huang W, Tian XL, Wu YL, Zhong J, Yu LF, Hu SP, Li B. Suppression of gastric cancer growth by baculovirus vector-mediated transfer of normal epithelial cell specific-1 gene. *World J Gastroenterol* 2008; 14(38): 5810-5815 Available from: URL: http://www.wjgnet.com/1007-9327/14/5810.asp DOI: http://dx.doi.org/10.3748/wjg.14.5810

INTRODUCTION

Gastric cancer (GC) remains one of the most common cancers and a major cause of cancer-related death worldwide. To date, tumor resection has remained the only curative therapy for GC. Since radiotherapy and chemotherapy have no significant therapeutic effect on GC, new therapeutic strategies are required[1]. Gene therapy represents an interesting alternative treatment for cancers, and a number of studies have demonstrated that gene therapy can effectively reduce tumor growth in animal models[2]. Studies aimed at probing the genetic changes that occur in GC have identified several genes as potential targets of gene therapy for GC[3]. Our previous study suggested that normal epithelial cell specific-1 (*NES1*) gene functions as a tumor suppressor gene in GC and contributes to the malignant progression of GC providing us a promising gene target of gene therapy[4].

The design of vectors for specific gene transfer is a major challenge in medical research. Viral vectors are the most efficient tools for genetic modification of the majority of somatic cells *in vitro* and *in vivo*[5]. Recombinant baculoviruses with a mammalian expression promoter have recently been viewed as a new generation of gene therapy vehicles holding a great promise[6,7]. The baculovirus genome is large and thus large transgenes can be accommodated. In addition, they are easy to scale up and obtain high levels of recombinant gene expression[8]. However, despite a good understanding of all these attractive features of baculovirus, gene therapy with the virus is still in its

infancy, and no practical application in cancer therapy, even in preclinical animal studies, has been reported[9]. In the present study, we developed a recombinant baculovirus vector encoding the *NES1* gene and tested whether intratumoral administration of *NES1* gene has anti-tumor activity in GC models of mice. Our results indicate that GC cells (SGC-7901) are permissive to baculovirus infection and baculovirus-mediated *NES1* gene can be used in the treatment of GC.

MATERIALS AND METHODS

Construction of recombinant vector

Baculovirus plasmid pFBGFPR was a gift from the Institute of Molecular Biology of Hong Kong University. pCMV-NES1 was a gift from Dr. Vimla Band from Division of Cancer Biology, Department of Radiotherapy, New England Medical Center. Recombinant baculoviruses were generated and propagated in spodoptera frugiperda (Sf-9) insect cells by a Bac-to-Bac system according to the standard manual (Invitrogen). Once the viruses were amplified in Sf-9 insect cells, GFP expression was observed under a fluorescent microscope for the CMV promoter activity in these cells. The viruses were amplified to a high titer by propagation in Sf-9 cells and stored in small aliquots at -80℃. Viral titers were determined by plaque assay on Sf-9 insect cells.

Cell line culture and incubation with the vector

SGC-7901 cells were preserved in our laboratory and maintained in RPMI 1640 with 10% FBS. Sf-9 cells were cultured at 27℃ in a spinner culture bottle containing Sf900Ⅱ (Gibco) supplemented with 10% FBS. SGC-7901 cells were plated at a density of 10^5 cells per well in 24-well plates. After 1 d, culture was infected with different multiplicities of infection (MOI) of Bac-GFP, which was defined as the number of virus particles per cell for 1 h. The viruses were then removed, and a fresh medium was added to the wells. Twenty-four hours after transduction, the culture was examined for GFP expression by fluorescence microscopy, and photos were taken with a Nikon coolpix990 digital camera. High levels of GFP expression could be detected in SGC-7901 cells transduced with Bac-GFP. The morphological characteristics and growth of SGC-7901 cells were normal during experiment.

Western blot analysis

Cell lysates were made with standard methods. The protein concentration of each sample was measured using a BCA kit. For SDS-PAGE, 20 μg of protein samples was loaded on 10% polyacrylamide gels. Proteins were transferred to a polyvinylidene difluoride membrane with a tank transfer system (Bio-Rad Laboratory), then blocked with a buffer containing 5% low fat skim milk and 0.1% Tween-20 in Tris-buffered saline (TBST) at room temperature for 1 h. Primary antibodies were diluted in TBST containing 5% skim milk. The membrane was incubated with primary antibodies overnight at 4℃. After washed three times with TBST, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (0.02 μg/mL in TBST) for 1 h at room temperature. Chemiluminescence was detected with an ECL Western blot detection kit (Amersham, Little Chalfont, UK) according to its manufacturer's instructions.

Proliferation assay of SGC-7901 cells

SGC-7901 cells were grown in RPMI 1640 medium containing 10% fetal serum. For cell growth measurement, 2×10^3 cells were reseeded into 96-well plates and incubated at 37℃ in 100 mL/L CO_2 for 24 h. The media were then replaced with 0.2 mL of RPMI 1640/10% FBS, and the test sample (Bac-NES1, Bac-GFP or PBS) was applied. The number of SGC-7901 cells was quantified by colorimetric 3,-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay with a minor modification, as previously described. The proliferation assay was repeated at least three times.

In vivo tumor xenograft studies

Inhibitory effect of the *NES1* gene on tumor growth was observed in xenografted nude mice with GC. Male athymic nude mice (Balb/c nude mice) at the age of five weeks were obtained from Shanghai Experimental Animals Centre of Chinese Academy of Sciences and had free access to sterilized food and autoclaved water. A suspension of SGC-7901 cells (1×10^7 cells in 0.2 mL PBS) was injected subcutaneously into the dorsal flank of each mouse. As the tumor grew larger, it was cut into small even pieces and replanted into the dorsal flanks of each mouse. Tumors were grown in mice to more than passage three. When the tumors were palpable, the mice were randomly divided into three groups (10 in each group). Mice in the two experimental groups were injected with 4×10^8 pfu baculoviruses containing the *NES1* gene (Bac-NES1) and PBS gene, respectively, directly into the tumors every three days. Mice in the control group were injected with 4×10^8 pfu baculoviruses containing the *GFP* (Bac-GFP) directly into the tumors every three days. Body weight and volume of xenografts were measured in a blinded fashion using callipers every three days during the 3 wk treatment period. Tumor size was calculated according to the formula $AB^2/2$, where A is the longest diameter and B is the shortest diameter of the tumor. After therapy, the mice were sacrificed, and tumor tissue was fixed in 10% formalin for subsequent immunohistochemistry examination.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections (4 μm) were deparaffinized, rehydrated, and washed with PBS. Tests were performed according to the two-step procedure. After incubated with 3% H_2O_2 for 10 min at room temperature, unmasked antigens were heated. The sections were covered with animal serum for 20 min, incubated with rabbit polyclonal NES1 antibody

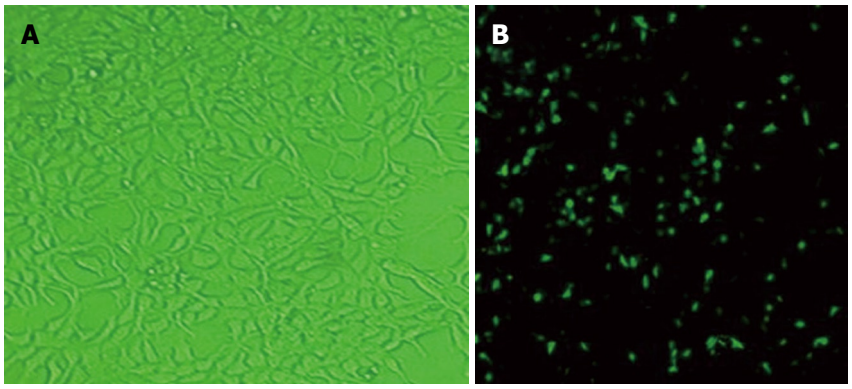


Figure 1 Microscopy (**A**) and fluorescence (**B**) photos of SGC-7901 cells infected with recombinant baculoviruses (Bac-GFP) (× 40).

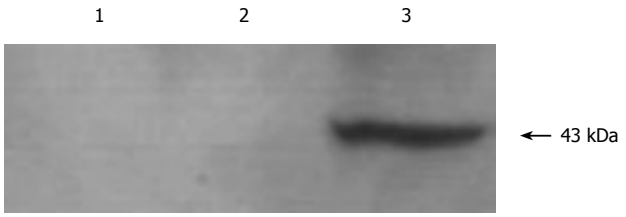


Figure 2 Western blot analysis showing NES1 protein expression in SGC-7901 cells. Lane 1: SGC-7901 cells + PBS; lane 2: SGC-7901 cells + Bac-GFP; lane 3: SGC-7901 cells + Bac-NES1.

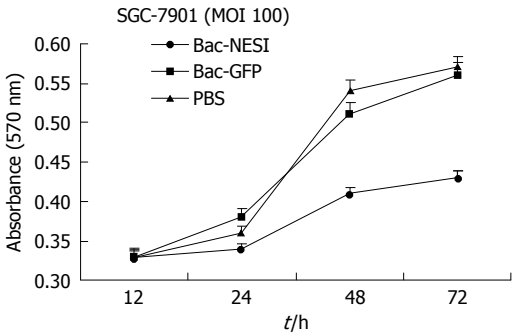


Figure 3 Effect of infection with Bac-NES1 on growth of GC *in vitro*.

(1:200) at 4℃ overnight, and further treated with an EnVision kit for 30 min at room temperature. The sections were visualized by diaminobenzidine (DAB) and counterstained with hematoxylin. TBS primary antibodies were replaced with TBS as a negative control. The sections were observed under a microscope after mounted. At least five thin sections of tumor tissue were used for quantitative immunohistochemistry. The results of staining were analyzed and evaluated with American Image-Pro Plus software.

Statistical analysis

Data were analyzed using the SPSS 10.0 software (Chicago, USA). Each experiment was done in triplicate. The data were presented as mean ± SD. Comparison among experimental groups was performed using ANOVA test. $P < 0.05$ was considered statistically significant. Nonparametric Kruskal-Wallis test and Spearman's correlation test were conducted to compare NES1 staining scores.

RESULTS

Construction of recombinant baculovirus vectors

We developed a baculovirus-derived vector, containing the *NES1* gene under control of the CMV promoter. Propagation of Bac-NES1 and Bac-GFP viruses in Sf-9 cells yielded viral stocks with a titer of 6×10^{12} PFU/mL, respectively. Bac-NES1 virus was purified and tittered as 2×10^{13} PFU/mL by standard end point dilution assay.

GFP expression in SGC-7901 cells transduced with recombinant baculoviruses

The efficiency of baculovirus gene transfer into SGC-7901 cells was assessed by fluorescence microscopy after infection with Bac-GFP. SGC-7901 cells transduced with Bac-GFP (MOI = 200) 24 h after transfection were examined by fluorescence microscopy, showing that SGC-7901 cells could be infected with baculovirus (Figure 1).

NES1 expression in SGC-7901 cells infected with Bac-NES1

Western blot analysis was performed using antibodies to NES1 was performed fro the expression of NES1 in SGC-7901 cells infected with recombinant baculoviruses (Bac-NES1). A polyclonal antibody to NES1 detected a single protein band at 43 kDa in the SGC-7901 cells + Bac-NES1 group (Figure 2).

Assay of SGC-7901 cell proliferation

SGC-7901 cells (2×10^3/well) were planted in 24 well plates and infected with Bac-NES1 or Bac-GFP at a multiplicity of infection (MOI) of 100 or treated with PBS. Proliferation of cells was quantified at 12 h, 24 h, 48 h, and 72 h, respectively by the modified MTT assay. Data are presented as mean ± SD of six wells. The proliferation of SGC-7901 cells infected with Bac-NES1 was significantly suppressed at MOI of 100 compared with PBS- and Bac-GFP- treated groups ($P < 0.05$), but there was no difference between the two groups at MOI = 100 (Figure 3). The data indicate that NES1 induced by baculovirus vectors had a significant *in vitro* inhibitory effect on proliferation of SGC-7901 cells.

GFP expression in gastric tumor xenografts

To investigate the possibility of using baculoviruses for *in vivo* GC gene therapy, we tested its expression in an

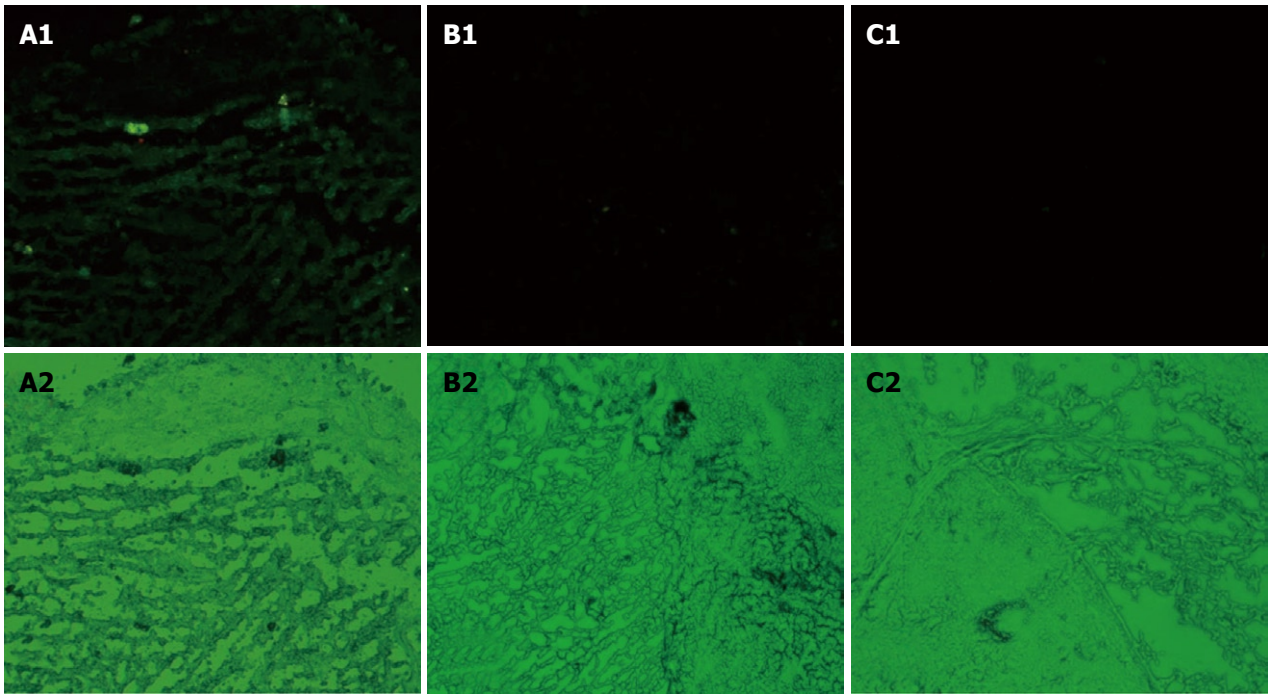


Figure 4 *In vivo* transgene expression in xenografted GC mediated by baculovirus carrying GFP (× 200) in Bac-NES1 treated group (**A**), Bac-GFP treated group (**B**), and PBS- treated group (**C**).

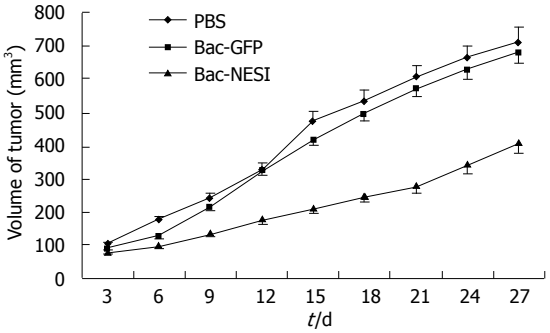


Figure 5 Inhibition of GC growth in nude mice. The mice were assigned to three groups (10 animals in each group). Growth of SGC-7901 xenografted tumor was measured at indicated time points. Relative tumor volume of the treatment groups was significantly different from that of the control group on day 27 of treatment ($P < 0.01$).

animal GC xenograft model. The GFP expression could be visually detected under a fluorescent microscope in the Bac-GFP-treated group (Figure 4). We did not observe any GFP positive cells in the Bac-NES1- and PBS- treated groups. The data indicate that gastric tumor xenografts could be infected with baculovirus.

Effect of NES1 expression on SGC-7901 gastric tumor xenografts

Xenografted GC was induced by injection of SGC-7901 cells into 5-week-old Balb/c nude mice. Treatment consisted of injection with 4×10^8 pfu Bac-NES1, 4×10^8 pfu Bac-GFP, or PBS directly into the tumor every three days. Tumor sizes were measured and presented as a mean. Tumor growth curves are shown in Figure 5. The growth of engrafted tumors was significantly inhibited in the Bac-NES1-treated groups (42.3%) compared with the control group on day 27 ($P < 0.01$). Significant differences in tumor volume were discovered between the control and Bac-NES1 treatment groups on day 9. However, the reduction rate was not significantly different between the Bac-GFP and PBS-treated groups throughout the treatment.

Effect of baculovirus vector-mediated transfer of NES1 gene on SGC-7901 gastric tumor xenografts

The expression of NES1 in SGC-7901 gastric tumor xenografts was assessed by immunostaining with NES1 antibody. Representative images of the three groups are shown in Figure 6. Tumors treated with Bac-NES1 exhibited a significantly higher NES1 expression (54.2 ± 15.7) than those treated with Bac-GFP (6.9 ± 1.7) and PBS (5.6 ± 1.4) ($P < 0.01$). The results suggest that reduced tumor growth and size might be associated with increased NES1 gene expression.

DISCUSSION

GC is one of the most common malignant tumors in the world, especially in Eastern Asia. Although the disease at its early stages is treatable with surgical resection, advanced GC does not generally respond to conventional chemotherapy or radiotherapy. Therefore, gene therapy represents an alternative modality for it[10]. Gene therapy is considered a promising therapeutic modality for cancer and has been successfully applied in animal models using various types of viral vector, gene expression regulation elements, and putative antitumor genes. Autographa californica multiple nucleopolyhedrovirus (AcMNPV)-based vector, traditionally used as a biopesticide to kill infected insects, is recently tested as a new type of

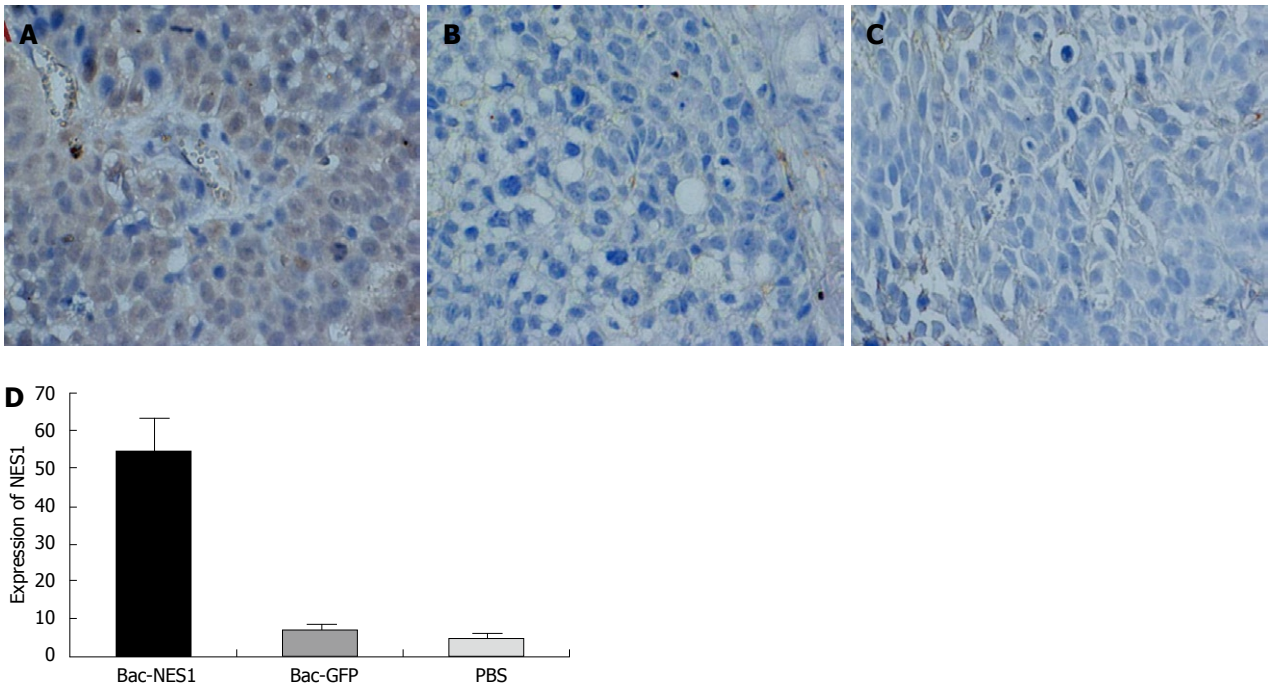


Figure 6 Immunohistochemical staining for tumors treated with Bac-NES1 (**A**), Bac-GFP (**B**), PBS (**C**), and the expression of NES1 protein in Bac-NES1-treated tumors (**D**). Data are presented as mean ± SD.

delivery vehicle for transgene expression in mammalian cells[11]. These viruses can enter but not replicate in mammalian cells. With mammalian expression promoters, recombinant baculoviruses provide a high transduction efficiency in different cells and tissues, including several tumor cell lines[12]. One of the attractive advantages of using AcMNPV as a cancer gene therapy vector is the large cloning capacity conferred by its 130-kb viral genome, which may be used to deliver a large functional gene or multiple genes from a single vector[13]. Other empirical advantages of baculovirus vectors include easy construction of a recombinant viral vector and simple procedure of purifying large quantities of viruses with high titers. It would be possible to scale up the less labor-intensive process to pharmaceutical levels[14].

Human kallikrein 10 (KLK10)/NES1 is a member of the human tissue kallikrein family of secreted serine proteases, encoded by a family of 15 genes clustered in tandem on chromosome 19q13.3-4[15]. The kallikrein gene family has been under intensive study due to its implications in carcinogenesis[16,17] and many members have been used as biomarkers for the diagnosis and monitoring of certain cancers[18-21]. Our previous study suggested that NES1, as a tumor suppressor gene in GC, contributes to the malignant progression of GC[2]. When the NES1 gene is transfected into tumorigenic breast cancer line MDA-MB-231, its anchorage-independent growth is reduced and when this cell line is inoculated into nude mice, tumor formation is significantly decreased[22]. The mechanism of how NES1 induces suppression of the tumorigenic phenotype is currently unknown. Given that NES1 is a secreted protein, it is likely that it functions extracellularly as a regulator of cell growth and/or differentiation in an autocrine or paracrine manner. In this study, the effect of Bac-NES1 on human GC cell lines was examined both *in vitro* and *in vivo*. The extent of baculovirus-mediated gene transfer was evaluated by measuring the expression of the GFP gene under fluorescence microscopy. Baculovirus vectors could transfer the GFP gene into over 90% of GC cell lines examined at MOI 100. Furthermore, Bac-NES1 could successfully deliver and express NES1 protein in Bac-NES1-transfected GC cell lines. These data show that baculovirus can transfer exogenous genes efficiently into GC cell lines. *In vitro*, GC cells infected with Bac-NES1 were significantly suppressed at MOI 100 compared with PBS treated and Bac-GFP infected cells. In order to observe the inhibitory effect of NES1 on tumor growth, a GC model was established. Baculovirus-mediated NES1 gene significantly inhibited the growth of SGC-7901 xenografted gastric tumors. Immunohistochemistry data showed that the reduced tumor growth and size might contribute to the reexpression of NES1 in xenografted gastric tumors.

In conclusion, NES1 gene transfection can inhibit the proliferation of SGC-7901 cells and suppress the transfected GC cells-derived tumor growth *in vivo*. Although further investigation is required to assess systemic *vs* regional issues, systemic adverse effects, and immunological response problems, baculovirus-mediated NES1 gene therapy may be a potent strategy for the treatment of GC.

COMMENTS

Background

Gastric cancer (GC) is one of the most common malignant tumors in the

world, especially in Eastern Asia. Although the disease at its early stages is treatable with surgical resection, advanced GC does not generally respond to conventional chemotherapy or radiotherapy. Therefore, gene therapy represents an alternative modality for GC. Normal epithelial cell specific-1 (*NES1*) gene functions as a tumor suppressor gene in GC and contributes to the malignant progression of GC, thus providing us a promising gene target of gene therapy.

Research frontiers

Gene therapy is considered a promising therapeutic modality for cancer and has been successfully applied in animal models using various types of viral vector, gene expression regulation elements, and putative antitumor genes. Autographa californica multiple nucleopolyhedrovirus (AcMNPV)-based vectors, traditionally used as a biopesticide to kill infected insects, has been recently tested as a new type of delivery vehicle for transgene expression in mammalian cells.

Innovations and breakthroughs

Recombinant baculovirus viruses with a mammalian expression promoter have recently been viewed as a new generation of gene therapy vehicles holding a great promise. The baculovirus genome is large and thus large transgenes can be accommodated. In addition, they are easy to scale up and obtain high levels of recombinant gene expression. Although further investigation is required to assess systemic vs regional issues, systemic adverse effect, and immunological response problems, baculovirus-mediated NES1 gene therapy may be a potent strategy for the treatment of GC.

Applications

Baculovirus-mediated expression of *NES1* inhibits the growth of xenografted GC by 42.3% compared with controls. Thanks to the highly efficient gene delivery, non-cytotoxicity and ease of scaling up, baculovirus may become a novel gene-delivery system for cancer gene therapy.

Terminology

Gene therapy is a promising molecular alternative treatment modality for cancer, including replacement of defective tumor suppressor genes, inactivation of oncogenes, introduction of suicide genes, genetic immunotherapy, anti-angiogenetic gene therapy, and virotherapy.

Peer review

The paper describes that baculovirus-mediated *NES1* gene can be used in treatment of GC in a mouse model. The study is well designed and interesting.

REFERENCES

1 **Foukakis T**, Lundell L, Gubanski M, Lind PA. Advances in the treatment of patients with gastric adenocarcinoma. *Acta Oncol* 2007; **46**: 277-285

2 **Vogiatzi P**, Cassone M, Claudio PP. Personalizing gene therapy in gastric cancer. *Drug News Perspect* 2006; **19**: 533-540

3 **Tamura G**. Alterations of tumor suppressor and tumor-related genes in the development and progression of gastric cancer. *World J Gastroenterol* 2006; **12**: 192-198

4 **Huang W**, Zhong J, Wu LY, Yu LF, Tian XL, Zhang YF, Li B. Downregulation and CpG island hypermethylation of NES1/hK10 gene in the pathogenesis of human gastric cancer. *Cancer Lett* 2007; **251**: 78-85

5 **Khalighinejad N**, Hariri H, Behnamfar O, Yousefi A, Momeni A. Adenoviral gene therapy in gastric cancer: a review. *World J Gastroenterol* 2008; **14**: 180-184

6 **Boyce FM**, Bucher NL. Baculovirus-mediated gene transfer into mammalian cells. *Proc Natl Acad Sci USA* 1996; **93**: 2348-2352

7 **Matilainen H**, Rinne J, Gilbert L, Marjomaki V, Reunanen H, Oker-Blom C. Baculovirus entry into human hepatoma cells. *J Virol* 2005; **79**: 15452-15459

8 **Jarvis DL**, Garcia A Jr. Long-term stability of baculoviruses stored under various conditions. *Biotechniques* 1994; **16**: 508-513

9 **Heideman DA**. Gene therapy and virotherapy of gastric cancer: preclinical results and clinical developments. *Dig Dis* 2004; **22**: 374-379

10 **Fumoto S**, Nishi J, Nakamura J, Nishida K. Gene therapy for gastric diseases. *Curr Gene Ther* 2008; **8**: 187-200

11 **Condreay JP**, Kost TA. Baculovirus expression vectors for insect and mammalian cells. *Curr Drug Targets* 2007; **8**: 1126-1131

12 **Hofmann C**, Sandig V, Jennings G, Rudolph M, Schlag P, Strauss M. Efficient gene transfer into human hepatocytes by baculovirus vectors. *Proc Natl Acad Sci USA* 1995; **92**: 10099-10103

13 **Kost TA**, Condreay JP. Recombinant baculoviruses as mammalian cell gene-delivery vectors. *Trends Biotechnol* 2002; **20**: 173-180

14 **Ghosh S**, Parvez MK, Banerjee K, Sarin SK, Hasnain SE. Baculovirus as mammalian cell expression vector for gene therapy: an emerging strategy. *Mol Ther* 2002; **6**: 5-11

15 **Luo L**, Herbrick JA, Scherer SW, Beatty B, Squire J, Diamandis EP. Structural characterization and mapping of the normal epithelial cell-specific 1 gene. *Biochem Biophys Res Commun* 1998; **247**: 580-586

16 **Yousef GM**, Luo LY, Diamandis EP. Identification of novel human kallikrein-like genes on chromosome 19q13.3-q13.4. *Anticancer Res* 1999; **19**: 2843-2852

17 **Diamandis EP**, Yousef GM, Luo LY, Magklara A, Obiezu CV. The new human kallikrein gene family: implications in carcinogenesis. *Trends Endocrinol Metab* 2000; **11**: 54-60

18 **Zhang Y**, Bhat I, Zeng M, Jayal G, Wazer DE, Band H, Band V. Human kallikrein 10, a predictive marker for breast cancer. *Biol Chem* 2006; **387**: 715-721

19 **Yunes MJ**, Neuschatz AC, Bornstein LE, Naber SP, Band V, Wazer DE. Loss of expression of the putative tumor suppressor NES1 gene in biopsy-proven ductal carcinoma in situ predicts for invasive carcinoma at definitive surgery. *Int J Radiat Oncol Biol Phys* 2003; **56**: 653-657

20 **Dhar S**, Bhargava R, Yunes M, Li B, Goyal J, Naber SP, Wazer DE, Band V. Analysis of normal epithelial cell specific-1 (NES1)/kallikrein 10 mRNA expression by in situ hybridization, a novel marker for breast cancer. *Clin Cancer Res* 2001; **7**: 3393-3383

21 **Luo LY**, Rajpert-De Meyts ER, Jung K, Diamandis EP. Expression of the normal epithelial cell-specific 1 (NES1; KLK10) candidate tumour suppressor gene in normal and malignant testicular tissue. *Br J Cancer* 2001; **85**: 220-224

22 **Li B**, Goyal J, Dhar S, Dimri G, Evron E, Sukumar S, Wazer DE, Band V. CpG methylation as a basis for breast tumor-specific loss of NES1/kallikrein 10 expression. *Cancer Res* 2001; **61**: 8014-8021

S- Editor Li DL **L- Editor** Wang XL **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5816

World J Gastroenterol 2008 October 14; 14(38): 5816-5822
World Journal of Gastroenterology ISSN 1007-9327

LIVER CANCER

Bioinformatics analysis of metastasis-related proteins in hepatocellular carcinoma

Pei-Ming Song, Yang Zhang, Yu-Fei He, Hui-Min Bao, Jian-Hua Luo, Yin-Kun Liu, Peng-Yuan Yang, Xian Chen

Pei-Ming Song, Jian-Hua Luo, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200030, China
Pei-Ming Song, Yang Zhang, Yu-Fei He, Hui-Min Bao, Yin-Kun Liu, Peng-Yuan Yang, Xian Chen, Institute of Biomedical Science, Fudan University, Shanghai 200320, China
Xian Chen, Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States
Author contributions: Song PM, He YF, Chen X, Liu YK, Yang PY and Luo JH designed the research; Song PM performed the proteomics analysis with the help of Bao HM; Song PM and Zhang Y analyzed the data; Song PM and He YF wrote the paper.
Supported by National Basic Research Priorities Program No. 2001CB510202; National Science and Technology Key Project No. 2002BAC11A11 and 2004BA711A19; National Natural Science Foundation of China No. 20328508; National High Technology Research Developing Program No. 02BAC11A11; Shanghai Science and Technology Development Program No. 03DZ14024
Correspondence to: Xian Chen, Professor, Institute of Biomedical Science, Fudan University, Shanghai 200320, China. xian_chen@med.unc.edu
Telephone: +86-21-54237443 Fax: +86-21-54237416
Received: May 14, 2008 Revised: August 26, 2008
Accepted: September 2, 2008
Published online: October 14, 2008

Abstract

AIM: To analyze the metastasis-related proteins in hepatocellular carcinoma (HCC) and discover the biomarker candidates for diagnosis and therapeutic intervention of HCC metastasis with bioinformatics tools.

METHODS: Metastasis-related proteins were determined by stable isotope labeling and MS analysis and analyzed with bioinformatics resources, including Phobius, Kyoto encyclopedia of genes and genomes (KEGG), online mendelian inheritance in man (OMIM) and human protein reference database (HPRD).

RESULTS: All the metastasis-related proteins were linked to 83 pathways in KEGG, including MAPK and p53 signal pathways. Protein-protein interaction network showed that all the metastasis-related proteins were categorized into 19 function groups, including cell cycle, apoptosis and signal transduction. OMIM analysis linked these proteins to 186 OMIM entries.

CONCLUSION: Metastasis-related proteins provide HCC cells with biological advantages in cell proliferation, migration and angiogenesis, and facilitate metastasis of HCC cells. The bird's eye view can reveal a global characteristic of metastasis-related proteins and many differentially expressed proteins can be identified as candidates for diagnosis and treatment of HCC.

Key words: Hepatocellular carcinoma; Metastasis; Bioinformatics; Protein-protein interaction; Kyoto encyclopedia of genes and genomes

Peer reviewer: Dr. Xin-Yuan Guan, Department of Clinical Oncology, University of Hong Kong, Room 109, Estate Building, 10 Sassoon Road, Hong Kong 852, China

Song PM, Zhang Y, He YF, Bao HM, Luo JH, Liu YK, Yang PY, Chen X. Bioinformatics analysis of metastasis-related proteins in hepatocellular carcinoma. *World J Gastroenterol* 2008; 14(38): 5816-5822 Available from: URL: http://www.wjgnet.com/1007-9327/14/5816.asp DOI: http://dx.doi.org/10.3748/wjg.14.5816

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world with 626 000 new cases occurred in 2002[1]. Surgical resection remains the primary treatment of choice, but because of the very high rate of metastasis and poor prognosis, the number of deaths is almost the same of new cases (598 000) occurred each year worldwide. Metastasis is a highly complicated biological process involving multiple proteins functioning in a coordinated manner and the molecular mechanism underlying the metastasis of HCC is not completely understood. Investigations on metastasis-related proteins and the molecular mechanism are urgent in the campaign against HCC both in the world and in China[2]. Technology advances in genomics and proteomics would facilitate our understanding of HCC metastasis. Genome analysis with DNA array can be used to scan different gene expressions in different HCC samples and valuable biomarkers have been discovered[3]. However, no well linear correlation has been found between gene and protein expression levels due to splice process of mRNA and post-transcriptional regulation[4,5]. Hence, differential expression profile analysis of a large number of proteins

is an essential step in understanding the mechanism of metastasis and in discovering the diagnostic markers and therapeutic targets for HCC.

Quantitative proteomics using stable isotope labeling has the necessary ability to rapidly identify and quantify differentially expressed proteins in two or more samples with a high throughout, and thus is currently used as the workhorse for discovering and validating proteins related with a special disease in clinical research. In our previous work, the proteome profiles of two HCC cell lines with different metastasis potentials were compared using stable isotope labeling and 223 differentially expressed proteins were identified confidently. In this study, we analyzed these proteins with bioinformatics tools to discover their biological role in the process of metastasis. Our results show that bioinformatics analysis can provide a valuable molecular basis for systematic interpretation of the mechanism underlying HCC metastasis where potential protein markers could be characterized.

MATERIALS AND METHODS

Chemicals and reagents

[5,5,5-d_3] leucine (leu-d_3) was purchased from Cambridge Isotope (Andover, MA). Formic acid (FA) and trifluoroacetic acid (TFA) were from FlukA (Switzerland) and acetonitrile (ACN) was from Merk (Darmastadt, Germany). Trypsins were purchased form Promega (Madison, WI). Dulbecco's modified Eagle's medium (DMEM) and dialyzed fetal bovine serum (FBS) were from Gibco-Invitrogen (Grand Island, NY, USA), and normal FBS was from PAA Laboratories GmbH. All others components of cell culture medium, amino acid kits, inorganic salts, vitamin solution, *etc*, were obtained from Sigma (St, Louis, MO). Other chemicals for SDS-PAGE gel electrophoresis, peptide extraction, and sample preparation for LC-ESI-MS were purchased from Sigma (St. Louis, MO, USA).

Cell culture, sample preparation

HCC cell line MHCC97H with a high metastasis potential was cultured in a normal DMEM, while MHCC97L with a low metastasis potential was cultured in a special DMEM, in which leu-d_3 was supplemented to substitute its unlabeled counterpart depleted in the normal DMEM[6]. Cells were harvested at the 80% confluence and treated with a lysis buffer containing 20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 3 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 10 mL/L NP-40, and 1 mmol/L PMSF. Samples were centrifuged at 10 000 r/min for 30 min at 4℃ and stored at -80℃ for electrophoresis. Samples from MHCC97H and MHCC97L were mixed at 1:1 of total protein mass and separated on SDS-PAGE. Trypsin digestion and peptide extraction were carried out as previously described[6].

LC separation and MS analysis

Peptides were resuspended in 5 μL of 1 mL/L TFA solution and directly loaded onto a 300 μm × 100 mm C18 column (Grace Vydac, Hesperia, CA) using an Agilent 1100 binary pump. The following gradients were used for peptide separation: 50-400 ml/L B from 0 min to 50 min, 400-800 ml/L B from 50 min to 70 min, 800-950 ml/L B from 70 min to 90 min, where solvent A was 1 mL/L FA and solvent B was 1 mL/L FA in ACN. Fractions were collected and deposited on a 12 × 16 array Applied Biosystems plate. Alpha-cyano-4-hydroxycinnamic acid (CHCA) was used as a matrix. Both MALDI-MS and MS/MS mass spectra were obtained with the Applied Biosystems 4700 proteomics analyzer (Framingham, MA, USA). MS/MS data were searched from the international protein index (IPI, version 3.07) human database with Mascot (version 1.9, Matrix Science, Boston, MA). Leu-d_3 modification was added into a modification file. Error tolerance of peptide MS was set at 0.3 U, MS/MS tolerance at 0.6 U. One missed cleavage per peptide was allowed.

Bioinformatics analysis of metastasis-related proteins

UniProt (Swiss-Prot) format file (ipi.HUMAN.dat) of IPI entries and cross-reference file were downloaded from IPI database (human, version 3.07, ftp://ftp.ebi.ac.uk/pub/databases/IPI/). GeneID, gene symbol and Swiss-Prot accession number for each metastasis-related protein were retried from the cross-reference file. Amino acid sequences of IPI entries in ipi.HUMAN.dat were used in prediction of transmembrane segments with Phobius (http://www.ebi.ac.uk/Tools/phobius/)[7].

Protein-protein interactions were extracted from human protein reference Database (HPRD)[8] and interaction network was demonstrated with Osprey (version 1.2.0, http://biodata.mshri.on.ca/osprey/index.html)[9]. GeneIDs were mapped to the KEGG pathway with a Perl script through the API service at the KEGG (http://www.genome.jp/kegg/soap/doc/keggapi_manual.html)[10].

Cross-references to each entry in the online mendelian inheritance in man (OMIM) database were obtained from the UniProtKB/Swiss-Prot subset of IPI entries through a Perl script. The detailed descriptions of OMIM entries were obtained from the OMIM web site (http://www.ncbi.nlm.nih.gov/omim).

Perl scripts were written in the environment of ActivePerl (version 5.8.8, http://www.activeperl.com).

RESULTS

Quantification of differentially expressed proteins between MHCC97H and MHCC97L

HCC cell lines MHCC97H and MHCC97L were subcloned from the same cell line MHCC97, with a high and low metastasis potential, respectively[11]. MHCC97L was cultured in a special medium for 10 passages and thus labeled completely with leu-d_3 while MHCC97H was cultured in a normal medium. Samples from normal (MHCC97H) and leu-d_3-labeled (MHCC97L) cells were combined and analyzed with off-line LC-MALDI-MS. Changes in expression of 506 proteins were quantified as previously described[12]. A high confidence as a single or multiple leucine-containing tryptic peptides

Table 1 Metastasis-related proteins in HCC

Protein name	IPI number	Ratio[1]	NO[2]	Coverage (%)[3]	Category
Filamin C	IPI00178352	2.49 ± 0.15	2	1.28	Cytoskeleton
Obscurin	IPI00549822	2.91 ± 0.18	2	1.16	Cytoskeleton
Keratin 8	IPI00554648	0.56 ± 0.15	4	25.1	Cytoskeleton
Titin	IPI00179357	1.73 ± 0.12	5	1.11	Cytoskeleton
Plectin 7	IPI00398776	2.49 ± 0.13	2	1.19	Cytoskeleton
MTA2	IPI00171798	0.24 ± 0.11	2	2.69	p53 regulation
Low density lipoprotein receptor-related protein 1 (LRP1)	IPI00020557	0.58 ± 0.15	2	2.27	Cell migration, proliferation, angiogenesis
Mos	IPI00018290	2.86 ± 0.2	2	6.36	Mitoses

[1]Ratio calculated as MHCC97H/MHCC97L, [2]number of quantified peptides, [3]protein coverage calculated as the number of amino acid residues for protein identification divided by the total number of amino acid residues of the protein.

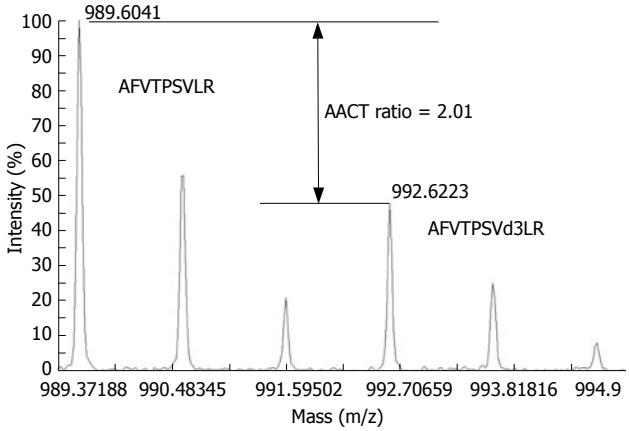


Figure 1 MS spectra of peptide AFVTPSVLR of Pr-domain zinc finger protein 5 (IPI00032997) and its labeled form AFVTPSVd3LR. Quantification was performed by comparing the intensities of peaks of AFVTPSVLR and AFVTPSVd3LR.

was detected for each of these proteins. Peptide AFVTPSVLR from Pr-domain zinc finger protein 5 (IPI00032997) contained one leucine, and the expression ratio was quantified as 2.01:1 (MHCC97H: MHCC97L) by comparing the intensity of peaks 989.6 and 992.6 (Figure 1). The results will be reported in another article in details and some important proteins are listed in Table 1. A total of 223 proteins with an expression ratio > 1.5 or < 0.67 were defined as metastasis-related proteins and further analyzed with bioinformatics tools.

The use of 2DE-MS for profiling the membrane proteins is limited due to the relative insolubility of these proteins under conditions suitable for 2-DE analysis[13,14]. However, membrane proteins are likely to be very import in understanding metastasis. For this reason, we undertook SDS-PAGE to identify more membrane proteins in our study. Phobius is a web tool to predict transmembrane topology of protein with a hidden Markov model[7]. Therefore, it was used to predict the transmembrane segments of metastasis-related proteins in our study. Several membrane proteins with multi-transmembrane segments were identified, such as sodium channel protein type 1 subunit alpha (IPI00216029) containing 24 transmembrane segments, brain calcium channel I (IPI00217499) containing 23 segments, HH1 (IPI00377006) containing 22 segments, hNaN (IPI00513973) containing 20 segments. It was difficult to detect these proteins with multi-transmembrane segments using the 2DE method due to their low solubility in 2DE lysis buffer. In our study, more membrane proteins were detected than in a previous report using 2DE[15]. The dataset of metastasis-related proteins here provided comprehensive biology information on the molecular mechanism of HCC and would facilitate the discovery of biomarkers for its diagnosis and therapy.

Biological pathways

Based on the API service of KEGG (http://www.genome.jp/kegg/soap/doc/keggapi_manual.html)[10], a Perl script was used to associate these metastasis-related proteins with known pathways and disease states (Table 2). All the proteins were linked to 83 biological pathways and human diseases in the KEGG, including antigen processing and presentation, regulation of actin cytoskeleton and signal pathways. Among these pathways, many biological processes were connected with metastasis in previous studies, including MAPK, p53 and ErbB, which are involved in regulating cell proliferation, angiogenesis and migration. In addition, several metastasis-related proteins were suggested to be involved in other types of cancer. For example, PAK7 and PAK 4 functioned in regulation of renal cell carcinoma according to the annotations in the KEGG.

Protein-protein interaction network

With the software Osprey and the protein-protein interaction data from HPRD, protein-protein interaction network of metastasis-related proteins was visualized (Figure 2, Table 3). The network contained 871 proteins (nodes) and 892 interactions (edges), and the edge/node was 1.02. Osprey also provides a functional tool to cluster proteins by their GO process, and proteins sharing the same GO process were grouped together. Based on the GO annotations in Osprey, all the proteins in the network were classed into 19 known biology function groups. For example, 120 proteins were involved in sig-

Table 2 KEGG pathway analysis of metastasis-related proteins

KEGG pathway	IPI accession	Protein description
Antigen processing and presentation	IPI00020984	Calnexin precusor
	IPI00025252	Protein disulfide-isomerase A3 precusor
	IPI00037070	Splice isoform 2 of heat shock cognate 71 kDa protein
	IPI00144014	MHC Cass I antigen
	IPI00514377	Heat shock 70 kDa protein
Regulation of actin cytoskeleton	IPI00001814	Serine/threonine- protein kinase PAK 7
	IPI00018290	Mos
	IPI00216691	Profiline-1
	IPI00384231	PAK4 protein
Renal cell carcinoma	IPI00001814	PAK 7
	IPI00384231	PAK4 protein
Thyroid cancer	IPI00022970	Translocated promoter region
Small cell lung cancer	IPI00217461	Splice isoform 3 of apoptotic protease activating factor 1
	IPI00377045	Laminin alpha 3 splice variant B1
Prostate cancer	IPI00219757	Glutahione S-transferase protein
p53 pathway	IPI00006160	Splice isoform alpha of tumor protein P73
	IPI00217461	Splice isoform 3 of apoptotic protease activating factor 1
MAPK pathway	IPI00018290	Mos
	IPI00037070	Splice isoform 2 of heat shock cognate 71 kDa protein (HSP70)
	IPI00155892	Voltage-dependent calcium channel gamma-5 subunit isoform B
	IPI00178352	Splice isoform 1 of filamin C
	IPI00382696	Splice isoform 1 of filamin B
	IPI00514377	Heat shock 70 kDa protein 1A
ErbB pathway	IPI00001814	PAK 7
	IPI00384231	PAK4

Table 3 Function classification of protein-protein interaction network

Cell function	Interactors	Percent (%)
Cell cycle	6	0.69
DNA repair	57	6.54
Stress response	14	1.61
Protein biosynthesis	9	1.03
Carbohydrate metabolism	26	2.99
DNA replication	3	0.34
Transcription	39	4.48
RNA processing	14	1.61
RNA localization	1	0.11
Signal transduction	120	13.78
Transport	41	4.71
Protein dephosphorylation	9	1.03
Cell organization and biogenesis	124	14.24
Metabolism	70	8.04
DNA damage response	3	0.34
Protein transport	59	6.77
Protein phosphorylation	60	6.89
Protein degradation	37	4.25
DNA repair	13	1.49
Unknown	166	19.06

nal transduction, 57 in DNA repair, and 6 in cell cycle (Table 3). This network could facilitate us to pinpoint the key proteins that play an important role in HCC metastasis and also help us to identify potential markers for the diagnosis and treatment of HCC.

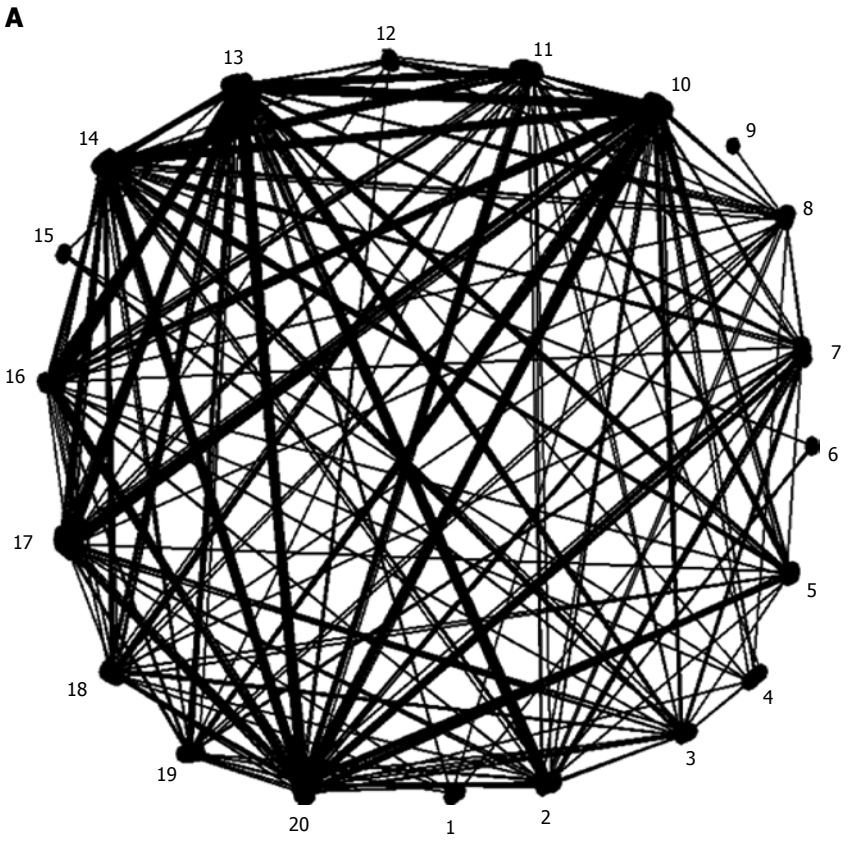
Disease association

OMIM is one of the most comprehensive resources of human genes and genetic disorders to support research in human genomics and proteomics[16]. A total of 145 proteins were linked to 186 OMIM entries, of which 141 described disease genes and 45 disease phenotypes. Among the disease phenotypes, one was cirrhosis (OMIM #215600) which would afford the development of HCC.

DISCUSSION

The accuracy of comparative proteomics using stable isotope labeling can be affected by various factors, such as isotope distribution[17]. When the peptide contains one leucine, M^+ ion peak of labeled peptide overlaps with the isotope distribution of the corresponding normal peptide. In order to eliminate the effect of isotope distribution and ensure the accuracy of quantification, the theoretical intensity of $(M+3)^+$ peak of normal peptide is subtracted from the labeled peak by calculating its isotope distribution[12]. Peaks with a signal-to-noise ratio < 30 are also excluded in quantification to attenuate the effect of noise on quantification. These modifications ensure the reliability of quantification with stable isotope labeling.

It was reported that some differentially expressed proteins function in the process of metastasis and plectin has an essential role in cell migration through MAP kinase signaling cascades[18,19]. Filamin C can organize actin polymerization and its dysregulation has been observed in some cancer samples[20]. Obscurin binding to titin and sarcomeric myosin[21,22] plays a role in the regular alignment of network sarcoplasmic reticulum developing sarcomeres[23]. The gene encoding this protein is interrupted by the translocation in Wilms' tumor and functions as a tumor suppressor[24]. Another study showed that obscurin is involved in gastrointestinal stromal tumor and leiomyosarcomas[25]. Proto-oncogene serine/threonine-protein kinase Mos acts as an up-



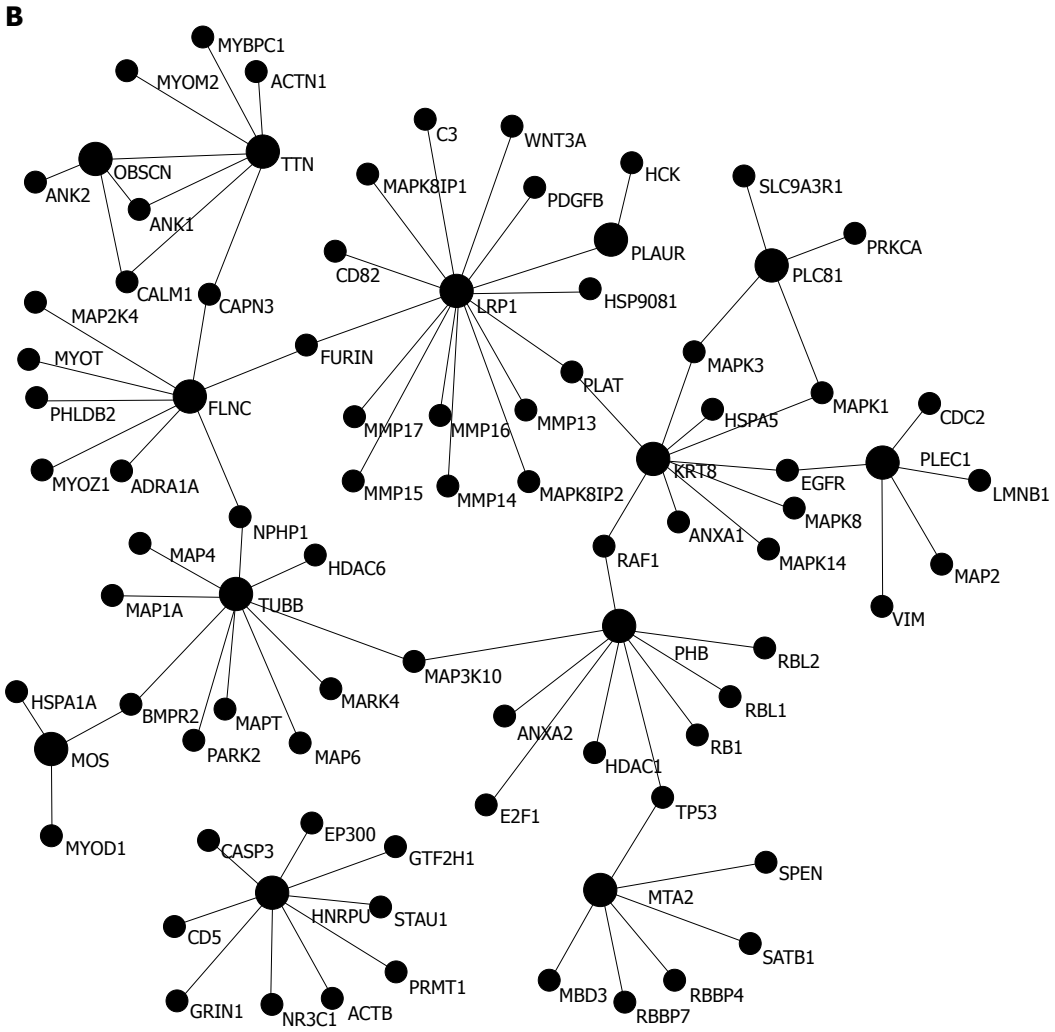


Figure 2 Protein-protein interaction network. **A**: Interactions of metastasis-related proteins. Based on the data from HPRD, protein-protein interactions were plotted using Osprey. Interactors are represented as nodes and grouped according to their biologic function based on Osprey annotation; **B**: A subnetwork displaying important proteins, such as Mos, LRP1, MTA2, and MAPK.

stream activator of the MEK/MAPK/p90Rsk pathway regulating M-phase and G2 arrest[26,27]. LRP1 can mediate growth inhibition by IGFBP-3 and cell migration inhibition by binding to apoE[28]. LRP1-mediated clearance of uPA is one of the mechanisms involved in the control of human thyroid carcinoma cell invasion[29]. It was reported that up-regulation of matrix metalloproteinase-9 by tPA in cell culture and *in vivo* is mediated by LRP1[30]. LRP1 also plays a role in determining the blood vessel structure and angiogenesis[31].

Two biological pathways discovered by KEGG analysis in our study were dysregulated and connected with HCC metastasis. The tumor suppressor protein p53 plays a pivotal role in the regulation of apoptosis and cell cycle arrest. In our study, two p53 regulators, p73 and apoptotic protease activating factor 1, were differentially expressed. MTA2 is a p53-interacting protein that induces p53 deacetylation[32,33]. Dysregulation of p53 function is linked with an unfavorable prognosis of a large number of more aggressive tumor types[34]. Eukaryotic cells possessing multiple MAPK pathways coordinately regulate diverse cellular activities including motility, survival, apoptosis and differentiation[35,36]. The Mos/MAPK/p90Rsk pathway regulates cell cycle progression in oocytes[37], whereas ectopic Mos expression in the early cleavage embryo induces M phase arrest[27]. HSP70 exhibits regulatory functions of c-Jun, ERK and the JNK pathway, thus inhibiting cell apoptosis[38]. The MAPK signaling pathway has long been identified as a convergence point for normal and pathologic signaling inputs, rendering it an appealing target for therapeutic intervention[39]. Various treatment modalities targeting p53 and MAPK pathway are currently under investigation, and dysregulation of p53 and MAPK pathway in HCC metastasis would facilitate finding targets for HCC therapy[39-41].

In conclusion, metastasis-related proteins are dysregulated in HCC metastasis. Biochemical alterations in cell proliferation and migration, angiogenesis and immune response confer selective biological advantages to HCC cells in the process of metastasis. Bioinformatics analysis of metastasis- related proteins provides valuable biological information on the molecular mechanism of metastasis and potential therapeutic targets for HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world with a high death rate. Metastasis is the major cause of HCC-related death. Discovering metastasis-related proteins would facilitate the diagnosis and treatment of HCC. Quantitative proteomics with stable isotope labeling is a powerful tool to analyze proteome differences between samples with different metastasis and to discover potential therapeutic targets for HCC.

Research frontiers

Our study showed the differential proteome profiles of two HCC cell lines with metastasis using stable isotope labeling. Based on the functional annotations with bioinformatics tools, metastasis-related proteins were functionally annotated with Kyoto encyclopedia of genes and genomes (KEGG) pathway, protein-protein interactions from human protein reference database (HPRD) and diseases from online mendelian inheritance in man (OMIM). Functional annotations showed that many proteins in the profile were clearly connected with the process of tumor metastasis.

Innovations and breakthrough

To the best of our knowledge, the present study showed the largest differential proteome profile of HCC metastasis. Functional annotations with bioinformatics tools showed that metastasis-related proteins were linked with 82 KEGG pathways, 892 interactions and 186 disease entries in OMIM, suggesting that they play a possible role in metastasis of HCC.

Applications

The differential proteome profile gives more valuable information on the molecular mechanism of metastasis of HCC and provides potential biomarkers for the diagnosis and treatment ofr HCC.

Peer review

This is a well conducted study. The manuscript describes the differential proteome profile that gives more information on the molecular mechanism of metastasis of HCC. The study also invested certain potential biomarkers that can be used in the diagnosis and treatment of HCC.

REFERENCES

1 **Parkin DM**, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74-108

2 **Tang ZY**, Ye SL, Liu YK, Qin LX, Sun HC, Ye QH, Wang L, Zhou J, Qiu SJ, Li Y, Ji XN, Liu H, Xia JL, Wu ZQ, Fan J, Ma ZC, Zhou XD, Lin ZY, Liu KD. A decade's studies on metastasis of hepatocellular carcinoma. *J Cancer Res Clin Oncol* 2004; **130**: 187-196

3 **Li Y**, Tang Y, Ye L, Liu B, Liu K, Chen J, Xue Q. Establishment of a hepatocellular carcinoma cell line with unique metastatic characteristics through in vivo selection and screening for metastasis-related genes through cDNA microarray. *J Cancer Res Clin Oncol* 2003; **129**: 43-51

4 **Gygi SP**, Rochon Y, Franza BR, Aebersold R. Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol* 1999; **19**: 1720-1730

5 **Futcher B**, Latter GI, Monardo P, McLaughlin CS, Garrels JI. A sampling of the yeast proteome. *Mol Cell Biol* 1999; **19**: 7357-7368

6 **Shui W**, Liu Y, Fan H, Bao H, Liang S, Yang P, Chen X. Enhancing TOF/TOF-based de novo sequencing capability for high throughput protein identification with amino acid-coded mass tagging. *J Proteome Res* 2005; **4**: 83-90

7 **Kall L**, Krogh A, Sonnhammer EL. Advantages of combined transmembrane topology and signal peptide prediction--the Phobius web server. *Nucleic Acids Res* 2007; **35**: W429-W432

8 **Peri S**, Navarro JD, Amanchy R, Kristiansen TZ, Jonnalagadda CK, Surendranath V, Niranjan V, Muthusamy B, Gandhi TK, Gronborg M, Ibarrola N, Deshpande N, Shanker K, Shivashankar HN, Rashmi BP, Ramya MA, Zhao Z, Chandrika KN, Padma N, Harsha HC, Yatish AJ, Kavitha MP, Menezes M, Choudhury DR, Suresh S, Ghosh N, Saravana R, Chandran S, Krishna S, Joy M, Anand SK, Madavan V, Joseph A, Wong GW, Schiemann WP, Constantinescu SN, Huang L, Khosravi-Far R, Steen H, Tewari M, Ghaffari S, Blobe GC, Dang CV, Garcia JG, Pevsner J, Jensen ON, Roepstorff P, Deshpande KS, Chinnaiyan AM, Hamosh A, Chakravarti A, Pandey A. Development of human protein reference database as an initial platform for approaching systems biology in humans. *Genome Res* 2003; **13**: 2363-2371

9 **Breitkreutz BJ**, Stark C, Tyers M. Osprey: a network visualization system. *Genome Biol* 2003; **4**: R22

10 **Kanehisa M**, Araki M, Goto S, Hattori M, Hirakawa M, Itoh M, Katayama T, Kawashima S, Okuda S, Tokimatsu T, Yamanishi Y. KEGG for linking genomes to life and the environment. *Nucleic Acids Res* 2008; **36**: D480-D484

11 **Li Y**, Tang ZY, Ye SL, Liu YK, Chen J, Xue Q, Chen J, Gao DM, Bao WH. Establishment of cell clones with different metastatic potential from the metastatic hepatocellular carcinoma cell line MHCC97. *World J Gastroenterol* 2001; **7**: 630-636

12 **Gu S**, Liu Z, Pan S, Jiang Z, Lu H, Amit O, Bradbury EM,

Hu CA, Chen X. Global investigation of p53-induced apoptosis through quantitative proteomic profiling using comparative amino acid-coded tagging. *Mol Cell Proteomics* 2004; **3**: 998-1008
13 **Carter P**, Smith L, Ryan M. Identification and validation of cell surface antigens for antibody targeting in oncology. *Endocr Relat Cancer* 2004; **11**: 659-687
14 **Flory MR**, Griffin TJ, Martin D, Aebersold R. Advances in quantitative proteomics using stable isotope tags. *Trends Biotechnol* 2002; **20**: S23-S29
15 **Ding SJ**, Li Y, Shao XX, Zhou H, Zeng R, Tang ZY, Xia QC. Proteome analysis of hepatocellular carcinoma cell strains, MHCC97-H and MHCC97-L, with different metastasis potentials. *Proteomics* 2004; **4**: 982-994
16 **Hamosh A**, Scott AF, Amberger JS, Bocchini CA, McKusick VA. Online Mendelian Inheritance in Man (OMIM), a knowledgebase of human genes and genetic disorders. *Nucleic Acids Res* 2005; **33**: D514-D517
17 **Thiede B**, Kretschmer A, Rudel T. Quantitative proteome analysis of CD95 (Fas/Apo-1)-induced apoptosis by stable isotope labeling with amino acids in cell culture, 2-DE and MALDI-MS. *Proteomics* 2006; **6**: 614-622
18 **Osmanagic-Myers S**, Gregor M, Walko G, Burgstaller G, Reipert S, Wiche G. Plectin-controlled keratin cytoarchitecture affects MAP kinases involved in cellular stress response and migration. *J Cell Biol* 2006; **174**: 557-568
19 **Boczonadi V**, McInroy L, Maatta A. Cytolinker cross-talk: periplakin N-terminus interacts with plectin to regulate keratin organisation and epithelial migration. *Exp Cell Res* 2007; **313**: 3579-3591
20 **Kaneda A**, Kaminishi M, Yanagihara K, Sugimura T, Ushijima T. Identification of silencing of nine genes in human gastric cancers. *Cancer Res* 2002; **62**: 6645-6650
21 **Bang ML**, Centner T, Fornoff F, Geach AJ, Gotthardt M, McNabb M, Witt CC, Labeit D, Gregorio CC, Granzier H, Labeit S. The complete gene sequence of titin, expression of an unusual approximately 700-kDa titin isoform, and its interaction with obscurin identify a novel Z-line to I-band linking system. *Circ Res* 2001; **89**: 1065-1072
22 **Kontrogianni-Konstantopoulos A**, Catino DH, Strong JC, Randall WR, Bloch RJ. Obscurin regulates the organization of myosin into A bands. *Am J Physiol Cell Physiol* 2004; **287**: C209-C217
23 **Kontrogianni-Konstantopoulos A**, Catino DH, Strong JC, Sutter S, Borisov AB, Pumplin DW, Russell MW, Bloch RJ. Obscurin modulates the assembly and organization of sarcomeres and the sarcoplasmic reticulum. *FASEB J* 2006; **20**: 2102-2111
24 **Vernon EG**, Malik K, Reynolds P, Powlesland R, Dallosso AR, Jackson S, Henthorn K, Green ED, Brown KW. The parathyroid hormone-responsive B1 gene is interrupted by a t(1;7)(q42;p15) breakpoint associated with Wilms' tumour. *Oncogene* 2003; **22**: 1371-1380
25 **Price ND**, Trent J, El-Naggar AK, Cogdell D, Taylor E, Hunt KK, Pollock RE, Hood L, Shmulevich I, Zhang W. Highly accurate two-gene classifier for differentiating gastrointestinal stromal tumors and leiomyosarcomas. *Proc Natl Acad Sci USA* 2007; **104**: 3414-3419
26 **Peter M**, Labbe JC, Doree M, Mandart E. A new role for Mos in Xenopus oocyte maturation: targeting Myt1 independently of MAPK. *Development* 2002; **129**: 2129-2139
27 **Wu JQ**, Hansen DV, Guo Y, Wang MZ, Tang W, Freel CD, Tung JJ, Jackson PK, Kornbluth S. Control of Emi2 activity and stability through Mos-mediated recruitment of PP2A. *Proc Natl Acad Sci USA* 2007; **104**: 16564-16569
28 **Zhu Y**, Hui DY. Apolipoprotein E binding to low density lipoprotein receptor-related protein-1 inhibits cell migration via activation of cAMP-dependent protein kinase A. *J Biol Chem* 2003; **278**: 36257-36263
29 **Sid B**, Dedieu S, Delorme N, Sartelet H, Rath GM, Bellon G, Martiny L. Human thyroid carcinoma cell invasion is controlled by the low density lipoprotein receptor-related protein-mediated clearance of urokinase plasminogen activator. *Int J Biochem Cell Biol* 2006; **38**: 1729-1740
30 **Wang X**, Lee SR, Arai K, Lee SR, Tsuji K, Rebeck GW, Lo EH. Lipoprotein receptor-mediated induction of matrix metalloproteinase by tissue plasminogen activator. *Nat Med* 2003; **9**: 1313-1317
31 **Gaultier A**, Salicioni AM, Arandjelovic S, Gonias SL. Regulation of the composition of the extracellular matrix by low density lipoprotein receptor-related protein-1: activities based on regulation of mRNA expression. *J Biol Chem* 2006; **281**: 7332-7340
32 **Yao YL**, Yang WM. The metastasis-associated proteins 1 and 2 form distinct protein complexes with histone deacetylase activity. *J Biol Chem* 2003; **278**: 42560-42568
33 **Luo J**, Su F, Chen D, Shiloh A, Gu W. Deacetylation of p53 modulates its effect on cell growth and apoptosis. *Nature* 2000; **408**: 377-381
34 **Levine AJ**, Hu W, Feng Z. The P53 pathway: what questions remain to be explored? *Cell Death Differ* 2006; **13**: 1027-1036
35 **Citri A**, Yarden Y. EGF-ERBB signalling: towards the systems level. *Nat Rev Mol Cell Biol* 2006; **7**: 505-516
36 **Huang D**, Ding Y, Luo WM, Bender S, Qian CN, Kort E, Zhang ZF, VandenBeldt K, Duesbery NS, Resau JH, Teh BT. Inhibition of MAPK kinase signaling pathways suppressed renal cell carcinoma growth and angiogenesis in vivo. *Cancer Res* 2008; **68**: 81-88
37 **Inoue D**, Ohe M, Kanemori Y, Nobui T, Sagata N. A direct link of the Mos-MAPK pathway to Erp1/Emi2 in meiotic arrest of Xenopus laevis eggs. *Nature* 2007; **446**: 1100-1104
38 **Lee JS**, Lee JJ, Seo JS. HSP70 deficiency results in activation of c-Jun N-terminal Kinase, extracellular signal-regulated kinase, and caspase-3 in hyperosmolarity-induced apoptosis. *J Biol Chem* 2005; **280**: 6634-6641
39 **Papatsoris AG**, Karamouzis MV, Papavassiliou AG. The power and promise of "rewiring" the mitogen-activated protein kinase network in prostate cancer therapeutics. *Mol Cancer Ther* 2007; **6**: 811-819
40 **Bell HS**, Ryan KM. iASPP inhibition: increased options in targeting the p53 family for cancer therapy. *Cancer Res* 2008; **68**: 4959-4962
41 **Prives C**, White E. Does control of mutant p53 by Mdm2 complicate cancer therapy? *Genes Dev* 2008; **22**: 1259-1264

S- Editor Zhong XY **L- Editor** Wang XL **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5823

World J Gastroenterol 2008 October 14; 14(38): 5823-5826
World Journal of Gastroenterology ISSN 1007-9327

Transcription factor PDX-1 in human colorectal adenocarcinoma: A potential tumor marker?

Nikiforos Ballian, Shi-He Liu, Francis Charles Brunicardi

Nikiforos Ballian, Shi-He Liu, Francis Charles Brunicardi, the Michael E. DeBakey Department of Surgery, Baylor College of Medicine, 1709 Dryden, Suite 1500 Houston, TX 77030, United States
Author contributions: Ballian N analyzed data, wrote manuscript; Liu SH performed research, analyzed data; Brunicardi FC treated physician, procured tissue specimens.
Correspondence to: Francis Charles Brunicardi, MD, FACS, the Michael E. DeBakey Department of Surgery, Baylor College of Medicine, 1709 Dryden, Suite 1500, Houston, TX 77030, United States. cbrunica@bcm.edu
Telephone: +1-713-7988020 Fax: +1-713-7986609
Received: March 23, 2008 Revised: August 20, 2008
Accepted: August 27, 2008
Published online: October 14, 2008

Abstract

AIM: To examine the expression of pancreatic duodenal homeobox-1 (PDX-1) transcription factor in human colorectal cancer.

METHODS: RT-PCR, Western blotting, and immunohistochemistry were performed to determine the expression pattern of transcription factor PDX-1 in primary colorectal tumor, hepatic metastasis, and benign colon tissue from a single patient.

RESULTS: The highest PDX-1 transcription levels were detected in the metastasis material. Lower levels of PDX-1 were found to be present in the primary tumor, while normal colon tissue failed to express detectable levels of PDX-1. Western blot data revealed a PDX-1 expression pattern identical to that of mRNA expression. Immunohistochemistry confirmed high metastasis PDX-1 expression, lower levels in the primary tumor, and the presence of only traces of PDX-1 in normal colon tissue.

CONCLUSION: These data argue for further evaluation of PDX-1 as a biomarker for colorectal cancer.

Key words: Colorectal cancer; Pancreatic duodenal homeobox-1; Tumor marker; Transcription factor; Diagnostics

Peer reviewer: Yutaka Saito, Division of Endoscopy, National Cancer Center Hospital, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

Ballian N, Liu SH, Brunicardi FC. Transcription factor PDX-1 in human colorectal adenocarcinoma: A potential tumor marker? *World J Gastroenterol* 2008; 14(38): 5823-5826 Available from: URL: http://www.wjgnet.com/1007-9327/14/5823.asp DOI: http://dx.doi.org/10.3748/wjg.14.5823

INTRODUCTION

In 2008, colorectal cancer is projected to be the third leading cause of cancer-related mortality in USA with an estimated 148 000 new cases and 50 000 deaths[1]. At least 40% of patients with colorectal cancer will experience metastases at some time during their illness[2]. Early detection of disease can improve prognosis, and survival varies significantly between patients with early-stage tumors and patients with metastases[3,4]. A number of studies have shown decreased mortality in populations undergoing colorectal cancer screening[5-8]. There is an increasing demand for colon cancer tumor markers for risk assessment and early diagnosis[9,10].

Pancreatic duodenal homeobox 1 (PDX-1) is a transcription factor with a critical role in pancreatic development[11]. PDX-1 regulates pancreatic cell proliferation and differentiation, and increased expression of this transcription factor has been described in human pancreatic adenocarcinoma and cell lines[12,13]. We recently found increased PDX-1 expression in benign tissues and malignant tumors from patients with pancreas, breast, colon, prostate, and renal cancers[14]. This indicates a possible role of PDX-1 as a tumor marker in patients with these malignancies.

In this report, levels of PDX-1 expression were quantified in a primary colorectal tumor, a metastasis, and in benign tissue from a single patient. Of particular interest were the expression pattern of PDX-1 and its potential use as a tumor marker in colorectal cancer.

MATERIALS AND METHODS

Samples

A 46-year-old male patient presented with a right-sided colorectal adenocarcinoma metastatic to the peritoneum and greater omentum. The patient underwent chemotherapy with capecitabine and oxaliplatin from August 2003 to February 2004 and right hemicolectomy in June 2004 for tumor-related bowel obstruction.

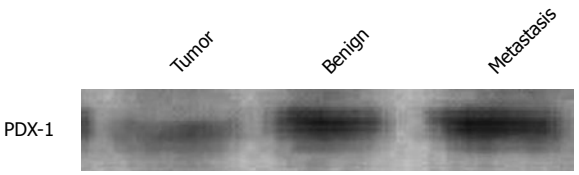


Figure 1 Western blotting results for PDX-1 in the primary tumor, benign colon tissue, and omental metastasis.

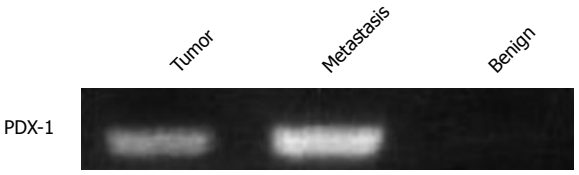


Figure 2 RT-PCR results for PDX-1 in the primary tumor, omental adenocarcinoma, and benign colon tissue.

Samples from the primary cecal tumor, omental metastases, and macroscopically normal colon mucosa from the distal end of the right hemicolectomy specimen (transverse colon) were collected during surgery (n = 16).

Immunohistochemistry

Tissues were fixed in 4% paraformaldehyde overnight at 4℃. Tissue processing, section preparation, and H&E staining were performed as described previously[14].

Western blot analysis

Western blotting was performed as described previously[15].

RNA preparations and RT-PCR

All samples were snap-frozen in liquid nitrogen. RNA was prepared according to procedures described in TRIzol Reagent manual (Cat. NO. 15596-026/-018). RT reactions were carried out according to the protocol of SuperScript Ⅲ First-Strand Synthesis System (Invitrogen Cat. No. 18080-051). PCR products were loaded on a 1.5% agarose gel and visualized and quantified by ethidium bromide staining using an UVP imaging system (UVP, Upland, CA).

RESULTS

As shown in Figure 1 (Western blot), high PDX-1 protein levels were found in the metastasis and the benign colon mucosa distant from the tumor. RT-PCR results in Figures 2 and 3 show that the highest PDX-1 mRNA levels were detected in the metastasis. Significant but lower levels were present in the primary tumor, while normal colon tissue had close to undetectable levels of PDX-1 mRNA. Immunohistochemistry (Figure 4) confirmed the high PDX-1 expression in the metastasis, the lower levels in the primary tumor, and the traces of PDX-1 in normal tissue.

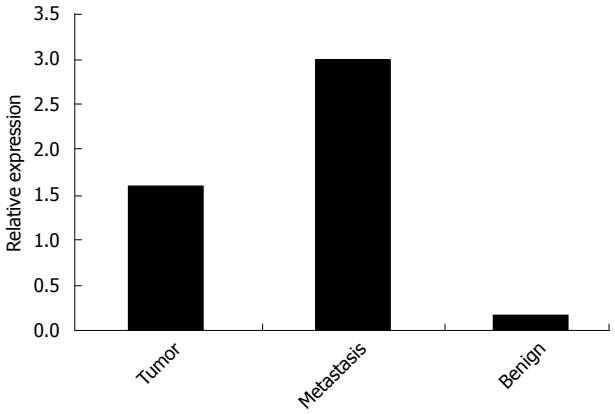


Figure 3 Quantitative RT-PCR results for PDX-1 in the primary tumor, omental metastasis, and benign colon tissue.

DISCUSSION

PDX-1 regulates pancreatic cell proliferation and differentiation[16-19]. Its aberrant expression in a number of human malignant tumors suggests a potential as a molecular marker. Among these malignancies, colorectal cancer is a common and frequently fatal disease for which screening programs are already being used in the United States[20]. Colonoscopy, the most accurate screening method, can cause significant patient discomfort and has risks of perforation and bleeding[21]. Perhaps for these reasons, participation in colorectal cancer screening programs is low[22]. Hence, safer and more acceptable screening methods are needed.

An important finding of this study is that malignant tissue was found to have significantly higher PDX-1 expression than normal colon mucosa outside the tumor. We have previously shown PDX-1 expression in 10 colon cancer specimens to be significantly elevated in both the nucleus and cytoplasm of malignant cells, compared to lower levels found in benign tissues[14]. In that study, six samples of colon tissue from colon cancer patients taken from sites outside the primary tumor were examined and found to express increased PDX-1 levels, although lower than that of tumors. In contrast, our current study showed colon mucosa distant from the primary tumor had nearly undetectable PDX-1 expression. These differences could be due to variations in the benign tissue sample distance from the primary tumor. Unfortunately, this was not recorded in our previous study and hence an accurate comparison is not possible.

Another significant observation is that, despite high levels of mRNA, PDX-1 protein levels are low in the primary tumor. This is consistent with posttranscriptional control of PDX-1 expression, which has been shown to occur in the pancreas[18]. In contrast, metastatic tissues retain high levels of both mRNA and protein expression. Although Western blotting showed lower levels of protein in the primary tumor compared to benign mucosa, immunohistochemistry did confirm high expression of PDX-1 protein in tumor cells. Hence, PDX-1 protein is present in tumor cells

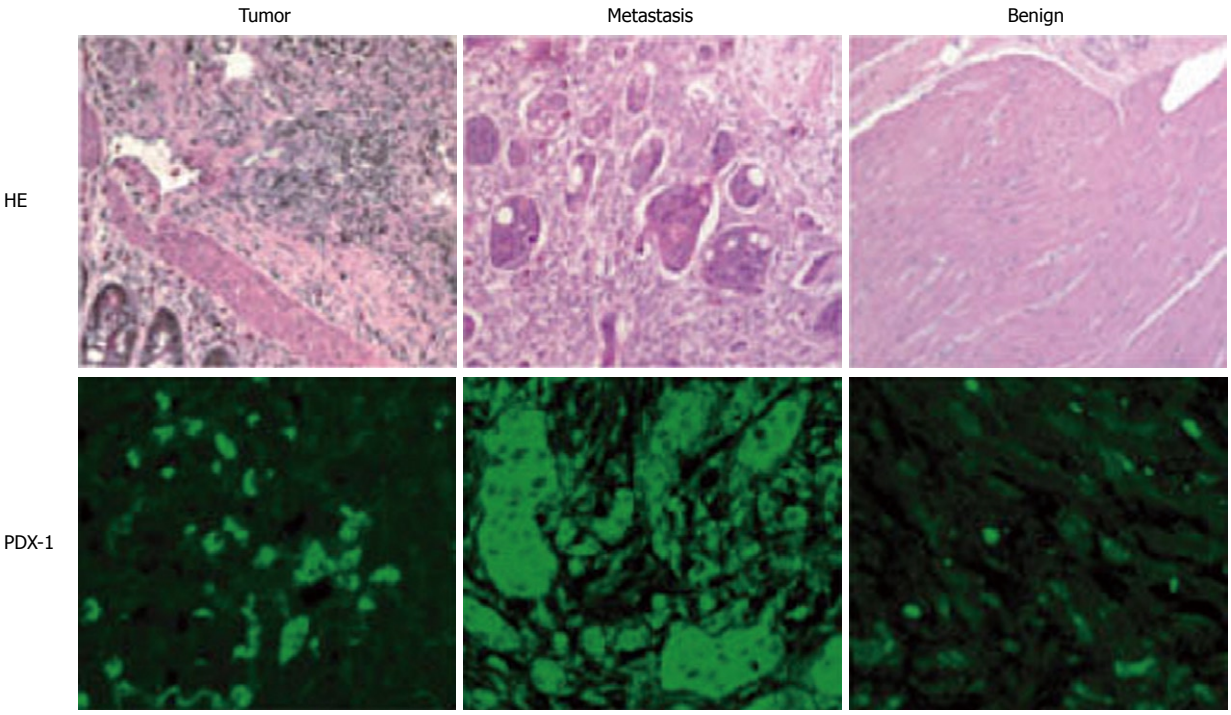


Figure 4 HE staining and immunohistochemistry for PDX-1 in the primary tumor, omental metastasis, and benign colon tissue.

and normal colonocytes, which are excreted in feces. This is a prerequisite for fecal screening for colorectal cancer. Interestingly, others have shown an upregulation of PDX-1 expression in colonic hyperplastic polyps and adenomas, the latter being precursors of adenocarcinomas[23].

Although the etiology of aberrant PDX-1 expression by colorectal cancer cells is unknown, a clue for this comes from its association with caudal homeobox 2 (Cdx-2). The genes for PDX-1 and Cdx-2 are closely linked on chromosome 5[24]. Cdx-2 is expressed in colon epithelium during embryonic development and has a central role in the differentiation of midgut endoderm[24]. It encodes for a transcription factor that is expressed in the proximal colon[25]. Cdx-2 expression is significantly reduced in colorectal adenocarcinoma proximal to the splenic flexure[26] and during the later stages of colorectal carcinogenesis[27]. *In vitro* studies show that PDX-1 physically interacts with and inhibits transcriptional activation by Cdx-2[28].

In this report, we have found expression of PDX-1 in a colorectal adenocarcinoma, its metastases, and macroscopically normal colonic mucosa from the same patient. This indicates a potential for use of this transcription factor as a molecular marker for colorectal cancer. Levels of PDX-1 in primary tumors and metastases from a large number of patients with and without colorectal cancer would need to be measured to confirm these observations. In addition, the levels of PDX-1 in stool samples from these patients need to be determined. This case report is an initial observation that PDX-1 expression could be indicative for colorectal carcinoma. A prospective study would be required to further evaluate its impact.

COMMENTS

Background

Colon cancer is a major cause of cancer-related morbidity and mortality and new diagnostic markers could improve the results of screening. Pancreatic duodenal homeobox-1 (PDX-1) is a transcription factor that regulates differentiation and proliferation. Increased PDX-1 levels have been found in colorectal adenocarcinoma compared to normal colon mucosa from a single patient.

Research frontiers

New molecular markers that could improve the accuracy of colorectal cancer screening are being sought. Development of molecular markers aims at developing non-invasive screening methods for colorectal cancer.

Innovations and breakthroughs

Detection of colorectal cancer-specific mutations in stool has been examined but is laborious and expensive. New molecular markers that will improve the efficiency and accuracy of non-invasive screening are needed.

Applications

Demonstrating overexpression of PDX-1 in the colon of patients with colorectal cancer is the first step in evaluating this molecule as a marker for colorectal cancer. If this observation is confirmed in a large sample of colorectal cancer patients, PDX-1 could prove valuable as a colorectal cancer marker.

Terminology

PDX-1 is a transcription factor essential for normal pancreatic organogenesis. Aberrant PDX-1 expression by a number of malignant tumors has been described.

Peer review

This study is very interesting. It suggests the possibility of PDX-1 as a biomarker for early diagnosis of colorectal cancer, so further study is needed to evaluate this potential.

REFERENCES

1 **Jemal A**, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. Cancer statistics, 2008. *CA Cancer J Clin* 2008; **58**: 71-96
2 **Labianca R**, Pessi MA, Zamparelli G. Treatment of colorectal cancer. Current guidelines and future prospects for drug therapy. *Drugs* 1997; **53**: 593-607
3 **Meyerhardt JA**, Mayer RJ. Systemic therapy for colorectal

cancer. *N Engl J Med* 2005; **352**: 476-487
4 National Center for Health Statistics, Division of Vital Statistics, Centers for disease control. Available from: URL: http://www.cdc.gov
5 **Mandel JS**, Bond JH, Church TR, Snover DC, Bradley GM, Schuman LM, Ederer F. Reducing mortality from colorectal cancer by screening for fecal occult blood. Minnesota Colon Cancer Control Study. *N Engl J Med* 1993; **328**: 1365-1371
6 **Kronborg O**, Fenger C, Olsen J, Jorgensen OD, Sondergaard O. Randomised study of screening for colorectal cancer with faecal-occult-blood test. *Lancet* 1996; **348**: 1467-1471
7 **Hardcastle JD**, Chamberlain JO, Robinson MH, Moss SM, Amar SS, Balfour TW, James PD, Mangham CM. Randomised controlled trial of faecal-occult-blood screening for colorectal cancer. *Lancet* 1996; **348**: 1472-1477
8 **Mandel JS**, Church TR, Bond JH, Ederer F, Geisser MS, Mongin SJ, Snover DC, Schuman LM. The effect of fecal occult-blood screening on the incidence of colorectal cancer. *N Engl J Med* 2000; **343**: 1603-1607
9 **Srivastava S**, Verma M, Henson DE. Biomarkers for early detection of colon cancer. *Clin Cancer Res* 2001; **7**: 1118-1126
10 **Mandel JS**. Screening for colorectal cancer. *Gastroenterol Clin North Am* 2008; **37**: 97-115, vii
11 **Ashizawa S**, Brunicardi FC, Wang XP. PDX-1 and the pancreas. *Pancreas* 2004; **28**: 109-120
12 **Koizumi M**, Doi R, Toyoda E, Masui T, Tulachan SS, Kawaguchi Y, Fujimoto K, Gittes GK, Imamura M. Increased PDX-1 expression is associated with outcome in patients with pancreatic cancer. *Surgery* 2003; **134**: 260-266
13 **Liu S**, Ballian N, Belaguli NS, Patel S, Li M, Templeton NS, Gingras MC, Gibbs R, Fisher W, Brunicardi FC. PDX-1 acts as a potential molecular target for treatment of human pancreatic cancer. *Pancreas* 2008; **37**: 210-220
14 **Wang XP**, Li ZJ, Magnusson J, Brunicardi FC. Tissue MicroArray analyses of pancreatic duodenal homeobox-1 in human cancers. *World J Surg* 2005; **29**: 334-338
15 **Tirone TA**, Wang XP, Templeton NS, Lee T, Nguyen L, Fisher W, Brunicardi FC. Cell-specific cytotoxicity of human pancreatic adenocarcinoma cells using rat insulin promoter thymidine kinase-directed gene therapy. *World J Surg* 2004; **28**: 826-833
16 **Dutta S**, Gannon M, Peers B, Wright C, Bonner-Weir S, Montminy M. PDX:PBX complexes are required for normal proliferation of pancreatic cells during development. *Proc Natl Acad Sci USA* 2001; **98**: 1065-1070
17 **Beattie GM**, Itkin-Ansari P, Cirulli V, Leibowitz G, Lopez AD, Bossie S, Mally MI, Levine F, Hayek A. Sustained proliferation of PDX-1+ cells derived from human islets. *Diabetes* 1999; **48**: 1013-1019
18 **Sharma A**, Zangen DH, Reitz P, Taneja M, Lissauer ME, Miller CP, Weir GC, Habener JF, Bonner-Weir S. The homeodomain protein IDX-1 increases after an early burst of proliferation during pancreatic regeneration. *Diabetes* 1999; **48**: 507-513
19 **Feanny MA**, Fagan SP, Ballian N, Liu SH, Li Z, Wang X, Fisher W, Brunicardi FC, Belaguli NS. PDX-1 expression is associated with islet proliferation in vitro and in vivo. *J Surg Res* 2008; **144**: 8-16
20 **Levin B**, Lieberman DA, McFarland B, Andrews KS, Brooks D, Bond J, Dash C, Giardiello FM, Glick S, Johnson D, Johnson CD, Levin TR, Pickhardt PJ, Rex DK, Smith RA, Thorson A, Winawer SJ. Screening and surveillance for the early detection of colorectal cancer and adenomatous polyps, 2008: a joint guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. *Gastroenterology* 2008; **134**: 1570-1595
21 **Imperiale TF**, Wagner DR, Lin CY, Larkin GN, Rogge JD, Ransohoff DF. Risk of advanced proximal neoplasms in asymptomatic adults according to the distal colorectal findings. *N Engl J Med* 2000; **343**: 169-174
22 **Peterson NB**, Murff HJ, Ness RM, Dittus RS. Colorectal cancer screening among men and women in the United States. *J Womens Health* (Larchmt) 2007; **16**: 57-65
23 **Mochizuka A**, Uehara T, Nakamura T, Kobayashi Y, Ota H. Hyperplastic polyps and sessile serrated 'adenomas' of the colon and rectum display gastric pyloric differentiation. *Histochem Cell Biol* 2007; **128**: 445-455
24 **Chawengsaksophak K**, Beck F. Chromosomal localization of cdx2, a murine homologue of the Drosophila gene caudal, to mouse chromosome 5. *Genomics* 1996; **34**: 270-271
25 **Beck F**. Homeobox genes in gut development. *Gut* 2002; **51**: 450-454
26 **James R**, Erler T, Kazenwadel J. Structure of the murine homeobox gene cdx-2. Expression in embryonic and adult intestinal epithelium. *J Biol Chem* 1994; **269**: 15229-15237
27 **Okon K**, Zazula M, Rudzki Z, Papla B, Osuch C, Stachura J. CDX-2 expression is reduced in colorectal carcinomas with solid growth pattern and proximal location, but is largely independent of MSI status. *Pol J Pathol* 2004; **55**: 9-14
28 **Ee HC**, Erler T, Bhathal PS, Young GP, James RJ. Cdx-2 homeodomain protein expression in human and rat colorectal adenoma and carcinoma. *Am J Pathol* 1995; **147**: 586-592

S- Editor Li DL **L- Editor** Mihm S **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5827

World J Gastroenterol 2008 October 14; 14(38): 5827-5833
World Journal of Gastroenterology ISSN 1007-9327

Antitumor bioactivity of adenovirus-mediated p27mt in colorectal cancer cell line SW480

Ze-Qun Sun, Chang-Sheng Deng, Shao-Yong Xu, Yong Du

Ze-Qun Sun, Chang-Sheng Deng, Gastroenterology Department of Zhongnan Hospital, Wuhan University, East Lake Road, Wuhan 430000, Hubei Province, China
Shao-Yong Xu, Yong Du, Gastroenterology Department of Renmin Hospital, Yunyang Medical College, Shiyan 442000, Hubei Province, China
Author contributions: Sun ZQ, Deng CS, Xu SY and Du Y designed the research; Sun ZQ and Du Y performed the research; Sun ZQ, Deng CS, Xu SY and Du Y analyzed data; and Sun ZQ and Deng CS wrote the paper.
Supported by Grant from the Natural Science Foundation of Hubei Province, NO.2003ABA193
Correspondence to: Chang-Sheng Deng, Gastroenterology Department of Zhongnan Hospital, Wuhan University, East Lake Road, Wuhan 430000, Hubei Province, China. wwwszq@163.com
Telephone: +86-27-87336067 Fax: +86-27-67813079
Received: April 9, 2008 Revised: August 12, 2008
Accepted: August 19, 2008
Published online: October 14, 2008

Abstract

AIM: To explore the antitumor bioactivity of adenovirus-mediated mutant type *p27^{kip1}* gene in a colorectal cancer cell line SW480.

METHODS: We constructed recombinant adenovirus vector expressing a mutant type *p27^{kip1}* gene (ad-p27mt), with mutation of Thr-187/Pro-188 (ACGCCC) to Met-187/Ile-188 (ATGATC), and transduced into SW480 cells. Then we detected expression of p27, Bcl-2 and Bax protein in the transductants by Western blotting, cell cycle of transductants by a digital flow cytometric system, migrating potential with Boyden Chamber and SW480 tumor cell growth inhibition *in vitro* and *in vivo*.

RESULTS: We found that a recombinant adenovirus vector of expressing ad-p27mt, with mutation of Thr-187/Pro-188 (ACGCCC) to Met-187/Ile-188 (ATGATC) has potent inhibition of SW480 tumor cell growth *in vitro* and *in vivo*. Furthermore, ad-p27mt induced cell apoptosis *via* regulating bax and bcl-2 expressions, and G_1/S arrest in SW480 cells and inhibited cell migration.

CONCLUSION: ad-p27mt has a strong anti-tumor bioactivity and has the potential to develop into new therapeutic agents for colorectal cancer.

Key words: *p27*; Adenovirus; Antitumor bioactivity; Colorectal cancer

Peer reviewer: Peter L Lakatos, MD, PhD, Assistant Professor, 1st Department of Medicine, Semmelweis University, Koranyi S 2A, Budapest H1083, Hungary

Sun ZQ, Deng CS, Xu SY, Du Y. Antitumor bioactivity of adenovirus-mediated p27mt in colorectal cancer cell line SW480. *World J Gastroenterol* 2008; 14(38): 5827-5833 Available from: URL: http://www.wjgnet.com/1007-9327/14/5827.asp DOI: http://dx.doi.org/10.3748/wjg.14.5827

INTRODUCTION

p27^{kip1}, a member of the Kip/Cip family of cyclin-dependent kinases inhibitors (CKIs), is a putative tumor suppressor gene[1], and promoter of apoptosis[2] that has been demonstrated in cancer cells as well as in normal cells[3]. Simultaneously, *p27^{kip1}* acts as a safeguard inflammatory injury[4] and plays a role in cell differentiation[5]. Furthermore, *p27^{kip1}* was identified as an inhibitor of cyclin E/CDK2 in cells arrested in the G_1 phase by lovastatin, transforming growth factor-beta (TGF-β), serum deprivation and contact inhibition[1,6].

Over the past years, p27^{kip1} protein has attracted our attention as an important prognostic factor in various malignancies. In short, lately, it has been reported that expression of p27^{kip1} protein is associated with poor prognosis in several types of malignancies, including breast, lung, gastric carcinoma[7-10] and colorectal adenocarcinoma[11-13].

The reduced expression of *p27^{kip1}* in cancer cells due to an increase in the rate of its degradation[14]. It is thought that the amount of p27^{kip1} protein is regulated by a posttranscriptional mechanism rather than *p27^{kip1}* gene aberrations because *p27^{kip1}* gene mutation seems to be uncommon in human malignancies[15]. It has been demonstrated that *p27^{kip1}* is poly-ubiquitinated both *in vivo* and *in vitro*, and *p27^{kip1}* ubiquitination requires its phosphorylation on threonine residue 187 (T187) both *in vivo* and *in vitro*[16,17].

Gene therapy is a promising approach to restore *p27* expression using adenoviral vectors[11,18-20]. These agents have induced cell-cycle arrest and loss of cyclinE-

CDK2 activity in cell lines and xenograft models and have triggered apoptosis in cancer cells[2,19,21-24]. The concentration of *p27* is thought to be regulated predominantly by the ubiquitin-dependent proteolytic pathway[25]. Degradation of *p27* triggered by its phosphorylation on Thr187 is required for the binding of *p27* to Skp2, the F-box protein component of an SCF ubiquitin ligase (E3) complex, and such interaction in turn results in the poly-ubiquitylation and degradation of p27[16,25]. Reduction of *p27* levels in various types of malignant tumors results from accelerated proteolytic degradation by this pathway[26].

However, so far no report has been published on the effects of adenovirus-mediated mutant type $p27^{kip1}$ gene on colorectal cancer cell. Thus, we constructed recombinant adenovirus vector expressing a mutant type $p27^{kip1}$ gene (ad-p27mt), with mutation of Thr-187/Pro-188 (ACGCCC) to Met-187/Ile-188 (ATGATC), which will inhibit degradation of p27 protein[11,16,24,25,27], and explored its antitumor bioactivity in a colorectal cancer cell line SW480.

MATERIALS AND METHODS

Cell and cell culture

Human colorectal cancer cell line SW480 was purchased from Shanghai Cell Lines Bank (Shanghai, China). Cells were maintained in RPMI 1640 (Beyotime, China) supplemented with 10% fetal bovine serum (FBS; Beyotime, China).

Preparation of ad-p27mt

Ad-p27mt was constructed in the Institute of Clinical Medicine of Yunyang Medical College (Hubei, China). Briefly, the cDNA of human *p27mt* gene was digested from plasmid of pORF9-hp27mt (Invitrogen) and subcloned into the plasmid of pBluescript Ⅱ SK (+) (Stratagene) and formed plasmid of pBluescript-hp27mt. Then human *p27mt* gene was digested from pBluescript-hp27mt and subcloned into shuttle vector pBluescript-CMV (Stratagene) and gained shuttle plasmid of pShuttle-CMV-hp27mt. Adenovirus genomic DNA plasmid of pAdeasy-1 (Stratagene) was transformed into BJ5183 bacterium (Stratagene) and prepared competent BJ5183 bacterium containing pAdeasy-1. PShuttle-CMV-hp27mt was linearized with Pmel (New England Bio labs) and transformed into competent BJ5183 bacterium containing pAdeasy-1 and positive clone of homologous recombination was selected. Identified recombinant adenovirus plasmid of pAd-p27mt was digested with Pacl and transfected into HEK293 cells (Stratagene) with liposome polyFect (Qiagen) to package adenovirus particles. HEK293 cells were maintained in DMEM with 10% FBS until the onset of the cytopathic effect. PCR technique was used to detect target gene and the titer of the recombinant adenovirus was determined by measuring the absorbance at 260 nm and 280 nm. Ad-p27mt was propagated in HEK293 cells, purified by two cesium chloride density centrifugations, titered and stored at -70℃. Recombinant adenovirus expressing β-galactosidase (ad-β-gal) without any therapeutic gene was used as the control virus in all experiments.

Transduction of SW480 cells with ad-p27mt

Exponential growing of SW480 cells were transduced with ad-p27mt or ad-β-gal at 20 multiplicity of infection for 1 h with gentle frequent shaking and then incubated with complete media for the experiment.

P27mt sequencing assay of transductant

Briefly, transductants were schizolysed and DNA was extracted using phenol/chloroform, and then dehydrated alcohol precipitated DNA. Sequence of DNA was assayed by Shanghai Sangon Company.

Western blotting

Cells lysates were prepared from transductants of ad-p27mt (SW480-ad-p27mt), transductants of ad-β-gal (SW480-ad-β-gal) and parental cells (SW480) as follows: cells were cultured for 96 h, washed three times with 100 mmol/L phosphate-buffered-saline (PBS, Beyotime, China), lysed with RIPA buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP-40, 0.1% SDS; Beyotime, China]. The protein concentration of the samples was determined by Bio-Rad protein assay (Bio-Rad, Amersham). Fifty micrograms of protein samples were electrophoresed on SDS-polyacrylamide gel. Proteins from gels were transferred to PVDF membrane (Sigma), and membrane was incubated with a 1:1000 dilution of the monoclonal anti-p27 antibody (Beyotime, China), anti-Bax antibody (Beyotime, China), and anti-Bcl-2 antibody (Beyotime, China), respectively. The blots were developed using the enhanced chemoluminescence (ECL) Western blotting system and protocol (Amersham). In all immunoblotting experiments, blots were reprobed with an anti-β-actin antibody (Sigma) for internal control.

MTT assay

Cells were seeded on 96-well plates (Beyotime, China) at 4×10^3 cells per well in RPMI 1640 supplemented with 10% FBS. After 2 d, 4 d and 6 d, the number of cells was quantitated by an assay in which MTT; 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) was used.

Cell cycle analysis

Cells were seeded in 75 cm^2 culture flask (Beyotime, China) in RPMI 1640 supplemented with 10% FBS. After 96 h, the stuck and floating cells were collected in conical tubes (Beyotime, China). Then, the cells were fixed with 70% cold ethanol and washed with PBS. After treatment with 0.1 mg/mL RNaseA (Sigma), the cells were stained with 40 μg/mL propidium iodine (Sigma), and the cell cycle was analyzed by a digital flow cytometric system (Beckman Coulter EPICS-XL).

Migration assay with Boyden chamber

Migrative potential was evaluated in the Boyden chamber apparatus (Kylin-bell, China). This assay was developed

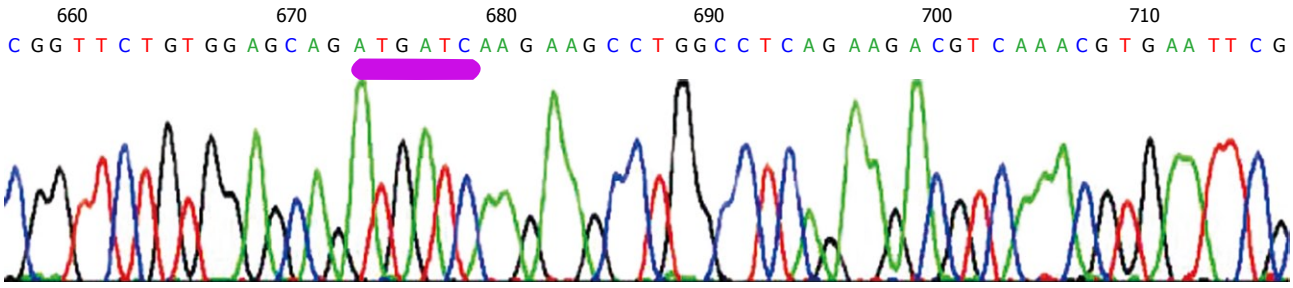


Figure 1 p27mt sequencing assay of transductants. Sequencing result was consistent with p27mt sequence in gene bank. Sequencing result was shown and the underline was mutant site Met-187/Ile-188 (ATGATC).

to facilitate analysis of aspects of cancer invasion and metastasis. Briefly, subconfluence cells were starved for 24 h and harvested with 0.05% trypsin (Sigma) containing 0.02% EDTA (Sigma), washed twice with PBS, and resuspended to a final concentration of 5×10^5/mL in serum-free medium with 0.1% fraction V bovine serum albumin. PVP filters (Kylin-bell, China) of 8 μm pore size were precoated with gelatin, rinsed in sterile water, and were used for assay. Bottom wells of the chamber were filled with 25 μL of RPMI 1640 supplemented with 10% FBS per well and covered with a gelatin coated membrane, and then 50 μL of cells suspension was added to the top wells. After 24 h of incubation, the membranes were stained with Giemsa solution. Cells on the upper surface of the filter were carefully removed with a cotton swab, and the cells that had migrated through the membrane to the lower surface were counted in six different fields under a light microscope at 400 magnification. Each experiment was performed in triplicate wells and repeated 3 times.

In vivo growth inhibition assay

BALB/c nude mice were purchased from Animal experiment center of Hubei (Hubei, China; Qualification number: SCXK: Hubei 2003-0005). All animal experimental procedures were conducted and approved by the Institutional Animal Care and Use Committee of Yunyang Medical College (Approval number: SYXK: Hubei 2004-0021). Briefly, SW480 cells were cultured in RPMI 1640 supplemented with 10% FBS. Cells were harvested through two consecutive trypsinizations, centrifuged at 300 × *g* for 5 min, washed twice, and resuspended in sterile PBS. Cells (1×10^7) in 0.2 mL were injected subcutaneously into a 6-week-old nude mouse between the scapulae of each nude mouse. After 14 days, tumors reached a mean size of 200 mm^3 in the mice's bodies. To test *in vivo* growth suppressive potential of ad-p27mt on nude mouse xenografts of SW480, intratumoral injections of ad-p27mt, ad-β-gal or PBS were made every other day for total 3 times, respectively. There were ten mice in each group. Twenty-eight days after inoculation, mice were sacrificed by cervical dislocation, and tumor specimens were taken, photographed and weighed.

Statistical analysis

Data are mean of at least 3 independent experiments ± SD. Results were compared by one-way analysis of variance (ANOVA). A two-tailed $P < 0.05$ was regarded as statistically significant. All calculations were performed using the SPSS for Windows version 13.0 statistical program on a personal computer.

RESULTS

p27mt sequencing assay of transductant

As shown in Figure 1, sequencing result was consistent with *p27mt* sequence in gene bank.

Expression of p27, Bax and Bcl-2 protein in the transductants

As shown in Figure 2, expression of p27 and Bax protein was increased significantly and Bcl-2 protein level was decreased in the SW480-ad-p27mt when compared with that in SW480-ad-β-gal and SW480. The expression of β-actin as an internal control was approximately the same in all of the cells.

Growth of cells *in vitro*

Relative cell number was evaluated by comparing the absorbance in each cell at day 2, day 4 and day 6. As shown in Figure 3, the growth of SW480-ad-p27mt was markedly inhibited compared with SW480-ad-β-gal and SW480.

Cells cycle analysis

To further determine whether up-regulation of p27 protein can induce apoptosis and or cell cycle arrest, flow cytometric analysis was performed on each transductant. It is suggested that cells containing a sub-G_1 content of DNA reflect the extent to which apoptosis is occurring. Flow cytometric analysis demonstrated that *p27mt* gene transduced into SW480 induced G_1/S arrest and apoptosis. A marked sub-G_1 peak and decreased percentage of cells in S phase were detected in SW480-ad-p27mt. Decreased percentage of cells in S phase suggested G_1/S arrest (Figure 4 and Table 1).

In vitro migration assay

SW480-ad-p27mt significantly showed the poor ability of migration when compared with that of SW480-ad-β-gal and SW480 (Figure 5).

In vivo growth inhibition assay

Twenty-eight days after inoculation, ten mice in each

Table 1 Cell cycle analysis of transductant by flow cytometry

	Sub-G_1 (n = 3)	G_0/G_1 (n = 3)	S (n = 3)	G_2/M (%) (n = 3)
SW480	3.88 ± 1.85	73.17± 5.10	11.42 ± 2.93	11.21 ± 4.70
SW480-ad-β-gal	4.30 ± 1.02	68.27 ± 3.15	11.1 ± 0.96	15.43 ± 1.98
SW480-ad-p27mt	39.13 ± 1.84[1]	49.40 ± 2.70	7.31 ± 0.70	3.92 ± 0.66

The percentage of cells of transductants in each phase. The values are mean of 3 independent experiments ± SD. [1]P = 0.001, *vs* control cells by one-way ANOVA.

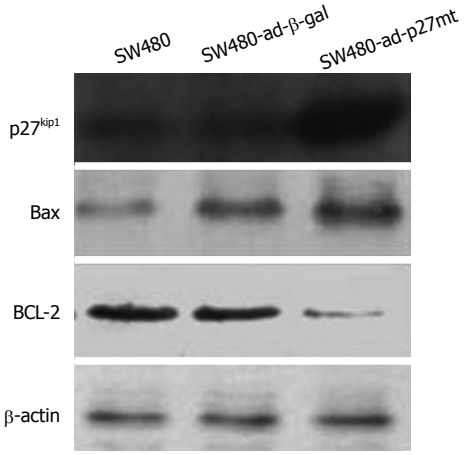


Figure 2 Expression of p27, Bcl-2 and Bax protein in each transductant by Western blot analysis.

group all survived. Intratumoral injection of ad-p27mt into established tumors induced marked growth suppression. As shown in Figure 6, the weight of tumors receiving ad-p27mt(SW480-ad-p27mt) was significantly lighter than that of ad-β-gal treated(SW480-ad-β-gal) or PBS treated(SW480).

DISCUSSION

In this study, we found that transduction of ad-p27mt into SW480 cells resulted in induction of overexpression of p27 protein, which suggests that the approach to restore *p27* expressing using adenoviral vectors is available and p27mt protein may be resistant to degradation by ubiquitin and more stable. Other studies also have shown that the p27 (T187A) mutant is not ubiquitinated[11,16,24,25,27], and the control of p27 protein levels is affected by ubiquitin-dependent degradation[17,25,26,28,29], in a ubiquitin-independent and Skp2-independent manner at G_1[30-32], and by Jab1-dependent degradation[33].

We also observed that ad-p27mt induced growth suppression, apoptosis, and G_1/S arrest in SW480 cells *in vitro* as well as the transplanted tumor growth inhibition *in vivo*, as was expected from the nature of *p27* as a CDKIs. These findings are consistent with those of another study in which ad-p27 (T187A) had a greater effect on cell cycle arrest and apoptosis induction because of its resistance to degradation, and suppressed the growth of established lung cancer xenografts[24]. In several studies, intratumoral injections of ad-p27 have

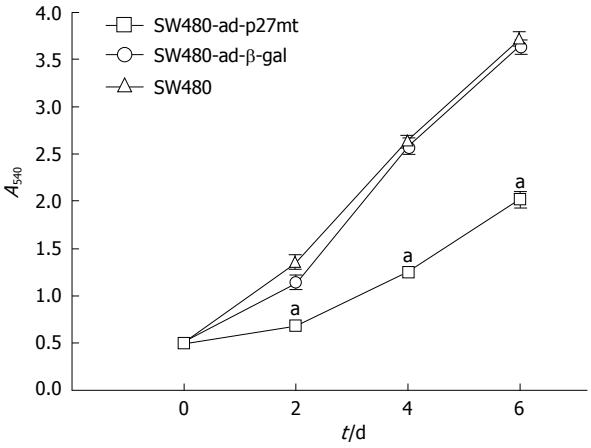


Figure 3 Growth of transductants *in vitro*. Relative cell number was evaluated by comparing the absorbance in each cell at day 2, 4 and 6. The values shown are the mean of eight determinations. [a]P < 0.05, *vs* control cells.

been shown to partially suppress tumor formation in animal models[18,23,24,34]. In other experiments, ad-p27 was the most potent of several cyclin kinase inhibitors in terms of inducing cell-cycle arrest, apoptosis and inhibiting tumorigenesis[2,18,21-23,27,34]. The effect of *p27* on the cell cycle is regulated mainly by its stability[35,36], but recent studies have shown that the function of *p27* is also associated with its subcellular localization[37-39]. Besides Thr187, there are three phosphorylation sites Ser10, Thr157 and Thr198 that are involved in cellular localization[40,41]. Phosphorylation at Ser10 stabilizes p27 protein in G_1[41]. Phosphorylation at Thr157 by protein kinase B/Akt impairs the nuclear import of *p27* but does not affect its stability in breast cancer and other cells[37,38]. Chu *et al*[42] indicating that the oncogenic kinase Src regulates *p27* stability through phosphorylation of *p27* at tyrosine 74 and tyrosine 88. Src inhibitors increase cellular p27 stability, and Src overexpression accelerates *p27* proteolysis.

The relationship between *p27* and induction of apoptosis is still unclear. Katayose *et al*[2] and Naruse *et al*[19] have suggested that the growth-inhibitory effect and apoptosis induction by overexpression of *p27* requires expression of pRb. The pRb-regulated checkpoint in G_1 phase is an important apoptotic checkpoint. CyclinE-CDK2 is the primary complex that phosphorylates pRb, which prevents interactions of it with the E2F transcription factor. To further understand the mechanism of ad-p27mt induced apoptosis in SW480 cells, we examined the expression of apoptosis-related genes Bax and Bcl-2 in each transductant. Results showed that ad-p27mt resulted in a marked increase in protein expression of Bax, a pro-apoptotic factor, and a marked decrease in protein expression of Bcl-2, an anti-apoptotic factor that binds to Bax and antagonizes its function. These results suggest that ad-p27mt-induced apoptosis in SW480 cells involves in induction of Bax.

Cell migration is an essential process involved in tumor invasion and metastasis. In this study, we tested the ability of cell migration in each transductant. We found that ad-p27mt resulted in strong migration

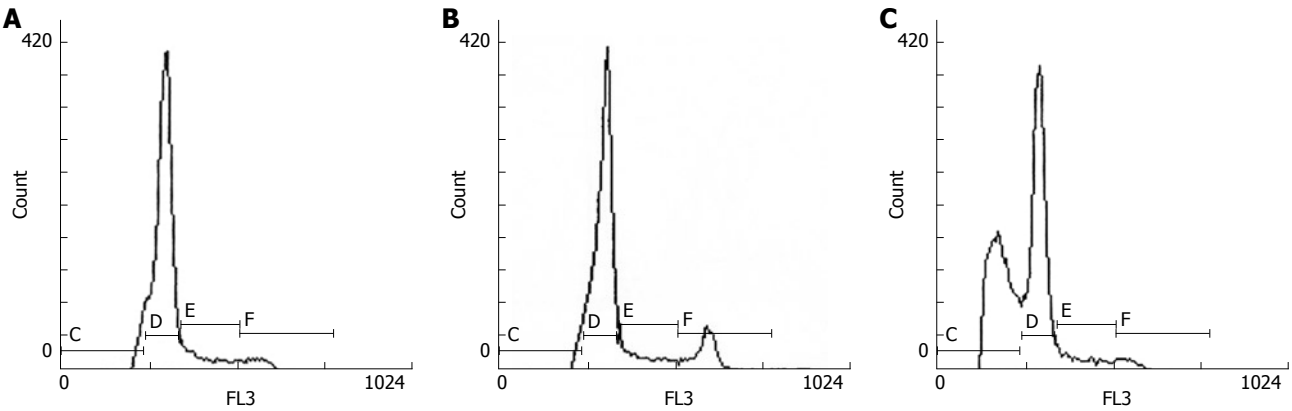


Figure 4 Cell cycle analysis of transductants by flow cytometry. **A**: SW480; **B**: SW480-ad-β-gal; **C**: SW480-ad-p27mt. C-F: Sub-G_1, G_0/G_1, S and G_2/M phase, respectively. The percentage of cells in sub-G_1, G_0/G_1, S and G_2/M phase is shown in Table 1.

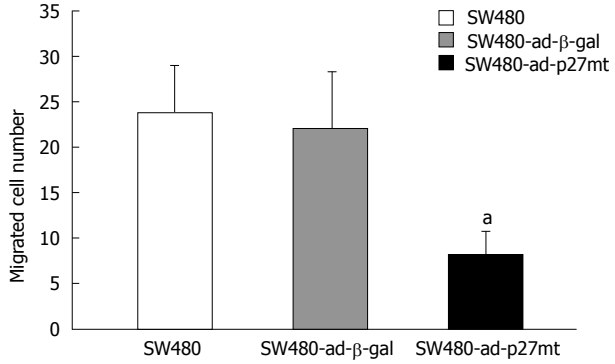


Figure 5 Cell migration assay by Boyden chamber. After 24 h of cell incubation, the migrated cells in each transductant were counted in six different fields under a light microscope. $^aP = 0.004$, *vs* control cells.

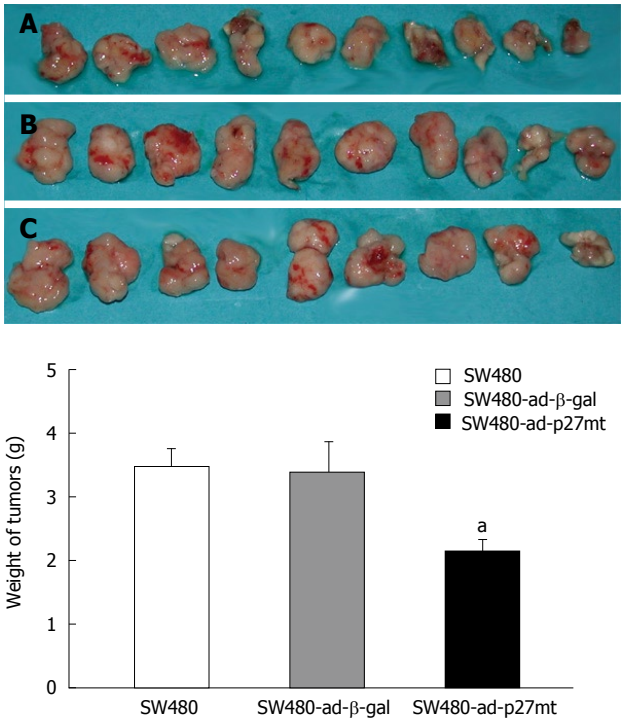


Figure 6 Upper: **A**, **B** and **C** indicated respectively the groups which received respectively intratumoral injections of ad-p27mt, ad-β-gal or PBS. Lower: *In vivo* tumor growth inhibition assay. The weight of tumors was measured. The values are mean of the weight of tumors of each group $^aP = 0.002$, *vs* control groups.

inhibition. Supriatno *et al*[11] have reported a similar result in oral cancer cell line. However, its mechanisms remain unclear and need further investigations. We speculate that it may be directly associated with decreased cell proliferation or/and alterations of structural proteins.

In conclusion, ad-p27mt shows a strong anti-tumor bioactivity in a colorectal cancer cell line SW480 and has the potential to develop into new therapeutic agents for colorectal cancer.

ACKNOWLEDGMENTS

We thank Dr. Jia-Ning Wang for his excellent technical assistance.

COMMENTS

Background

The incidence of colorectal cancer is increasing all over the world. However it is short of effective therapeutic approach. Gene therapy to restore *p27* expressing has been promising, furthermore, a mutant type *p27* gene, with mutant of Thr-187/Pro-188 to Met-187/Ile-188, can inhibit degradation of p27 protein by ubiquitin-mediated pathway.

Research frontiers

p27, as a cyclin-dependent kinases inhibitor, tumor suppressor gene, and promoter of apoptosis, has been widely investigated. Furthermore, antitumor activity of *p27* has been demonstrated in breast, lung, and oral cancer. But the antitumor bioactivity of *p27* mt has not been studied on colorectal cancer.

Innovations and breakthroughs

The study indicates that ad-p27mt has a strong anti-tumor bioactivity in a colorectal cancer cell line SW480.

Applications

This will develop into new therapeutic agents for colorectal cancer.

Peer review

This is an interesting manuscript on the antitumor activity of the adenovirus mediated mutant $p27^{kip}$ gene in a colorectal cancer (CRC) cell line. Major finding of the study was that the tumor cell growth was inhibited both *in vitro* and *in vivo* by the gene transfer.

REFERENCES

1 **St Croix B**, Florenes VA, Rak JW, Flanagan M, Bhattacharya N, Slingerland JM, Kerbel RS. Impact of the cyclin-dependent kinase inhibitor p27Kip1 on resistance of tumor cells to anticancer agents. *Nat Med* 1996; **2**: 1204-1210

2 **Katayose Y**, Kim M, Rakkar AN, Li Z, Cowan KH, Seth P. Promoting apoptosis: a novel activity associated with the cyclin-dependent kinase inhibitor p27. *Cancer Res* 1997; **57**: 5441-5445

3 **Polyak K**, Lee MH, Erdjument-Bromage H, Koff A, Roberts JM, Tempst P, Massague J. Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* 1994; **78**: 59-66

4 **Ophascharoensuk V**, Fero ML, Hughes J, Roberts JM, Shankland SJ. The cyclin-dependent kinase inhibitor p27Kip1 safeguards against inflammatory injury. *Nat Med* 1998; **4**: 575-580

5 **Durand B**, Gao FB, Raff M. Accumulation of the cyclin-dependent kinase inhibitor p27/Kip1 and the timing of oligodendrocyte differentiation. *EMBO J* 1997; **16**: 306-317

6 **Polyak K**, Kato JY, Solomon MJ, Sherr CJ, Massague J, Roberts JM, Koff A. p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes Dev* 1994; **8**: 9-22

7 **Catzavelos C**, Bhattacharya N, Ung YC, Wilson JA, Roncari L, Sandhu C, Shaw P, Yeger H, Morava-Protzner I, Kapusta L, Franssen E, Pritchard KI, Slingerland JM. Decreased levels of the cell-cycle inhibitor p27Kip1 protein: prognostic implications in primary breast cancer. *Nat Med* 1997; **3**: 227-230

8 **Esposito V**, Baldi A, De Luca A, Groger AM, Loda M, Giordano GG, Caputi M, Baldi F, Pagano M, Giordano A. Prognostic role of the cyclin-dependent kinase inhibitor p27 in non-small cell lung cancer. *Cancer Res* 1997; **57**: 3381-3385

9 **Mori M**, Mimori K, Shiraishi T, Tanaka S, Ueo H, Sugimachi K, Akiyoshi T. p27 expression and gastric carcinoma. *Nat Med* 1997; **3**: 593

10 **Mineta H**, Miura K, Suzuki I, Takebayashi S, Amano H, Araki K, Harada H, Ichimura K, Wennerberg JP, Dictor MR. Low p27 expression correlates with poor prognosis for patients with oral tongue squamous cell carcinoma. *Cancer* 1999; **85**: 1011-1017

11 **Supriatno**, Harada K, Kawaguchi S, Onoue T, Yoshida H, Sato M. Characteristics of antitumor activity of mutant type p27Kip1 gene in an oral cancer cell line. *Oral Oncol* 2004; **40**: 679-687

12 **Moore HG**, Shia J, Klimstra DS, Ruo L, Mazumdar M, Schwartz GK, Minsky BD, Saltz L, Guillem JG. Expression of p27 in residual rectal cancer after preoperative chemoradiation predicts long-term outcome. *Ann Surg Oncol* 2004; **11**: 955-961

13 **Lin LC**, Lee HH, Hwang WS, Li CF, Huang CT, Que J, Lin KL, Lin FC, Lu CL. p53 and p27 as predictors of clinical outcome for rectal-cancer patients receiving neoadjuvant therapy. *Surg Oncol* 2006; **15**: 211-216

14 **Loda M**, Cukor B, Tam SW, Lavin P, Fiorentino M, Draetta GF, Jessup JM, Pagano M. Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. *Nat Med* 1997; **3**: 231-234

15 **Kawamata N**, Morosetti R, Miller CW, Park D, Spirin KS, Nakamaki T, Takeuchi S, Hatta Y, Simpson J, Wilcyznski S. Molecular analysis of the cyclin-dependent kinase inhibitor gene p27/Kip1 in human malignancies. *Cancer Res* 1995; **55**: 2266-2269

16 **Montagnoli A**, Fiore F, Eytan E, Carrano AC, Draetta GF, Hershko A, Pagano M. Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. *Genes Dev* 1999; **13**: 1181-1189

17 **Vlach J**, Hennecke S, Amati B. Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27. *EMBO J* 1997; **16**: 5334-5344

18 **Katner AL**, Hoang QB, Gootam P, Jaruga E, Ma Q, Gnarra J, Rayford W. Induction of cell cycle arrest and apoptosis in human prostate carcinoma cells by a recombinant adenovirus expressing p27(Kip1). *Prostate* 2002; **53**: 77-87

19 **Naruse I**, Hoshino H, Dobashi K, Minato K, Saito R, Mori M. Over-expression of p27kip1 induces growth arrest and apoptosis mediated by changes of pRb expression in lung cancer cell lines. *Int J Cancer* 2000; **88**: 377-383

20 **Ishii T**, Fujishiro M, Masuda M, Goshima Y, Kitamura H, Teramoto S, Matsuse T. Effects of p27Kip1 on cell cycle status and viability in A549 lung adenocarcinoma cells. *Eur Respir J* 2004; **23**: 665-670

21 **Wang X**, Gorospe M, Huang Y, Holbrook NJ. p27Kip1 overexpression causes apoptotic death of mammalian cells. *Oncogene* 1997; **15**: 2991-2997

22 **Craig C**, Wersto R, Kim M, Ohri E, Li Z, Katayose D, Lee SJ, Trepel J, Cowan K, Seth P. A recombinant adenovirus expressing p27Kip1 induces cell cycle arrest and loss of cyclin-Cdk activity in human breast cancer cells. *Oncogene* 1997; **14**: 2283-2289

23 **Schreiber M**, Muller WJ, Singh G, Graham FL. Comparison of the effectiveness of adenovirus vectors expressing cyclin kinase inhibitors p16INK4A, p18INK4C, p19INK4D, p21(WAF1/CIP1) and p27KIP1 in inducing cell cycle arrest, apoptosis and inhibition of tumorigenicity. *Oncogene* 1999; **18**: 1663-1676

24 **Park KH**, Seol JY, Kim TY, Yoo CG, Kim YW, Han SK, Shim YS, Lee CT. An adenovirus expressing mutant p27 showed more potent antitumor effects than adenovirus-p27 wild type. *Cancer Res* 2001; **61**: 6163-6169

25 **Carrano AC**, Eytan E, Hershko A, Pagano M. SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. *Nat Cell Biol* 1999; **1**: 193-199

26 **Pagano M**, Tam SW, Theodoras AM, Beer-Romero P, Del Sal G, Chau V, Yew PR, Draetta GF, Rolfe M. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science* 1995; **269**: 682-685

27 **Zhang Q**, Tian L, Mansouri A, Korapati AL, Johnson TJ, Claret FX. Inducible expression of a degradation-resistant form of p27Kip1 causes growth arrest and apoptosis in breast cancer cells. *FEBS Lett* 2005; **579**: 3932-3940

28 **Sheaff RJ**, Groudine M, Gordon M, Roberts JM, Clurman BE. Cyclin E-CDK2 is a regulator of p27Kip1. *Genes Dev* 1997; **11**: 1464-1478

29 **Sutterluty H**, Chatelain E, Marti A, Wirbelauer C, Senften M, Muller U, Krek W. p45SKP2 promotes p27Kip1 degradation and induces S phase in quiescent cells. *Nat Cell Biol* 1999; **1**: 207-214

30 **Hara T**, Kamura T, Nakayama K, Oshikawa K, Hatakeyama S, Nakayama K. Degradation of p27(Kip1) at the G(0)-G(1) transition mediated by a Skp2-independent ubiquitination pathway. *J Biol Chem* 2001; **276**: 48937-48943

31 **Kamura T**, Hara T, Matsumoto M, Ishida N, Okumura F, Hatakeyama S, Yoshida M, Nakayama K, Nakayama KI. Cytoplasmic ubiquitin ligase KPC regulates proteolysis of p27(Kip1) at G1 phase. *Nat Cell Biol* 2004; **6**: 1229-1235

32 **Shapira M**, Ben-Izhak O, Linn S, Futerman B, Minkov I, Hershko DD. The prognostic impact of the ubiquitin ligase subunits Skp2 and Cks1 in colorectal carcinoma. *Cancer* 2005; **103**: 1336-1346

33 **Tomoda K**, Kubota Y, Kato J. Degradation of the cyclin-dependent-kinase inhibitor p27Kip1 is instigated by Jab1. *Nature* 1999; **398**: 160-165

34 **Rakkar AN**, Li Z, Katayose Y, Kim M, Cowan KH, Seth P. Adenoviral expression of the cyclin-dependent kinase inhibitor p27Kip1: a strategy for breast cancer gene therapy. *J Natl Cancer Inst* 1998; **90**: 1836-1838

35 **Boehm M**, Yoshimoto T, Crook MF, Nallamshetty S, True A, Nabel GJ, Nabel EG. A growth factor-dependent nuclear kinase phosphorylates p27(Kip1) and regulates cell cycle progression. *EMBO J* 2002; **21**: 3390-3401

36 **Ishida N**, Hara T, Kamura T, Yoshida M, Nakayama K, Nakayama KI. Phosphorylation of p27Kip1 on serine 10 is required for its binding to CRM1 and nuclear export. *J Biol Chem* 2002; **277**: 14355-14358
37 **Liang J**, Zubovitz J, Petrocelli T, Kotchetkov R, Connor MK, Han K, Lee JH, Ciarallo S, Catzavelos C, Beniston R, Franssen E, Slingerland JM. PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. *Nat Med* 2002; **8**: 1153-1160
38 **Shin I**, Yakes FM, Rojo F, Shin NY, Bakin AV, Baselga J, Arteaga CL. PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. *Nat Med* 2002; **8**: 1145-1152
39 **Ogino S**, Kawasaki T, Ogawa A, Kirkner GJ, Loda M, Fuchs CS. Cytoplasmic localization of p27 (cyclin-dependent kinase inhibitor 1B/KIP1) in colorectal cancer: inverse correlations with nuclear p27 loss, microsatellite instability, and CpG island methylator phenotype. *Hum Pathol* 2007; **38**: 585-592
40 **Fujita N**, Sato S, Tsuruo T. Phosphorylation of p27Kip1 at threonine 198 by p90 ribosomal protein S6 kinases promotes its binding to 14-3-3 and cytoplasmic localization. *J Biol Chem* 2003; **278**: 49254-49260
41 **Rodier G**, Montagnoli A, Di Marcotullio L, Coulombe P, Draetta GF, Pagano M, Meloche S. p27 cytoplasmic localization is regulated by phosphorylation on Ser10 and is not a prerequisite for its proteolysis. *EMBO J* 2001; **20**: 6672-6682
42 **Chu I**, Sun J, Arnaout A, Kahn H, Hanna W, Narod S, Sun P, Tan CK, Hengst L, Slingerland J. p27 phosphorylation by Src regulates inhibition of cyclin E-Cdk2. *Cell* 2007; **128**: 281-294

S- Editor Zhong XY **L- Editor** Ma JY **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5834

World J Gastroenterol 2008 October 14; 14(38): 5834-5841
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Evidence for the involvement of NOD2 in regulating colonic epithelial cell growth and survival

Sheena M Cruickshank, Louise Wakenshaw, John Cardone, Peter D Howdle, Peter J Murray, Simon R Carding

Sheena M Cruickshank, Molecular and Cellular Biology, University of Leeds, Leeds LS2 9JT, United Kingdom; Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, United Kingdom
Louise Wakenshaw, Simon R Carding, Institute of Molecular and Cellular Biology, University of Leeds, Leeds LS2 9JT, United Kingdom; The Institute of Food Research, Norwich Research Park, Norwich NR4 7UA, United Kingdom
John Cardone, Institute of Molecular and Cellular Biology, University of Leeds, Leeds LS2 9JT, United Kingdom
Peter D Howdle, Section of Medicine, Surgery & Anaesthesia, Leeds Institute of Molecular Medicine, University of Leeds, Leeds LS2 9JT, United Kingdom
Peter J Murray, Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis TN 38105, United States
Author contributions: Cruickshank SM and Wakenshaw L contributed equally to this work; Cruickshank SM, Wakenshaw L and Cardone J performed research; Howdle PD and Carding SR contributed funding; Murray PJ contributed new reagents; Carding SR designed research; Cruickshank SM and Carding SR analyzed data and wrote the paper.
Supported by (in part) Grants from Action Medical Research (SRC, SMC); The Leeds Teaching Hospitals Charitable Foundation (SRC and PH), NIH (PJM); The European Union "Leonardo da Vinci" Scholarship Program (JC); BBSRC sponsored postgraduate studentship (LW); The American Lebanese Syrian Associated Charities (PJM)
Correspondence to: Simon R Carding, Professor, The Institute of Food Research, Norwich Research Park, Norwich NR4 7UA, United Kingdom. simon.carding@bbsrc.ac.uk
Telephone: +44-1603-251410 Fax: +44-1603-255288
Received: December 21, 2007 Revised: July 14, 2008
Accepted: July 21, 2008
Published online: October 14, 2008

Abstract

AIM: To investigate the function of NOD2 in colonic epithelial cells (CEC).

METHODS: A combination of *in vivo* and *in vitro* analyses of epithelial cell turnover in the presence and absence of a functional NOD2 protein and, in response to enteric *Salmonella typhimurium* infection, were used. shRNA interference was also used to investigate the consequences of knocking down *NOD2* gene expression on the growth and survival of colorectal carcinoma cell lines.

RESULTS: In the colonic mucosa the highest levels of NOD2 expression were in proliferating crypt epithelial cells. Muramyl dipeptide (MDP), that is recognized by NOD2, promoted CEC growth *in vitro*. By contrast, the growth of NOD2-deficient CECs was impaired. *In vivo* CEC proliferation was also reduced and apoptosis increased in *Nod2*$^{-/-}$ mice, which were also evident following enteric *Salmonella* infection. Furthermore, neutralization of NOD2 mRNA expression in human colonic carcinoma cells by shRNA interference resulted in decreased survival due to increased levels of apoptosis.

CONCLUSION: These findings are consistent with the involvement of NOD2 protein in promoting CEC growth and survival. Defects in proliferation by CECs in cases of CD may contribute to the underlying pathology of disrupted intestinal homeostasis and excessive inflammation.

Key words: Colon; Epithelial cells; NOD2; Growth

Peer reviewers: Jian-Ying Wang, Professor, University of Maryland School of Medicine, Baltimore VA Medical Center (112), 10N. Greene St, Baltimore, MD 21201, United States; Marc Basson, MD, PhD, MBA, Chief of Surgery, John D. Dingell VA Medical Center, 4646 John R. Street, Detroit, MI 48301, United States

Cruickshank SM, Wakenshaw L, Cardone J, Howdle PD, Murray PJ, Carding SR. Evidence for the involvement of NOD2 in regulating colonic epithelial cell growth and survival. *World J Gastroenterol* 2008; 14(38): 5834-5841 Available from: URL: http://www.wjgnet.com/1007-9327/14/5834.asp DOI: http://dx.doi.org/10.3748/wjg.14.5834

INTRODUCTION

The intestinal epithelium both acts as a physical barrier and senses and responds to commensal bacteria *via* expression of pattern recognition receptors (PRRs) that recognize microbe associated molecular patterns (MAMPs)[1]. There are two distinct groups of PRRs; the Toll-like receptor family (TLRs) and the NOD-like (nucleotide-binding oligomerisation domain) receptors. The leucine rich repeat sequences of the NOD2 protein are implicated in recognition of fragments of bacterial peptidoglycan (PGN) including muramyl dipeptide (MDP)[2,3].

NOD2 is expressed in the cytosol of professional antigen presenting cells and epithelial cells exposed to

microorganisms containing PGN[3-6]. In cell-based models of NOD2 overexpression, MDP stimulation results in NF-κB activation[4,7]. This together with the ability of pro-inflammatory cytokines to influence NOD2 expression[8] suggests NOD2 contributes to the innate immune response to microbial pathogens. As intestinal epithelial cells are generally refractory to TLR signals in the absence of inflammation, NOD2 may have additional functions[9]. In the small intestine NOD2 appears to contribute to Peyer's patch development[10] and paneth cell production of anti-microbial proteins[11], linking NOD2 and host defense at the epithelial interface.

By contrast, little is known about NOD2 function in the colon. It has been proposed that TLRs control epithelial homeostasis[12]. In considering the cross talk between NOD2 and TLR signaling pathways[13], NOD2 expression in IBD[14] and the central role CARD domain-containing proteins play in regulating apoptosis[15], we determined if activation of NOD2 in CECs is important for promoting CEC turnover and maintaining the integrity of the epithelial barrier. We found that NOD2 contributes to regulating CEC proliferation and survival.

MATERIALS AND METHODS

Animals and infections

Six to nine wk old C57BL/6-*Nod2*$^{+/+}$ and C57BL/6-*Nod2*$^{-/-}$ (F8)[16] mice bred and maintained in the same animal facility were infected by oral gavage with 10^6 cfu luciferase-expressing *Salmonella enterica serovar typhimurium* (SL1344-Tn5lux). Biophotonic imaging (Xenogen Corp. Alameda, CA) was used to determine bacterial cfu in tissue homogenates[17]. All animal experiments were conducted in full accordance with the Animal Scientific Procedures Act 1986 under Home Office approval.

CEC isolation and culture

Segments of colon were sequentially incubated three times in dissociation buffer (130 mmol/L NaCl, 10 mmol/L HEPES, pH 7.4, 10% FCS and 1 mmol/L DTT) containing first 1 mmol/L, then 5 mmol/L and finally 10 mmol/L EDTA at 37℃ for 15 min[18]. Aliquots of cells were stained with Wright-Giemsa (Baxter, Miami, FL), CD45 (Caltag Labs, Burlingame, CA), cytokeratin (Sigma-Aldrich, Poole, UK) and Ki67 (Dako, Carpinteria, CA) antibodies and incubated with alkaline phosphatase (AlkP) substrate (Vector Labs, Burlingame, CA) to establish CEC purity and identify proliferating (cytokeratin$^+$, Ki67$^+$, CD45$^-$) and differentiated (cytokeratin$^+$, AP$^+$, CD45$^-$) CECs. CEC monolayer cultures, established from dispase-digested fragments of colonic mucosa[19] were incubated with 1-10 mg/mL MDP (Ac-muramyl-Ala-Disoglutamine) for up to 4 d. Cell growth and viability were assessed by trypan blue exclusion. For NF-κB activation, nuclear extracts of CECs cultured for 2 h with MDP (1 mg/mL) or media alone were analyzed by ELISA (BD-Pharmingen) using specific inhibitors to block NF-κB activation as per the manufacturers' instructions. Recombinant human TNF α (R&D Systems) was added to HT-29 and SW480 human colonic carcinoma cell lines (provided by Prof. Mark Hull, Univ. Leeds) to induce NOD2 expression[8].

Histology

Villous crypt height was determined by measuring the distances from the base of the crypt to the villous tip of at least 20 villi from 3 HE-stained sections of colon from 5 mice of each strain prior to and following infection. Axiovision software (Imaging Associates Ltd, Bicester, UK) was used for scaling and measurements.

Flow cytometry

Antibody staining (cytokeratin and CD45) and flow cytometry was used to assess CEC purity. Apoptotic cells were quantified by Annexin V and propidium iodide (PI) or 7AAD staining[20,21]. Levels of caspase 3 activity in cultured CECs were determined using the NucViewTM 488 substrate (Biotium, Hayward, CA) according to the manufacturers' recommendation. Stained cells were analyzed using a FACSCalibur and CellQuest software (BD).

Immunohistochemistry

Paraffin (5 μm) sections were incubated with Ki67 (Dako), caspase 3 (BD-Pharmingen), BrdU (Oxford Biotechnology Ltd, Oxford, UK) or isotype matched control antibodies followed by biotinylated secondary antibodies (Vector Labs) and streptavidin-horseradish peroxidase plus DAB (Vector Labs) or anti-rabbit EnVision™ labeled polymer (Dako). For BrdU detection sections were pre-treated with 2 mol/L HCl for 30 min followed by neutralization in 0.1 mol/L $Na_2B_4O_7$ for 5 min to denature DNA. Stained cells in sections were enumerated using a Zeiss Axiovert 200 M microscope (Zeiss, Welwyn Garden City, UK) equipped with Axiovision software.

qRT-PCR

NOD2 mRNA was quantified in freshly isolated primary CECs and in HT-29 and SW480 cells using pre-optimized primer sets (Applied Biosystems, Foster City, CA) and an ABI prism 7900HT Sequence Detection System (Applied Biosystems). Threshold cycle (Ct) numbers were determined with Sequence Detection Software (Applied Biosystems) and analysed using the delta Ct comparative method. β-actin was used as a reference gene.

RNAi

NOD2 and scrambled NOD2 shRNA sequences (Dharamacon, Lafayette, CO) were cloned into GFP-expressing lentiviral vector, pLL 3.7. shRNA expressing lentiviruses were prepared using the ViraPower Expression System (Invitrogen). HT-29 and SW480 human colonic carcinoma cells were infected with lentiviruses and 10 mg/mL polybrene (Sigma) for 16 h. Quantitative (Taqman) RT-PCR was used to assess CARD15 mRNA knockdown. Viability and growth of infected (GFP$^+$) and non-infected (GFP$^-$) cells was assessed by flow cytometry as described above.

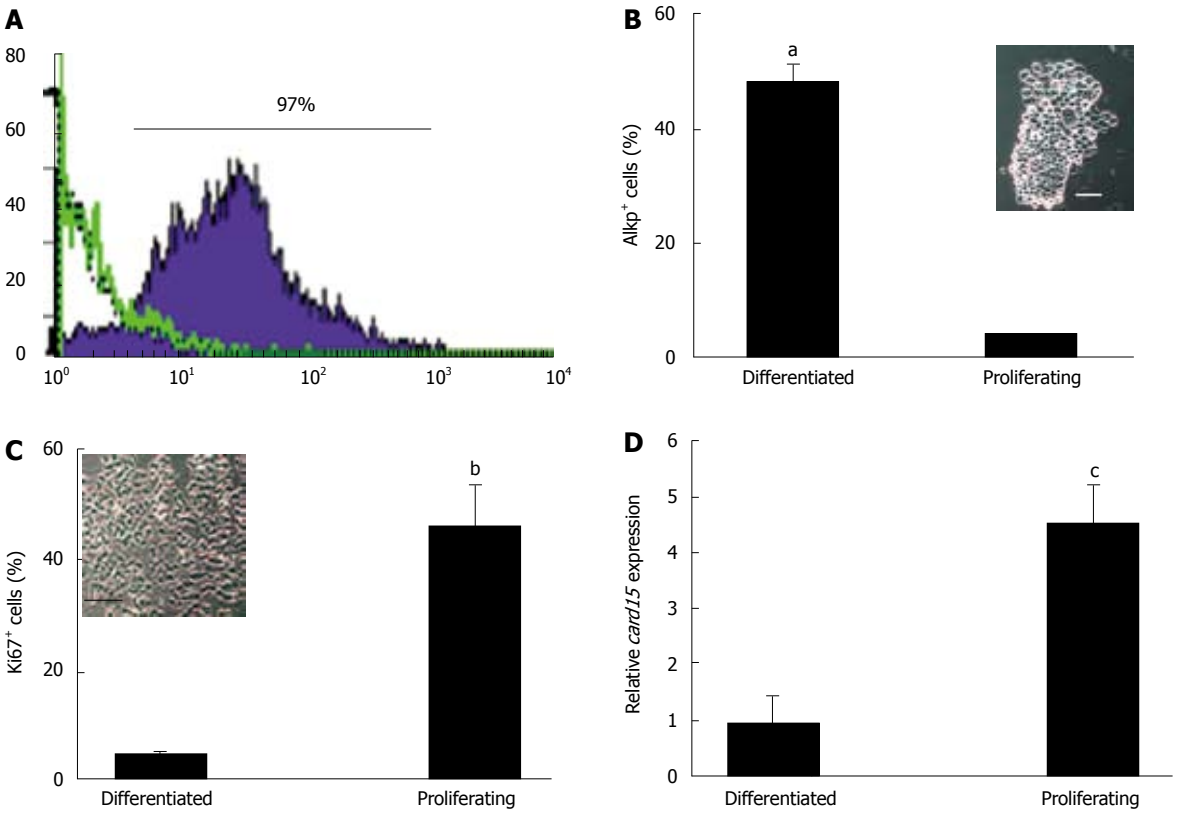


Figure 1 NOD2 expression by CECs. **A**: Colonic epithelial cells were isolated by incubating segments of colon with increasing concentrations of EDTA. Purity was assessed by staining with anti-cytokeratin (filled profile) and -CD45 (green open profile) antibodies and by flow cytometry using isotype matched antibodies to determine levels of background staining (dotted open profile). The % value shown represents the frequency of cytokeratin$^+$ cells in a representative 10 mmol/L EDTA fraction. **B** and **C**: 1 mmol/L and 10 mmol/L EDTA fractions of CECs were evaluated for the presence of differentiated and proliferating epithelial using alkaline phosphatase (AlkP) activity and Ki67 expression, respectively, as described in the Methods section. $^aP < 0.03$ in **B** and $^bP < 0.015$ in **C**. The morphology of cells in 1 mmol/L and 10 mmol/L EDTA fractions is depicted in the phase contrast photomicrograph insets in **B** (10 mmol/L) and **C** (1 mmol/L). Magnification is 160. Scale bar in **B** = 10 μm and in **C** = 40 μm. **D**: The relative levels of Card15 mRNA in fractions of colonic crypts enriched for proliferating and differentiated epithelial cells were determined by real time RT-PCR using β-actin as a reference gene. The data represents averaged values (± SE) of four experiments. $^cP < 0.05$.

Statistical analysis

All data were assessed for normal distribution using a Shapiro-Wilk test. For parametric and non-parametric data, analysis was performed using Student *t*-test and Mann Whitney test, respectively using the Student Package for the Social Sciences software (SPSS). *P* values < 0.05 were considered significant.

RESULTS

Cell turnover is altered in NOD2-deficient CECs

In view of previous reports of NOD2 expression in human colonic epithelial cell lines, isolated colonic crypts and among individual CECs[5,8,14] we sought evidence of NOD2 expression in primary murine colonic crypt epithelia. Initial immunohistochemical studies identified weak immunoreactivity among epithelial cells at the base of colonic crypts in wild type mice with a NOD2 antiserum (data not shown). To verify this finding, quantitative RT-PCR was used to measure NOD2 mRNA levels in CECs from different regions of the crypt. Sequential incubations in EDTA-containing buffers provided two morphologically distinct populations of epithelial cells of high purity (> 97% cytokeratin$^+$) enriched for cells expressing markers of proliferation (Ki67) or differentiation (alkaline phosphatase, AlkP; Figure 1A-C). Preparations enriched for proliferating cells (about 50% Ki67$^+$, < 5% AP$^+$) contained significantly higher (0.95 ± 0.5 *vs* 4.5 ± 0.7, $P < 0.05$.) levels of NOD2 mRNA than fractions enriched for differentiated CECs (about 50% AP$^+$, < 5% Ki67$^+$; Figure 1D).

Consistent with NOD2 involvement in epithelial homeostasis, the length of colonic crypts in adult *Nod2*$^{-/-}$ mice was significantly shorter (143 ± 10 *vs* 93 ± 5, $P < 0.05$) compared to that in *Nod2*$^{+/+}$ mice (Figure 2 and 3A). These differences were also evident after oral infection with a non-lethal dose of *Salmonella enterica serovar typhimurium*. The average length in *Nod2*$^{-/-}$ mice was increased upon infection, but was still significantly shorter (134.74 ± 4.22 *vs* 170.97 ± 6.55, $P < 0.05$) than in infected *Nod2*$^{+/+}$ mice at 32 h.

Nod2$^{-/-}$ mice were more susceptible than wild type mice to invasion by *Salmonella*. During the first 24 h of *Salmonella* infection the colonic bacterial burden of *Nod2*$^{-/-}$ mice (cfu at 4 h: 840 ± 161; at 8 h: 1123 ± 187, at 24 h: 631 ± 202) was significantly higher ($P < 0.0005$) than in *Nod2*$^{+/+}$ mice (cfu at 4 h: 78 ± 52; at 8 h: 88 ± 55; at 24 h: 101 ± 33). *Nod2*$^{-/-}$ animals also manifested severe diarrhea compared to *Nod2*$^{+/+}$ mice, which persisted for 36 h, although they fully recovered after 3 d.

CEC turnover *in vivo* was investigated further by

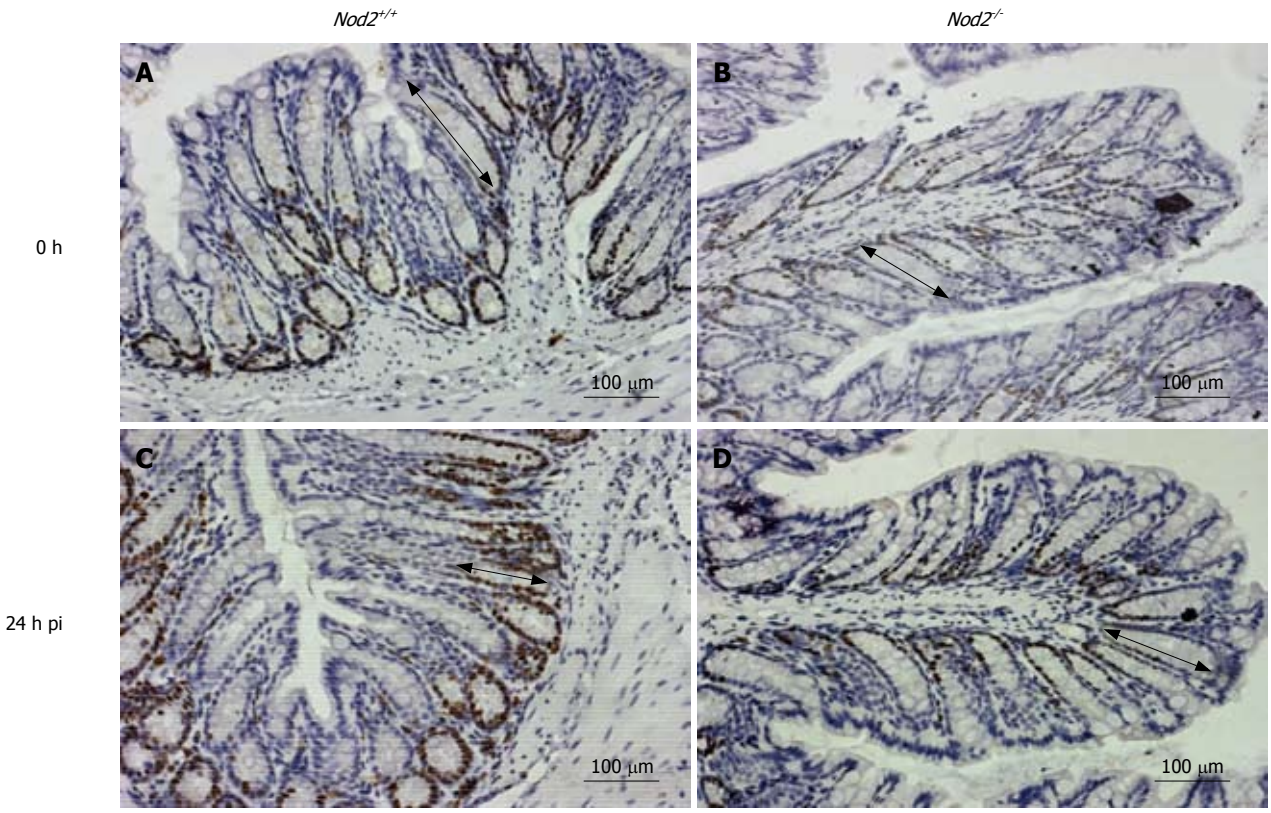


Figure 2 Decreased proliferation of NOD2-deficient CECs. Sections of colon from *Nod2*$^{+/+}$ (wild type: WT) and *Nod2*$^{-/-}$ mice obtained prior to (0 h) and 24 h after enteric *Salmonella* infection were stained with an anti-Ki67 antibody and DAB and then counterstained with H&E. Ki67-reactive cells within the crypt regions are identified by the darkly stained nuclei. The arrows indicate the crypt-villous height with the scale bar representing 100 μm.

BrdU uptake and Ki67 expression. Significantly fewer Ki67$^+$ and BrdU$^+$ CECs were present in *Nod2*$^{-/-}$ mice compared to *Nod2*$^{+/+}$ mice, both prior to and after *Salmonella* infection (Figures 2, 3B and 3C). Proliferating (BrdU$^+$) epithelial cells also migrated shorter distances from the crypts in *Nod2*$^{-/-}$ mice compared to *Nod2*$^{+/+}$ mice (unpublished observations), consistent with reduced CEC proliferation. *Nod2*$^{-/-}$ mice also had significantly higher numbers of apoptotic (caspase3$^+$) CECs compared to *Nod2*$^{+/+}$ mice after *Salmonella* infection (1.52 ± 0.37 *vs* 2.75 ± 0.48, $P < 0.001$, Figure 3D). These *in vivo* studies do not, however, exclude the possibility that the effects of NOD2 deficiency on CEC growth and apoptosis are secondary to changes in the activity of mucosal immunocompetent cells.

Growth and apoptosis of *Nod2*$^{-/-}$ CECs in culture following isolation was analyzed. Primary cultures of *Nod2*$^{-/-}$ CECs (see Methods section) contained significantly higher (11.5 ± 1.1 *vs* 17.7 ± 1.4, $P < 0.0105$) numbers of apoptotic cells compared to *Nod2*$^{+/+}$ CECs (Figure 4A) with consistently more cells expressing caspase3 (Figure 4B). The growth of *Nod2*$^{-/-}$ CEC *in vitro* was also significantly less than *Nod2*$^{+/+}$ CECs at 24 h (10.7 ± 0.4 *vs* 8.3 ± 0.2, $P < 0.026$, Figure 4C). Of note, the addition of MDP to cultures of *Nod2*$^{+/+}$ CEC increased cell numbers, whereas no increase in *Nod2*$^{-/-}$ CEC number was seen in response to MDP (Figure 4C). The defect in *Nod2*$^{-/-}$ CEC growth *in vitro* was correlated with defective MDP-mediated NF-κB activation (Figure 4D).

Neutralization of CARD15 by shRNA reduces human colonic epithelial carcinoma cell survival

Further evidence for the direct involvement of NOD2 in regulating epithelial cell proliferation was sought using RNA interference (RNAi) to knockdown expression of *NOD2* in HT-29 and SW480 human colonic carcinoma cells.

After transfection with lentiviral vectors expressing short hairpin RNA (shRNA) *NOD2* sequences, the level of CARD15 mRNA in both HT-29 and SW480 cells, as determined by quantitative RT-PCR, was reduced by 75%-80%, compared to both non-treated cells and cells infected with control lentivirus expressing scrambled *NOD2* sequences (Figure 5A and data not shown). *NOD2* shRNA treatment led to a significant decline in HT-29 cell survival with < 20% of GFP$^+$ (virus infected) cells surviving beyond 6 days (Figure 5B). The decline in GFP$^+$ cells was explained by decreased cell viability and increased apoptosis (Figure 5C). Similar to primary *Nod2*$^{+/+}$ and *Nod2*$^{-/-}$ CEC, knockdown of *NOD2* mRNA had no effect on cell cycle progression, although the number of cycling cells was reduced (unpublished observations) reflecting the increased levels of cell death. By comparison, the growth and survival of HT-29 cells infected with lentiviral vectors expressing scrambled *NOD2* shRNA sequences was unaffected (Figure 5B). A similar outcome of *NOD2* shRNA on epithelial cell growth was seen in SW480 cells (Figure 5D), although the loss of GFP$^+$ SW480 cells occurred over a longer time period with about 60% reduction in GFP$^+$ cells seen after 4 wk (Figure 5D).

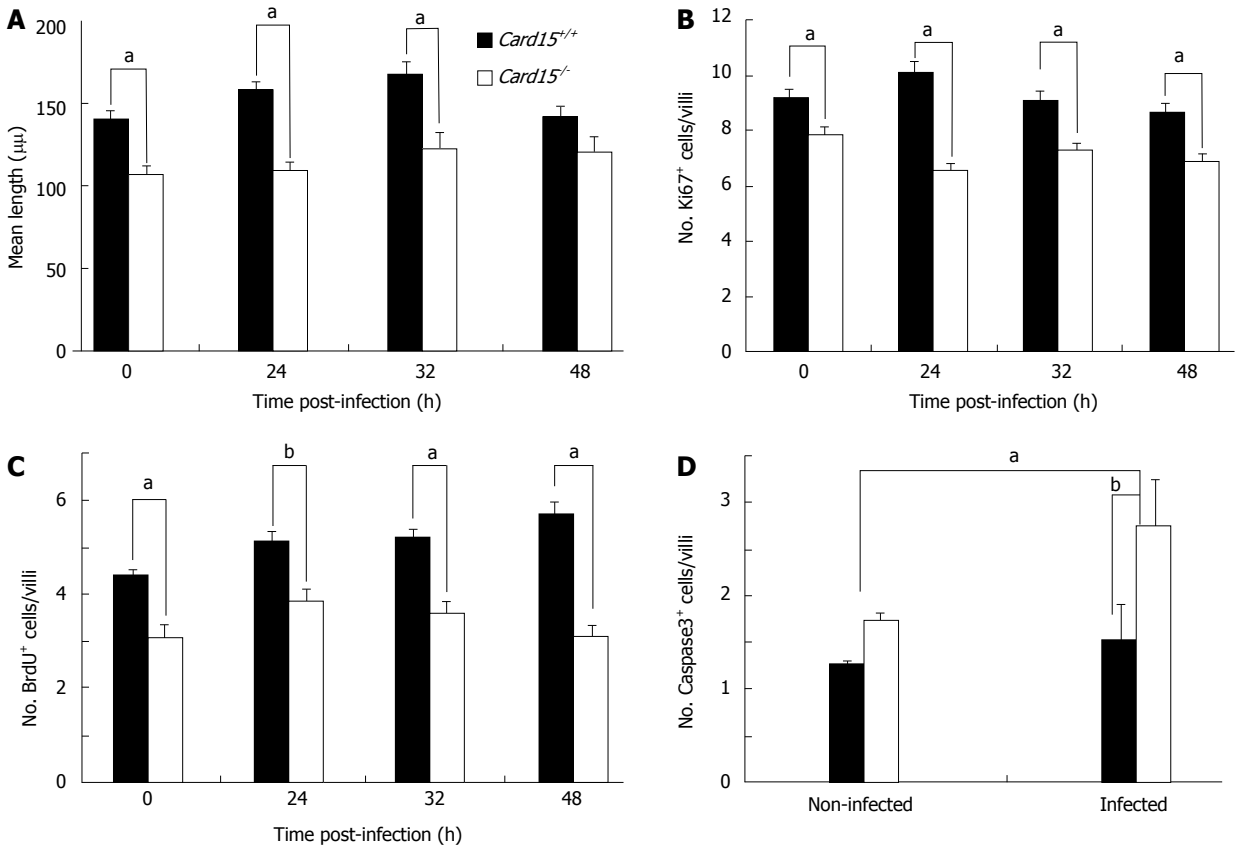


Figure 3 Decreased proliferation of *Nod2*$^{-/-}$ CECs *in vivo*. **A**: Mean crypt-villous height measurements were determined by measuring distances from the base to the tip of the crypt of at least 20 crypts from sections taken at the distal, mid and proximal parts of the colon of *Nod2*$^{+/+}$ and *Nod2*$^{-/-}$ mice (n = 5) prior to (0 h) and at different times after peroral *Salmonella* infection. The mean (± SE) of counting at least 20 crypts in 3-4 sections of 4-5 mice of each strain is shown. **B**: Sections of *Nod2*$^{+/+}$ and *Nod2*$^{-/-}$ colon were stained with anti-Ki67 antibody with the mean (± SE) number of stained cells in 5-6 sections from three mice of each strain shown. $^{a}P < 0.05$. **C**: BrdU-incorporation was assessed by injecting *Nod2*$^{+/+}$ and *Nod2*$^{-/-}$ mice with BrdU 1 h prior to removing the colons, which were then sectioned and stained with an anti-BrdU antibody. The graph represents the mean (± SE) number of BrdU^{+} epithelial cells per crypt as determined by counting stained cells in > 20 crypts from 5-6 sections taken from proximal, mid and distal regions of the colons from three mice of each strain. $^{a}P < 0.05$, $^{b}P < 0.001$. **D**: Sections of colon from *Nod2*$^{+/+}$ and *Nod2*$^{-/-}$ mice were stained with anti-active caspase3 antibodies with the mean (± SE) number of caspase3^{+} cells in 4-5 sections from three mice of each strain shown. $^{a}P < 0.05$, $^{b}P < 0.001$.

DISCUSSION

The integrity of the colonic epithelium is maintained by the continual renewal of epithelial cells as a result of the accelerated division of crypt cells that migrate upwards from the base of the crypts. Little is known about the origin and nature of the factors that regulate these processes. Here, we provide evidence for the involvement of NOD2 in regulating murine CEC turnover.

Expression of NOD2 in preparations of CECs enriched for proliferating cells described here, together with prior accounts of increased epithelial expression under conditions of rapid proliferation *in vitro* and in the inflamed and infected intestine[6,11,14,22] and, the increased severity of chemical-induced colonic mucosal damage in NOD2-deficient mice[10], suggests NOD2 contributes to regulating epithelial cell turnover and promoting epithelial repair. The low amount of NOD2 expression in the colonic mucosa, under steady-state conditions described here and previously[14], is consistent with this hypothesis and that altered epithelial cell proliferation and death[23] and increased epithelial permeability[24] contribute to the development of IBD. The absence of intestinal inflammation in *Nod2*$^{-/-}$ mice[10,11,16,25], or in mice with a knock-in mutation corresponding to the predominant disease-associated mutant form of human *NOD2* in Crohn's disease[25], suggests that while *NOD2* gene mutations and altered epithelial turnover are a prerequisite for developing Crohn's disease, they are not sufficient in themselves[26]. Other additional genetic factors or environmental triggers that disrupt the epithelial barrier[10] must also occur for the development of disease.

The absence of NOD2 in CECs does not completely abolish cellular proliferation. NOD2 may, therefore, function as part of a network of interacting and interdependent factors that includes other PRRs and various cytoprotective and repair factors that collectively regulate epithelial homeostasis with other components partially compensating for the absence of NOD2. NOD2 does, however, appear to play a more essential role in promoting the growth and survival of immortalized HT-29 and SW480 colonic carcinoma cells. Differences in the survival curves of these cell lines after *NOD2* shRNA treatment may relate to differences in the genetic mutations and their impact on the requirement for NOD2 dependent-survival signals. Additional studies, using primary human CECs and colonic specimens from patients bearing CD-associated

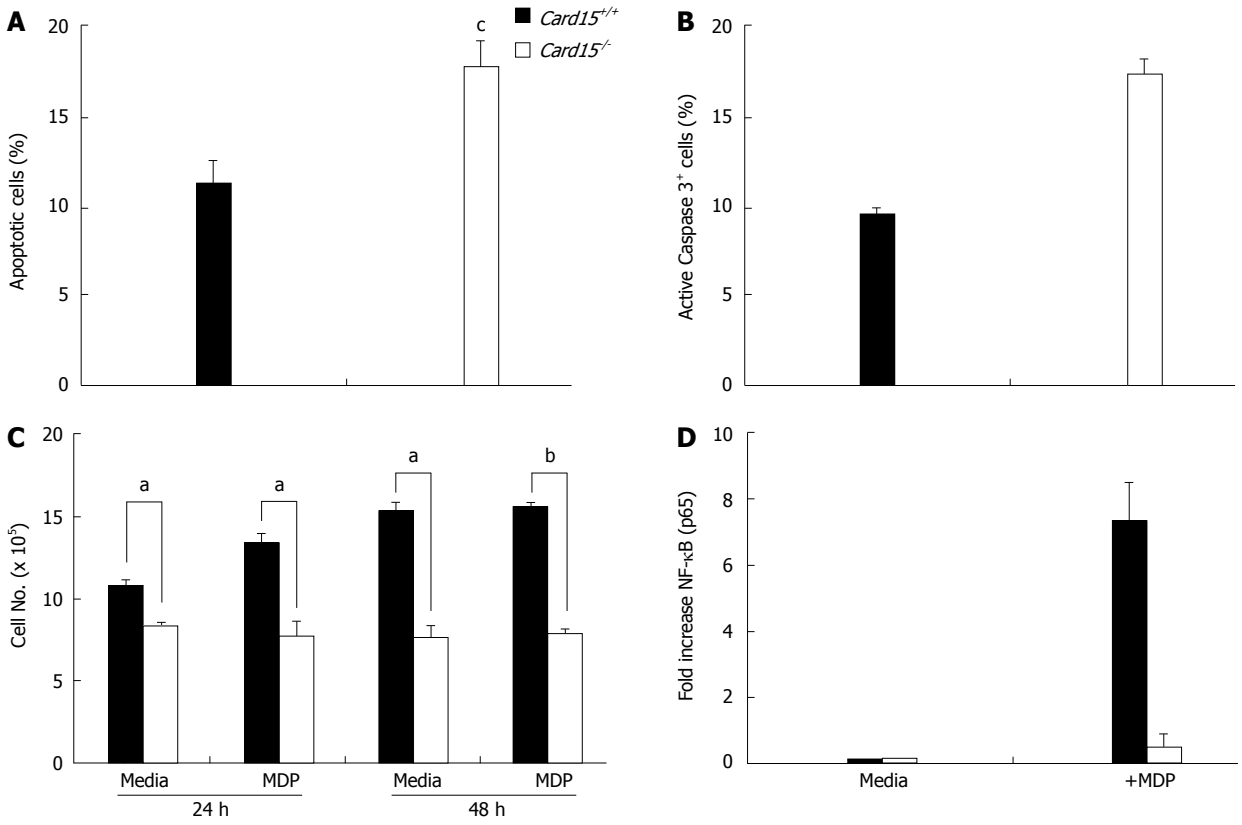


Figure 4 Decreased survival of *Nod2*$^{-/-}$ CECs *in vitro*. **A**: The level of apoptotic cells in cultures of primary CECs from *Nod2*$^{+/+}$ and *Nod2*$^{-/-}$ mice was determined by annexin V and propidium iodide (PI) staining and flow cytometry. Data was collected from 5 experiments and shows the mean (± SE) values. $^{c}P < 0.01$ **B**: The frequency of *Nod2*$^{+/+}$ and *Nod2*$^{-/-}$ CECs containing caspase3 activity was determined using the flow cytometry based NucViewTM488 Caspase-3 assay. The data represents the mean (± SE) values collected from two experiments. **C**: The growth of *Nod2*$^{+/+}$ and *Nod2*$^{-/-}$ CECs in the absence (Media) or presence of 10 μg/mL muramyl dipeptide (MDP) was determined by comparing the number of viable CEC at the initiation of culture and after 24 h and 48 h. The data shown represents the mean (± SE) of 3 experiments. $^{a}P < 0.05$, $^{b}P < 0.001$. **D**: NF-κB activation in *Nod2*$^{+/+}$ (filled bars) and *Nod2*$^{-/-}$ (open bars) CEC after exposure to muramyl dipeptide (MDP) was determined by quantitating NF-κB p65 levels in nuclear extracts using a Transfactor Kit as described in Methods section. Values were normalized to control values of cells grown in media alone. The data shown represents the mean (± SE) values from 3 independent experiments.

NOD2 mutations, are required to substantiate these findings. However, the inability of HT-29 and SW480 cells to survive NOD2 RNAi treatment is consistent with the disrupted growth of *Nod2*$^{-/-}$ primary CECs *in vitro* and *in vivo* and, that the sustained expression of NOD2 is required to maintain high rates of CEC proliferation. Identifying the pathways and mediators of NOD2 signaling in CECs will help establish how NOD2 contributes to CEC and tumor cell survival.

The proposed role of NOD2 in colonic epithelial homeostasis does not necessarily contradict the notion that it is a bacterial sensor and contributes to innate immunity[27,28]. The importance of NOD2 in innate anti-bacterial responses is demonstrated by the increased bacterial burden and inflammation seen here in *Nod2*$^{-/-}$ mice after enteric *Salmonella* infection and previously after infection with *Listeria*[11], both of which disrupt epithelial barrier function. Cell type specific influences and microenvironmental factors may account for contrasting roles for NOD2 in different studies. The importance of environmental influences is perhaps best demonstrated by its divergent role(s) in the very different environment of the small intestine, where it regulates anti-microbial protein production[11] and GALT development[10], and the colon where it regulates epithelial cell turnover. In addition, differences in the expression of NOD2-regulatory proteins[29,30], or endogenous inhibitors of NOD2[31], could also explain different outcomes of NOD2 activation in different cell types. The recent identification of a NOD2 target gene (DMBT1) that is predominantly expressed in epithelial cells[32] is also consistent with cell type specific NOD2 responses.

How NOD2 carries out its diverse array of functions in different regions of the gastrointestinal tract remains to be determined. In the colon, NOD2's effect on CEC growth could be direct by, for example, regulating expression of genes and/or proteins involved in cell growth. mRNA profiling of wild type and *Nod2*$^{-/-}$ CECs by microarray analysis has identified securin (pituitary tumor transforming gene-1) that is required for maintaining appropriate cell division[33] as one of the most underrepresented genes in *Nod2*$^{-/-}$ CECs (unpublished observation). Alternatively, the involvement of PRRs in the production of cytoprotective and reparative cytokines in the colon[12] and reports of altered patterns of cytokine production by NOD2-deficient cells[34-36] suggest an indirect mechanism of action of NOD2. The growth-promoting effect of NOD2 in CECs contrasts with MDP-induced apoptosis of rabbit kidney epithelial cells, which may reflect cell type specific differences in the response to MDP and its interaction

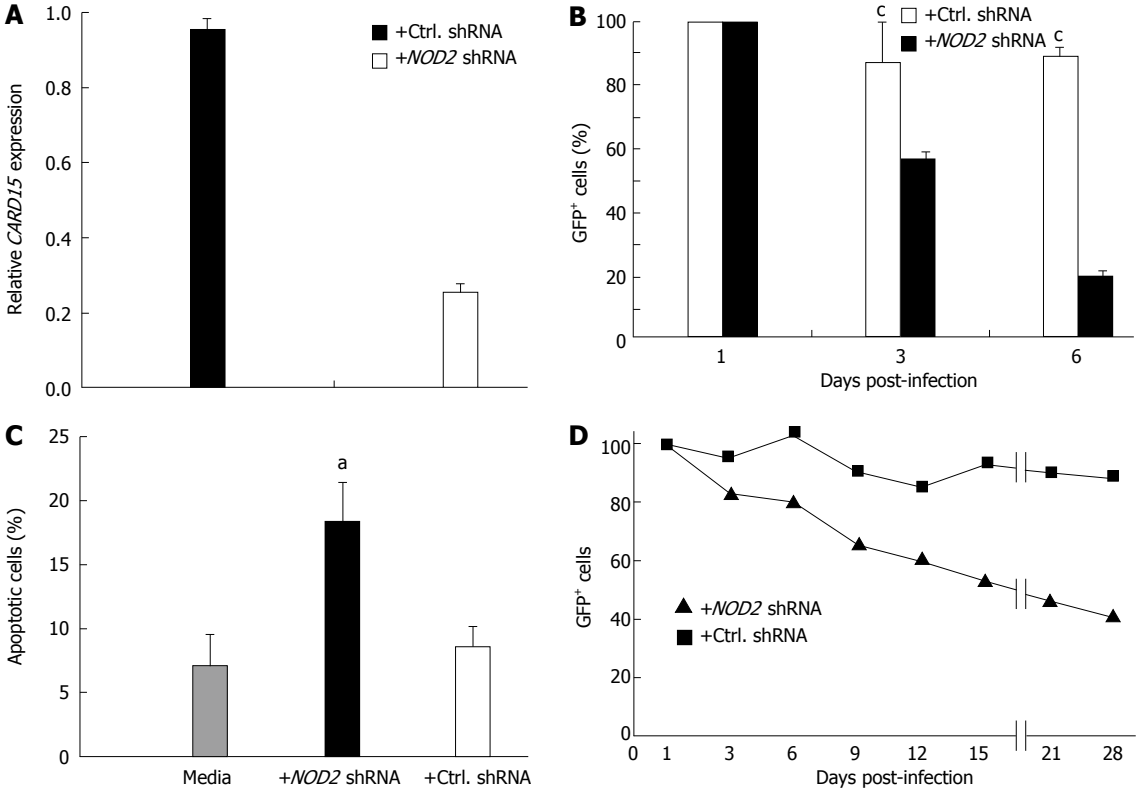


Figure 5 Neutralization of *CARD15* gene expression in colon carcinoma cells reduces their survival. **A**: The level of *NOD2* mRNA in HT-29 cells transfected with green fluorescent (GFP) containing lentiviral vectors expressing either *NOD2* (*NOD2* shRNA) or scrambled *NOD2* shRNA sequences (Ctrl. shRNA) was determined by real time RT-PCR after 4 h stimulation with TNFα. Levels were compared to that of nontreated cells (= 1.0). Data are averaged values (± SE) of 3 experiments. **B**: The impact of CARD15 and control shRNA on the growth of HT-29 cells was assessed by determining the frequency of GFP$^+$ and GFP-cells by flow cytometry at 1 d, 3 d and 6 d post infection. Data are averaged values (± SE) of 3 experiments. $^cP < 0.01$. **C**: Frequency of apoptotic cells in cultures of non-infected HT-29 cells (Media) and in cells 2 d after infection with *NOD2* shRNA or Ctrl shRNA was determined by staining with Annexin V and 7AAD and flow cytometry. The graph represents the averaged frequency (± SE) of apoptotic cells from three experiments. $^aP < 0.05$. **D**: The survival of SW480 cells after transfection with lentiviral vectors expressing either *NOD2* or scrambled shRNA sequences was analysed by determining the frequency of GFP$^+$ cells remaining at various times after infection by flow cytometry. The data is representative of two independent experiments.

with different cellular proteins including NOD2[37]. Further studies aimed at identifying NOD2 signaling pathways in CECs will be important in determining how this protein functions in intestinal epithelial cells.

In summary, we have shown that NOD2 contributes to maintaining epithelial cell homeostasis in the colon. Compromised barrier repair may, therefore, underlie aspects of Crohn's disease where mutant *NOD2* alleles contribute to disease.

COMMENTS

Background

Mutations in *NOD2* alleles are associated with an increased risk of developing Crohn's disease (CD), an Inflammatory Bowel Disease (IBD). However, it is unclear how *NOD2* mutations contribute to the development of CD and in particular to intestinal epithelial homeostasis.

Research Frontiers

Our study has identified a novel function for NOD2 in the regulation of colonic epithelial cell growth and in the survival of colonic epithelial tumor cell lines.

Innovations and breakthroughs

This study is the first to provide direct evidence of a role for NOD2 in epithelial cell growth and survival. Compromised epithelial barrier restitution and repair after injury may, therefore, contribute to the pathogenesis of CD in patients with mutant *NOD2* alleles.

Applications

Further understanding of the function of NOD2 and the role it plays in the pathogenesis of CD will be of benefit in developing new therapies for CD.

Peer review

This interesting study was to determine the molecular mechanisms regulating NOD2 function in colonic epithelial cells. The authors conducted both *in vivo* and *in vitro* studies to show the specific functions of the NOD2 protein. This is an outstanding and clearly written paper that provides strong evidence for a role for NOD2 in murine colonic epithelial survival.

REFERENCES

1 **Sansonetti PJ**. War and peace at mucosal surfaces. *Nat Rev Immunol* 2004; **4**: 953-964
2 **Inohara N**, Ogura Y, Fontalba A, Gutierrez O, Pons F, Crespo J, Fukase K, Inamura S, Kusumoto S, Hashimoto M, Foster SJ, Moran AP, Fernandez-Luna JL, Nunez G. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J Biol Chem* 2003; **278**: 5509-5512
3 **Girardin SE**, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, Philpott DJ, Sansonetti PJ. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem* 2003; **278**: 8869-8872
4 **Ogura Y**, Inohara N, Benito A, Chen FF, Yamaoka S, Nunez G. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *J Biol Chem* 2001; **276**: 4812-4818
5 **Hisamatsu T**, Suzuki M, Reinecker HC, Nadeau WJ, McCormick BA, Podolsky DK. CARD15/NOD2 functions as an antibacterial factor in human intestinal epithelial cells. *Gastroenterology* 2003; **124**: 993-1000
6 **Lala S**, Ogura Y, Osborne C, Hor SY, Bromfield A, Davies S, Ogunbiyi O, Nunez G, Keshav S. Crohn's disease and the

NOD2 gene: a role for paneth cells. *Gastroenterology* 2003; **125**: 47-57
7 **Kobayashi K**, Inohara N, Hernandez LD, Galan JE, Nunez G, Janeway CA, Medzhitov R, Flavell RA. RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* 2002; **416**: 194-199
8 **Rosenstiel P**, Fantini M, Brautigam K, Kuhbacher T, Waetzig GH, Seegert D, Schreiber S. TNF-alpha and IFN-gamma regulate the expression of the NOD2 (CARD15) gene in human intestinal epithelial cells. *Gastroenterology* 2003; **124**: 1001-1009
9 **Melmed G**, Thomas LS, Lee N, Tesfay SY, Lukasek K, Michelsen KS, Zhou Y, Hu B, Arditi M, Abreu MT. Human intestinal epithelial cells are broadly unresponsive to Toll-like receptor 2-dependent bacterial ligands: implications for host-microbial interactions in the gut. *J Immunol* 2003; **170**: 1406-1415
10 **Barreau F**, Meinzer U, Chareyre F, Berrebi D, Niwa-Kawakita M, Dussaillant M, Foligne B, Ollendorff V, Heyman M, Bonacorsi S, Lesuffleur T, Sterkers G, Giovannini M, Hugot JP. CARD15/NOD2 is required for Peyer's patches homeostasis in mice. *PLoS ONE* 2007; **2**: e523
11 **Kobayashi KS**, Chamaillard M, Ogura Y, Henegariu O, Inohara N, Nunez G, Flavell RA. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* 2005; **307**: 731-734
12 **Rakoff-Nahoum S**, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 2004; **118**: 229-241
13 **Strober W**, Murray PJ, Kitani A, Watanabe T. Signalling pathways and molecular interactions of NOD1 and NOD2. *Nat Rev Immunol* 2006; **6**: 9-20
14 **Berrebi D**, Maudinas R, Hugot JP, Chamaillard M, Chareyre F, De Lagausie P, Yang C, Desreumaux P, Giovannini M, Cezard JP, Zouali H, Emilie D, Peuchmaur M. Card15 gene overexpression in mononuclear and epithelial cells of the inflamed Crohn's disease colon. *Gut* 2003; **52**: 840-846
15 **Ting JP**, Davis BK. Caterpiller: a novel gene family important in immunity, cell death, and diseases. *Annu Rev Immunol* 2005; **23**: 387-414
16 **Pauleau AL**, Murray PJ. Role of nod2 in the response of macrophages to toll-like receptor agonists. *Mol Cell Biol* 2003; **23**: 7531-7539
17 **Burns-Guydish SM**, Olomu IN, Zhao H, Wong RJ, Stevenson DK, Contag CH. Monitoring age-related susceptibility of young mice to oral Salmonella enterica serovar Typhimurium infection using an in vivo murine model. *Pediatr Res* 2005; **58**: 153-158
18 **Inan MS**, Tolmacheva V, Wang QS, Rosenberg DW, Giardina C. Transcription factor NF-kappaB participates in regulation of epithelial cell turnover in the colon. *Am J Physiol Gastrointest Liver Physiol* 2000; **279**: G1282-G1291
19 **Baumgart DC**, Olivier WA, Reya T, Peritt D, Rombeau JL, Carding SR. Mechanisms of intestinal epithelial cell injury and colitis in interleukin 2 (IL2)-deficient mice. *Cell Immunol* 1998; **187**: 52-66
20 **Cruickshank SM**, McVay LD, Baumgart DC, Felsburg PJ, Carding SR. Colonic epithelial cell mediated suppression of CD4 T cell activation. *Gut* 2004; **53**: 678-684
21 **Singh JC**, Cruickshank SM, Newton DJ, Wakenshaw L, Graham A, Lan J, Lodge JP, Felsburg PJ, Carding SR. Toll-like receptor-mediated responses of primary intestinal epithelial cells during the development of colitis. *Am J Physiol Gastrointest Liver Physiol* 2005; **288**: G514-G524
22 **Ogura Y**, Lala S, Xin W, Smith E, Dowds TA, Chen FF, Zimmermann E, Tretiakova M, Cho JH, Hart J, Greenson JK, Keshav S, Nunez G. Expression of NOD2 in Paneth cells: a possible link to Crohn's ileitis. *Gut* 2003; **52**: 1591-1597
23 **Dignass AU**. Mechanisms and modulation of intestinal epithelial repair. *Inflamm Bowel Dis* 2001; **7**: 68-77
24 **Rask-Madsen J**, Hammersgaard EA, Knudsen E. Rectal electrolyte transport and mucosal permeability in ulcerative colitis and Crohn's disease. *J Lab Clin Med* 1973; **81**: 342-353
25 **Maeda S**, Hsu LC, Liu H, Bankston LA, Iimura M, Kagnoff MF, Eckmann L, Karin M. Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing. *Science* 2005; **307**: 734-738
26 **Watanabe T**, Kitani A, Murray PJ, Wakatsuki Y, Fuss IJ, Strober W. Nucleotide binding oligomerization domain 2 deficiency leads to dysregulated TLR2 signaling and induction of antigen-specific colitis. *Immunity* 2006; **25**: 473-485
27 **Murray PJ**. NOD proteins: an intracellular pathogen-recognition system or signal transduction modifiers? *Curr Opin Immunol* 2005; **17**: 352-358
28 **Fritz JH**, Ferrero RL, Philpott DJ, Girardin SE. Nod-like proteins in immunity, inflammation and disease. *Nat Immunol* 2006; **7**: 1250-1257
29 **Kufer TA**, Kremmer E, Banks DJ, Philpott DJ. Role for erbin in bacterial activation of Nod2. *Infect Immun* 2006; **74**: 3115-3124
30 **McDonald C**, Chen FF, Ollendorff V, Ogura Y, Marchetto S, Lecine P, Borg JP, Nunez G. A role for Erbin in the regulation of Nod2-dependent NF-kappaB signaling. *J Biol Chem* 2005; **280**: 40301-40309
31 **Rosenstiel P**, Huse K, Till A, Hampe J, Hellmig S, Sina C, Billmann S, von Kampen O, Waetzig GH, Platzer M, Seegert D, Schreiber S. A short isoform of NOD2/CARD15, NOD2-S, is an endogenous inhibitor of NOD2/receptor-interacting protein kinase 2-induced signaling pathways. *Proc Natl Acad Sci USA* 2006; **103**: 3280-3285
32 **Rosenstiel P**, Sina C, End C, Renner M, Lyer S, Till A, Hellmig S, Nikolaus S, Folsch UR, Helmke B, Autschbach F, Schirmacher P, Kioschis P, Hafner M, Poustka A, Mollenhauer J, Schreiber S. Regulation of DMBT1 via NOD2 and TLR4 in intestinal epithelial cells modulates bacterial recognition and invasion. *J Immunol* 2007; **178**: 8203-8211
33 **Wang Z**, Yu R, Melmed S. Mice lacking pituitary tumor transforming gene show testicular and splenic hypoplasia, thymic hyperplasia, thrombocytopenia, aberrant cell cycle progression, and premature centromere division. *Mol Endocrinol* 2001; **15**: 1870-1879
34 **Netea MG**, Kullberg BJ, de Jong DJ, Franke B, Sprong T, Naber TH, Drenth JP, Van der Meer JW. NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease. *Eur J Immunol* 2004; **34**: 2052-2059
35 **Netea MG**, Ferwerda G, de Jong DJ, Jansen T, Jacobs L, Kramer M, Naber TH, Drenth JP, Girardin SE, Kullberg BJ, Adema GJ, Van der Meer JW. Nucleotide-binding oligomerization domain-2 modulates specific TLR pathways for the induction of cytokine release. *J Immunol* 2005; **174**: 6518-6523
36 **van Heel DA**, Ghosh S, Hunt KA, Mathew CG, Forbes A, Jewell DP, Playford RJ. Synergy between TLR9 and NOD2 innate immune responses is lost in genetic Crohn's disease. *Gut* 2005; **54**: 1553-1557
37 **Chen D**, Texada DE, Duggan C, Liang C, Reden TB, Kooragayala LM, Langford MP. Surface calreticulin mediates muramyl dipeptide-induced apoptosis in RK13 cells. *J Biol Chem* 2005; **280**: 22425-22436

S- Editor Tian L **L- Editor** Rippe RA **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5842

World J Gastroenterol 2008 October 14; 14(38): 5842-5850
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Therapy with bone marrow cells reduces liver alterations in mice chronically infected by *Schistosoma mansoni*

Sheilla Andrade Oliveira, Bruno Solano Freitas Souza, Carla Adriana Guimarães-Ferreira, Elton Sá Barreto, Siane Campos Souza, Luiz Antonio Rodrigues Freitas, Ricardo Ribeiro-dos-Santos, Milena Botelho Pereira Soares

Sheilla Andrade Oliveira, Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão 121, Brotas, 40295-001 Salvador, BA, Brazil and Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz, Recife, Av Moraes Rego s/n, Cidade Universitária, Recife 50670-420, PE, Brazil
Bruno Solano Freitas Souza, Carla Adriana Guimarães-Ferreira, Elton Sá Barreto, Siane Campos Souza, Luiz Antonio Rodrigues Freitas, Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão 121, Brotas, Salvador 40295-001, BA, Brazil
Ricardo Ribeiro-dos-Santos, Milena Botelho Pereira Soares, Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão 121, Brotas, 40295-001 Salvador, BA, Brazil and Hospital São Rafael, Av São Rafael, 2152, São Marcos, Salvador 41256-900, BA, Brazil
Author contributions: Oliveira SA designed, conducted the study and analyzed the data; Souza BSF and Barreto ES conducted the morphometric and immunofluorescence studies; Guimarães-Ferreira CA and Souza SC performed FISH and PCR analyses; Freitas LAR performed the histopathological analyses; Ribeiro-dos-Santos R and Soares MBP were involved in designing the study, reviewing and interpreting the results. All authors were involved in drafting the manuscript.
Supported by Instituto do Milênio de Bioengenharia Tecidual (IMBT, CNPq/MCT), Rede Nordeste de Biotecnologia (RENORBIO, FINEP/MCT), Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB) and Fundação Oswaldo Cruz (FIOCRUZ)
Correspondence to: Milena Botelho Pereira Soares, PhD, Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão 121, Brotas, Salvador 40295-001, BA, Brazil. milena@bahia.fiocruz.br
Telephone: +55-71-31762260 Fax: +55-71-31762292
Received: July 8, 2008 Revised: August 21, 2008
Accepted: August 28, 2008
Published online: October 14, 2008

Abstract

AIM: To investigate the potential of bone marrow mononuclear cells (BM-MCs) in the regeneration of hepatic lesions induced by *Schistosoma mansoni* (*S.mansoni*) chronic infection.

METHODS: Female mice chronically infected with *S.mansoni* were treated with BM-MCs obtained from male green fluorescent protein (GFP) transgenic mice by intravenous or intralobular injections. Control mice received injections of saline in similar conditions. Enzyme-linked immunosorbent assay (ELISA) assay for transforming growth factor-beta (TGF-β), polymerase chain reaction (PCR) for GFP DNA, immunofluorescence and morphometric studies were performed.

RESULTS: Transplanted GFP$^+$ cells migrated to granuloma areas and reduced the percentage of liver fibrosis. The presence of donor-derived cells was confirmed by Fluorescence in situ hybridization (FISH) analysis for detection of cells bearing Y chromosome and by PCR analysis for detection of GFP DNA. The levels of TGF-β, a cytokine associated with fibrosis deposition, in liver fragments of mice submitted to therapy were reduced. The number of oval cells in liver sections of *S.mansoni*-infected mice increased 3-4 fold after transplantation. A partial recovery in albumin expression, which is decreased upon infection with *S.mansoni*, was found in livers of infected mice after cellular therapy.

CONCLUSION: In conclusion, transplanted BMCs migrate to and reduce the damage of chronic fibrotic liver lesions caused by *S.mansoni*.

Key words: *Schistosoma mansoni*; Liver injury; Fibrosis; Bone marrow cells; Transplantation; Albumin; Oval cells

Peer reviewers: Natalia A Osna, Liver Study Unit, Research Service (151), VA Medical Center, 4101 Woolworth Avenue, Omaha NE 68105, United States; Maurizio Parola, Professor, Department of Dip. Medicina e Oncologia Sperimentale, Universita degli Studi di torino, Corso Raffaello 30, Torino 10125, Italy

Oliveira SA, Souza BSF, Guimarães-Ferreira CA, Barreto ES, Souza SC, Freitas LAR, Ribeiro-dos-Santos R, Soares MBP. Therapy with bone marrow cells reduces liver alterations in mice chronically infected by *Schistosoma mansoni*. *World J Gastroenterol* 2008; 14(38): 5842-5850 Available from: URL: http://www.wjgnet.com/1007-9327/14/5842.asp DOI: http://dx.doi.org/10.3748/wjg.14.5842

INTRODUCTION

Chronic infection by *Schistosoma mansoni* (*S.mansoni*) is one of the experimental models of hepatic fibrosis

used to elucidate the mechanisms involved in the fibrogenic processes. In schistosomiasis, the main immune-inflammatory response is directed against the parasite eggs, which, when led to portal circulation, may become lodged into hepatic portal venules, eliciting a granulomatous response. In the mouse model of schistosomiasis, the persistence of the stimulus leads to the development of two pathological patterns; isolated granulomas or periportal fibrosis, the latter resembling the pipe-stem fibrosis found in the severe hepatosplenic form of human disease[1].

Liver fibrosis occurs in the setting of chronic injury caused by different etiologies constituting a serious worldwide public health problem. Whereas in acute hepatic injury nonviable cells are replaced by normal tissue, during chronic injuries a persistent repair response may lead in fibrosis and scar formation as a result of an imbalance between proliferation and degradation of the extracellular matrix components[2]. In addition to schistosomiasis, hepatopathies due to alcohol, viral hepatitis, drugs, metabolic and autoimmune diseases, and congenital abnormalities are important causes of liver fibrosis[2].

New therapeutic strategies aiming to minimize damages caused by hepatic fibrogenesis in chronic liver diseases are of great interest. Adult bone marrow contains pluripotent stem cells with the ability to differentiate into diverse cell types, including hepatocytes[3]. The regenerative potential of bone marrow stem cells has been tested in experimental models of hepatic injury, demonstrating the ability of bone marrow cells (BMCs) to generate hepatocytes under tissue stress in mice and human[4,5]. In this context, regenerative medicine has emerged as an alternative therapy to improve damaged liver function[5-7].

In this report, we used an experimental model of hepatic fibrogenesis caused by chronic infection with *S.mansoni* in order to evaluate the contribution of cellular therapy in hepatic diseases. We investigated the potential of syngeneic bone marrow mononuclear cells in the modulation of fibrosis, albumin expression and cellular alterations.

MATERIALS AND METHODS

Animals

Female or male C57Bl/6 wild-type and enhance green fluorescence protein (EGFP) transgenic mice (4-6 wk old) were used as recipients and as donors of BMCs, respectively. All the animals weighting 20-23 g were raised and maintained at the Gonçalo Moniz Research Center/ FIOCRUZ in rooms with controlled temperature (22 ± 2℃) and humidity (55% ± 10%) and continuous air renovation. Animals were housed in a 12 h light/12 h dark cycle (6 am-6 pm) and provided with rodent diet and water *ad libitum*. Animals were handled according to the NIH guidelines for animal experimentation. All procedures described here had prior approval from the local animal ethics committee.

Infection with Schistosoma mansoni

C57Bl/6 mice were infected by transcutaneous route with 30 *S.mansoni* cercariae of the Feira de Santana strain[8]. This strain was maintained through successive passages in laboratory-raised *Biomphalaria glabrata* (*B.glabrata*). Two weeks later the animals were exposed to reinfection with 15 cercariae to increase the hepatic injury. The infection was confirmed 40 d after the primary infection by parasitological exam of feces. Only mice presenting viable eggs in the stools were used. For the retreat of the injury stimulus mice were treated with praziquantel (Farmanguinhos, Fiocruz, Rio de Janeiro, Brazil) by gavage in a single dose of 400 mg/kg per wk 4 mo after the primary infection.

Transplantation of BMCs

BMCs were obtained from femurs and tibiae of 4-6 wk old C57Bl/6 EGFP transgenic mice. BMC were purified by centrifugation in Ficoll gradient at 1000 *g* for 15 min (Histopaque 1119 and 1077, 1:1; Sigma, St. Louis, MO). After two washings in RPMI medium (Sigma), the mononuclear cell fraction was suspended in saline, filtered over nylon wool and used for therapy. BMC population was analyzed by flow cytometry using the following conjugated antibodies from Becton Dickinson (San Diego, CA, USA): Sca 1-PE/Cy5, CD45-APC, CD44-PE, CD34-PE, CD11b-PE, and CD117-PE. Acquisition and analysis were performed in a FACScalibur flow cytometer (Becton Dickinson). The following percentages were obtained: 96.51 ± 1.32 for GFP^+ cells; 0.11 ± 0.032 for Sca 1^+ cells; 96.35 ± 3.12 for $CD45^+$ cells; 92.72 ± 3.23 for $CD44^+$ cells; 0.02 ± 0.05 for $CD34^+$ cells; 60.22 ± 5.71 for $CD11b^+$ cells; and 0.17 ± 0.04 for $CD117^+$ cells. For therapy, one group of mice received one administration of BMC (3×10^7 cells/ mouse) directly into the left hepatic lobe (intralobular). In the other experiments, mice were treated with BMC (3×10^7 cells/mouse) intravenous (iv, by retroorbital plexus), once a week, during 3 wk. Control mice received injections of saline in similar conditions to the respective experimental groups. Mice were submitted to euthanasia at different times after therapy, under anesthesia with ketamine and xylazine.

Transforming growth factor-beta (TGF-β) assessment

TGF-β levels were measured by enzyme-linked immunosorbent assay (ELISA) in total protein of homogenized liver tissue (50 mg) in 100 μL PBS containing 0.4 mol/L NaCl, 0.05% Tween 20 and protease inhibitors (0.1 mmol/L PMSF, 0.1 mmol/L benzethonium chloride, 10 mmol/L EDTA and 20 KI aprotinin A/100 mL). The samples were centrifuged at 10 000 r/min for 10 min at 4℃ and the supernatant was frozen at -80℃ for later quantification. TGF-β levels were measured using a sandwich ELISA assay following the manufacturers' instructions (R&D Systems, Minneapolis, MN).

Morphological and morphometrical analyses

Mice were perfused under anesthesia through the heart with 50 mL of PBS followed by 200 mL of 16 g/L paraformaldehyde at 4℃. Liver slices were fixed in

Bouin or formalin at 10% and, after paraffin embedding, 5 μm-thick sections were obtained and stained with conventional hematoxylin-eosin or with picrosirius-red for collagen[9]. Quantification of fibrosis was carried out in sections stained with picrosirius-red examined by optical microscopy, in 10 fields per liver in 5-14 mice per group. Images were digitalized using a color digital video camera (CoolSnap, Montreal, Canada) adapted to a BX41 microscope (Olympus, Tokyo, Japan) and analyzed using Image Pro program (version 6.1; Media Cybernetics, San Diego, CA). For morphometric measurements of granuloma area, a total sectional area of 723255 μm^2 per animal was evaluated. All periocular granulomas were included. Albumin granules and OV-6$^+$ cells were quantified in five liver sections of seven or five animals per group (normal, treated and untreated), respectively, by fluorescence microscopy (BX61 Olympus) and analyzed using Image Pro program. Albumin granules were automatically quantified and the number of hepatocytes was estimated manually by nuclear staining in order to determine the average number of albumin granules/cell. All the analyses were done double-blinded.

Immunofluorescence analyses

Five micrometer frozen sections obtained at various times after transplantation were prepared and fixed in 16 g/L cold paraformaldehyde in 0.1 mol/L phosphate buffer. The presence of transplanted GFP$^+$ BMC in the liver tissue was analyzed by direct fluorescence. Oval cells were stained using a biotinylated anti-OV-6 antibody (R&D Systems, Minneapolis, MN) followed by streptavidin Alexa 568 (Molecular Probes, Carlsbad, CA). For albumin visualization and quantification, liver sections were stained using a rabbit anti-human albumin (DAKO, Glostrup, Denmark) followed by anti-rabbit IgG conjugated with Alexa fluor 568 (Molecular Probes). For collagen I quantification, sections were stained with rabbit anti-human collagen (Novatec, Saint Martin La Garenne, France) followed by anti-rabbit IgG conjugated with Alexafluor 568 (Molecular Probes). Nuclei were stained with 4,6-diamidino-2-phenylindole (VectaShield Hard Set mounting medium with DAPI H-1500; Vector Laboratories, Burlingame, CA). The presence of fluorescent cells was determined by observation in a BX61 microscope with epifluorescence system plus grid to enhance the fluorescence resolution (Optigrid, Structured-light Imaging System, Thales Optem inc, Fairport, NY) using appropriate filters (Olympus). Images were captured using a color digital video SPOT flex camera (15.2, 64 Mp, Shifting Pixel, Diagnostic Instruments inc, Sterling Heights, MI).

Fluorescence in situ hybridization (FISH) in paraffin-embedded tissue sections

Liver sections 3-6 μm thick were prepared on glass slides and dried overnight at 37℃. After dewaxing in xylene and rehydration, sections were incubated with sodium thiocyanate solution for 10 min at 80℃, washed in PBS followed by incubation with pepsin solution for 10 min at 37℃. Sections were washed in PBS, post-fixed in paraformaldehyde solution for 2 min, washed in PBS, and dehydrated through graded alcohols before air drying. Detection of Y chromosome was done using Y-paint probe kit (Cambio, Cambridge, UK), according to the manufacturers' instructions. Slides were mounted with coverslips using Vectashield mounting set with DAPI (Vector), and analyzed by fluorescence microscopy.

Polymerase chain reaction (PCR) for GFP

The presence of donor-derived DNA in liver tissue was analyzed 2 mo after BMC transplantation in *S.mansoni*-infected mice. DNA was extracted from liver tissue using a DNA extraction kit (QIAamp® DNA Mini Kit-50, Qiagen, Hilden, Germany) according to the manufacturers' instructions. DNA concentrations were quantified in 1 μL in a spectrophotometer ND-1000 (Nanodrop Technologies, Wilmington, DE). All samples were diluted to 100-200 ng/μL. PCR amplification of GFP cDNA was performed using Taq polymerase (Invitrogen, Carlsbad, CA) using standard procedures and the following primers pair (Invitrogen): forward, 5'-CGTCGCCGTCCAGCTCGACCAG-3', reverse, 5'-CATGGTCCTGCTGGAGTTCGTG-3'.

Statistical analyses

Data were analyzed using Student's *t*-test, Mann Whitney, ANOVA and Newman-Keuls multiple comparison test with the aid of Prism Software (version 3.0, GraphPad Software, San Diego, CA). Differences were considered significant if $P \leqslant 0.05$.

RESULTS

Transplanted GFP$^+$ BMC are found in livers of S.mansoni-infected mice

Mice chronically infected with *S.mansoni* were treated with GFP$^+$ mononuclear BMC by injection into the left hepatic lobe. Liver sections were obtained at different times after transplantation. Bright GFP$^+$ cells were found around periocular granulomas, in the injected lobe, 2 h after cell infusion (Figure 1A to C). After 24 h of injection, GFP$^+$ cells were also found inside granulomas in sections of injected (Figure 1D) as well as in non-injected lobes (not shown). Five days after transplantation, elongated, spindle shape GFP$^+$ cells were mainly found inside granuloma areas (Figure 1E and F). Bright GFP$^+$ cells were not found in sections of BMC-treated mice 1 or 2 mo after transplantation by iv route, although faint fluorescent cells co-expressing albumin were found in the hepatic parenchyma at this later timepoint (Figure 1G). The presence of donor-derived hepatocyte-like cells was confirmed by detection of Y-chromosome by FISH analysis in parenchymatous cells in liver sections of BMC-treated mice (Figure 1H). Furthermore, the presence of GFP DNA was detected by PCR in liver samples of BMC-treated mice 2 mo after therapy (Figure 2).

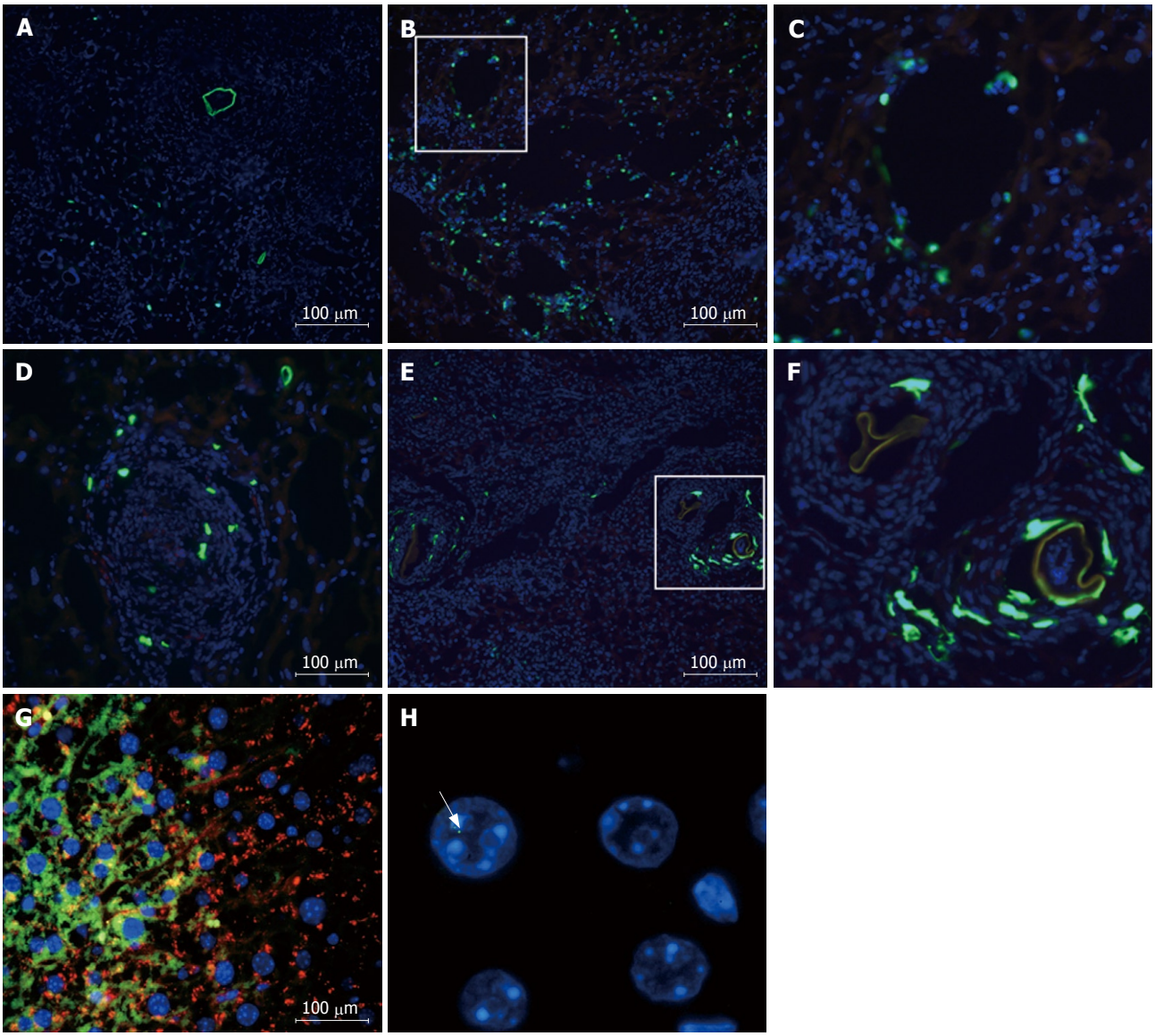


Figure 1 Visualization of donor-derived cells in liver sections of *S.mansoni*-infected mice. Mice chronically infected with *S.mansoni* were treated with GFP$^+$ BMC by injection into the left hepatic lobe (**A**-**F**) or by iv route (**G** and **H**) and sacrificed after different time points for evaluation by fluorescence microscopy. For visualization of GFP$^+$ cells (green), sections were mounted with the nuclear counterstaining with DAPI (blue). **A** and **B**: Sections of injected lobe of mice sacrificed 2 h after transplantation; **C**: Magnification of square area of image **B**. Sections of livers obtained from mice sacrificed 24 h (**D**) or 5 d (**E**) after intralobular BMC injection. **F**: Magnification of square area of image **E**; **G**: GFP$^+$ albumin$^+$ cells found in liver sections 2 mo after iv injection of BMC; **H**: Detection of Y chromosome (arrow) in liver section of BMC-treated mice 1 mo after transplantation by iv route.

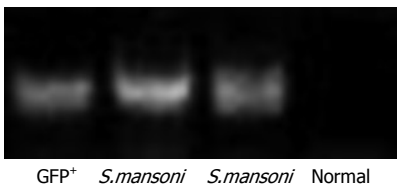


Figure 2 Detection of donor cell DNA in livers of BMC-transplanted mice. *S.mansoni*-infected mice treated with BMC by iv route were sacrificed 2 mo after therapy. Liver fragments were used for DNA extraction and PCR amplification of *GFP* gene. Liver fragments of normal wild-type and GFP mice were used as controls.

Therapy with BMC reduces liver fibrosis induced by S.mansoni infection

Liver sections of *S.mansoni*-infected mice were analyzed, showing a granulomatous inflammatory process in all groups, with periocular granulomas well delimited and homogeneous in shape and cellular infiltrate with predominance of mononuclear cells and with regular and concentric deposits of collagen fibers (Figure 3A to C). After treatment with BMC, we observed a reduction of fibrosis (Figure 3B and D), compared to saline-treated animals. In animals treated by intralobular injection of BMC, a statistically significant reduction of fibrosis was observed in treated lobes 2 mo after treatment (Figure 4A). In addition, a reduction of fibrosis was also found in sections of untreated lobes (% of fibrosis in untreated lobes: 14.1 ± 0.7 compared to livers of saline-treated mice: 17.9 ± 0.7; $P < 0.001$). Fibrosis reduction was also observed when BMC were injected by iv route (Figure 4B). Morphometric evaluation of granuloma area showed a significant reduction after

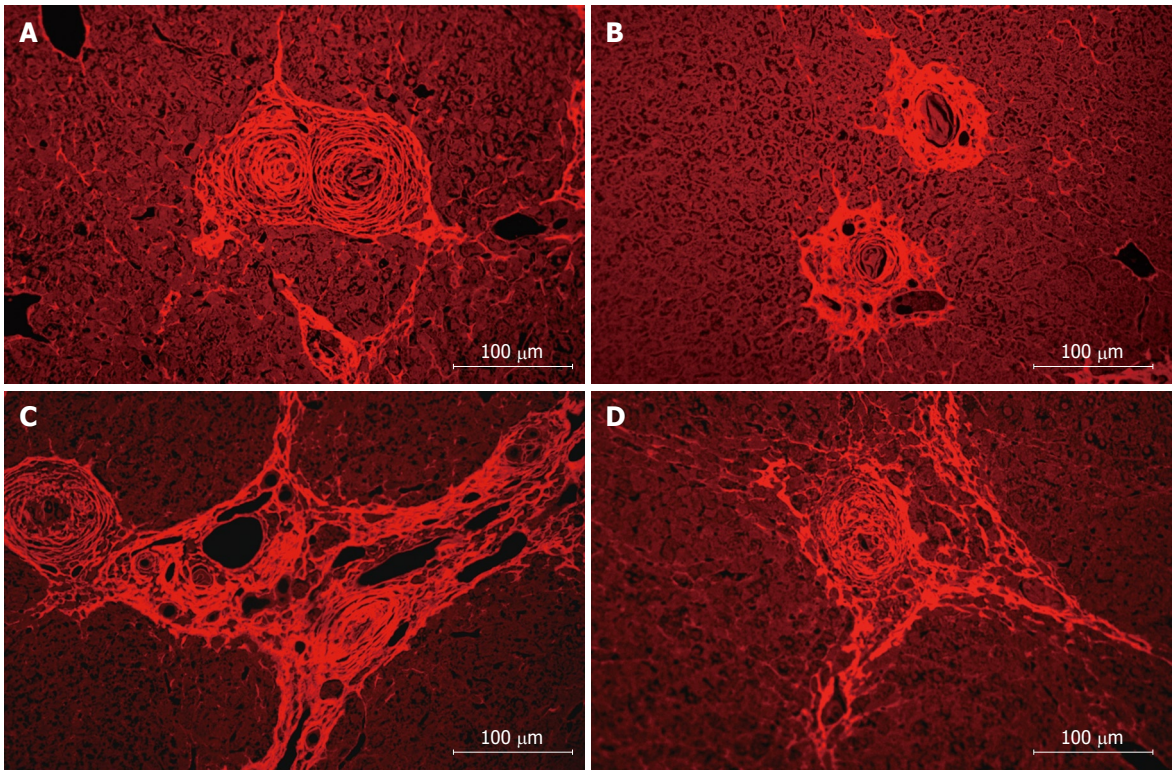


Figure 3 Alterations in liver fibrosis after BMC therapy in *S.mansoni*-infected mice. The morphological aspects of liver sections were detected by fluorescence analyses. Representative images of liver sections stained by Sirius red-Fast green of *S.mansoni*-infected mice 2 mo after saline (**A** and **B**) or BMC (**C** and **D**) treatment by iv route.

therapy in animals treated with BMC by intralobular (Figure 4C) as well as by iv injection (Figure 4D). This reduction was confirmed in sections stained with anti-collagen I antibodies, by morphometry (saline-treated: 7.14% ± 0.45% compared to BMC-treated: 4.20% ± 0.25%; $P < 0.0004$).

TGF-β levels in liver fragments were assessed 2 mo after BMC therapy by iv route. A significant decrease of TGF-β levels was observed in livers of BMC-treated mice compared to saline-treated *S.mansoni*-infected mice. The levels of TGF-β in BMC-treated mice were similar to those of normal mice (Figure 5).

Increase in oval cell numbers after BMC transplantation in S.mansoni-infected mice

The presence of oval cells in liver sections of normal, *S.mansoni*-infected mice treated or not with BMC was evaluated by immunofluorescence using an anti-OV-6 antibody. Sections of normal livers had none or few OV-6$^+$ cells (Figure 6A). Infection with *S.mansoni* caused an increase in the number of OV-6$^+$ cells, which were mainly found in zone 1 areas of the hepatic lobe in saline-treated (Figure 6B), as well as in BMC-treated animals (Figure 4B) 2 mo after iv route. The number of OV-6$^+$ cells, however, was 3-4 times higher in sections of BMC-treated mice (Figure 6C; $P = 0.0027$).

BMC treatment increases albumin production in S.mansoni-infected mice

Immunostaining for albumin in liver sections of mice chronically infected with *S.mansoni* showed an abnormal pattern of albumin expression. A bright intracytoplasmic granular staining is found in sections of uninfected mice (Figure 7A), whereas liver sections of untreated *S.mansoni*-infected mice had few and abnormal albumin granules, preferentially located in the periphery of hepatocytes (Figure 7B). Liver sections of BMC-treated mice also had an altered albumin staining pattern, although more intense than untreated mice (Figure 7C). When the number of albumin$^+$ granules was evaluated, a significant difference between uninfected and infected mice was found, showing a 3-fold higher number of granules in normal mice compared to saline-treated infected mice (Figure 7D; $P < 0.001$). BMC therapy caused a significant increase in the number of granules, compared to saline-treated, *S.mansoni*-infected mice ($P < 0.05$), although the levels where still below those of normal livers (Figure 7D; $P < 0.001$).

DISCUSSION

Cellular therapy for liver regeneration has been intensively investigated mainly in models of hepatic diseases caused by drug administration, surgical interventions and by genetic disorders[10-13]. A number of reports have demonstrated that transplantation of BMCs improves liver function, decreases fibrosis and contributes to parenchyma regeneration[11,14,15]. In this report, we describe for the first time the effects of BMC therapy in a model of chronic liver disease caused by a parasitic infection.

The recruitment of BMCs to liver lesions has

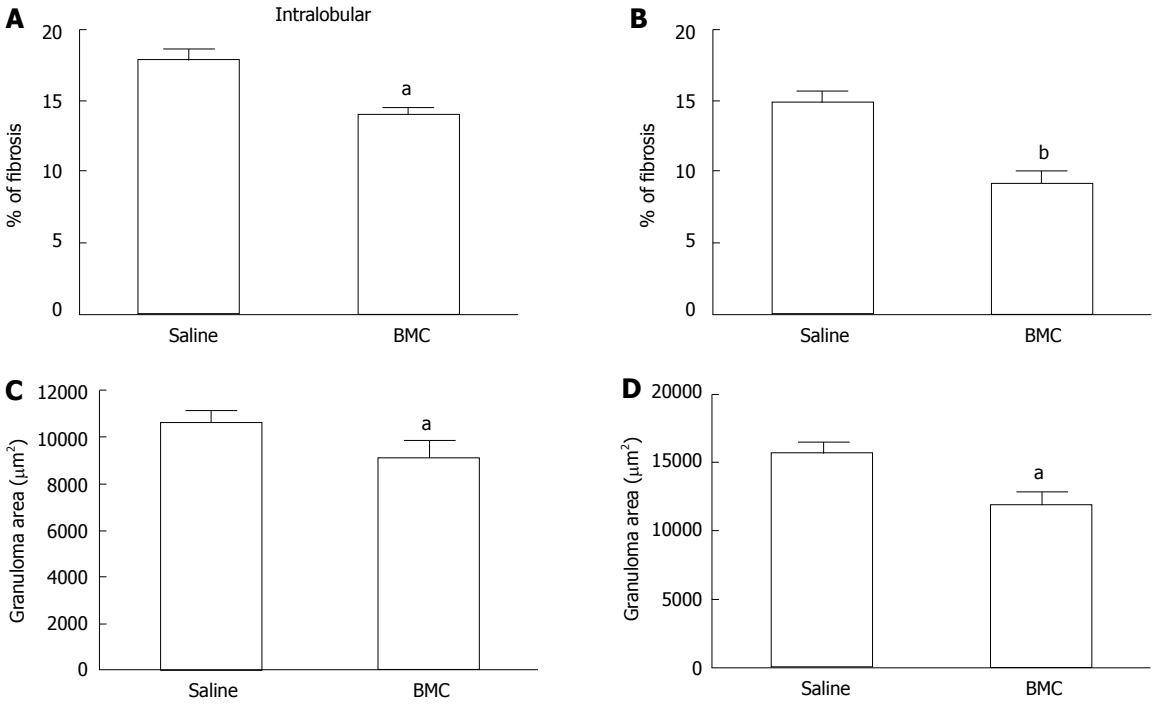


Figure 4 Reduction of fibrosis and granuloma area after BMC therapy. Mice infected with *S.mansoni* and treated with saline or BMC by intralobular (**A** and **C**) or iv **B** and **D** routes were sacrificed 2 mo after treatment. Liver sections sampled randomly and stained with Sirius red-Fast green were examined by optical microscopy. Images were digitalized and analyzed by morphometry. The percentage of fibrosis (**A** and **B**) and the area of periocular granulomas (**C** and **D**) were evaluated. Data were represented graphically as mean ± SE of 5-14 animals per group. $^{a}P < 0.05$ and $^{b}P < 0.01$ compared to saline-treated group.

Figure 5 BMC therapy decreases the levels of TGF-β in the liver. TGF-β levels were assessed in fragments of hepatic tissue of uninfected, saline-treated or BMC-treated *S. mansoni*-infected mice 2 mo after BMC infusion by iv route, by sandwich ELISA. Data represents the mean ± SE of 4-8 animals per group. $^{a}P < 0.05$ compared to uninfected group; $^{b}P < 0.01$ compared to saline-treated group.

been well documented in several models of liver diseases[10-12,14]. In our study, we also found that liver injury caused by *S.mansoni* infection elicits the migration of BMCs, which were able to reach the granuloma areas. Many of the cells observed in the liver sections are probably leukocytes (found in the mononuclear cell fraction used in the cellular therapy), that are chemoattracted by the intense inflammatory reaction around periocular granulomas. Most GFP^{+} cells that migrated inside the granulomas, however, are larger than leukocytes and could be spindle-shaped like fibroblasts or myofibroblasts. Both of these cells are well known to participate in the fibrogenesis associated with *S.mansoni* infection and also to take part in collagen degradation during fibrosis modulation[16-18]. The differentiation of BMCs into myofibroblasts and/or fibroblasts may be an important step in the modulation of fibrosis observed in the present study. Recently, it has been shown that a significant number of myofibroblasts in human hepatic fibrosis are of bone marrow origin[19].

In latter time points, cells expressing weak GFP fluorescence co-expressing albumin were observed. The presence of transplanted BMC-derived parenchymatous cells was confirmed by observation of donor DNA by PCR analysis (for detection of GFP DNA) and FISH (for detection of Y chromosomes in nuclei). These results reinforce a contribution of BMCs in the reconstitution of liver parenchyma, as described before in other models of liver diseases[20,21]. In our study, we found few hepatocyte-like cells bearing Y chromosome (1-2 cells/section). In addition, we did not find binucleated cells with Y chromosome in one nucleus. Although we cannot rule out that fusion of transplanted BMC with resident hepatocytes occurs, these findings indicate that the few hepatocyte-like cells derived from transplanted BMC observed in our model are generated by transdifferentiation. In addition to the population of stem cells, the potential of transdifferentiation into hepatocytes has been recently extended to another cell type present in the bone marrow, the monocytes[22]. Thus, it is possible that more than one cell type present in the cell preparation used in our study take part in the generation of hepatocyte-like cells. It is also likely that the transplanted cells take part in the immune regulation of the liver[23], causing the modulation of fibrosis,

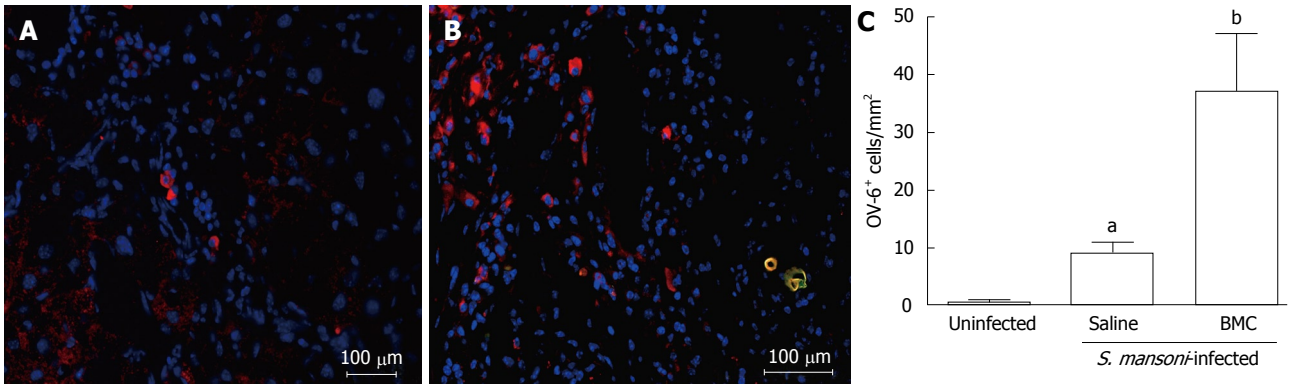


Figure 6 Increased numbers of oval cells after BMC therapy. Oval cells (OV-6$^+$ stained in red) in sections of saline (**A**) or BMC (**B**) treated mice 2 mo after transplantation by iv route. **C**: The number of oval cells in liver sections of uninfected, saline-treated or BMC-treated *S. mansoni*-infected mice 2 mo after cellular infusion by iv route was determined by immunofluorescence analysis using an anti-OV-6 antibody. Data represents the mean ± SE of 5-6 animals per group. $^aP < 0.05$ compared to uninfected group; $^bP < 0.001$ compared to saline-treated group.

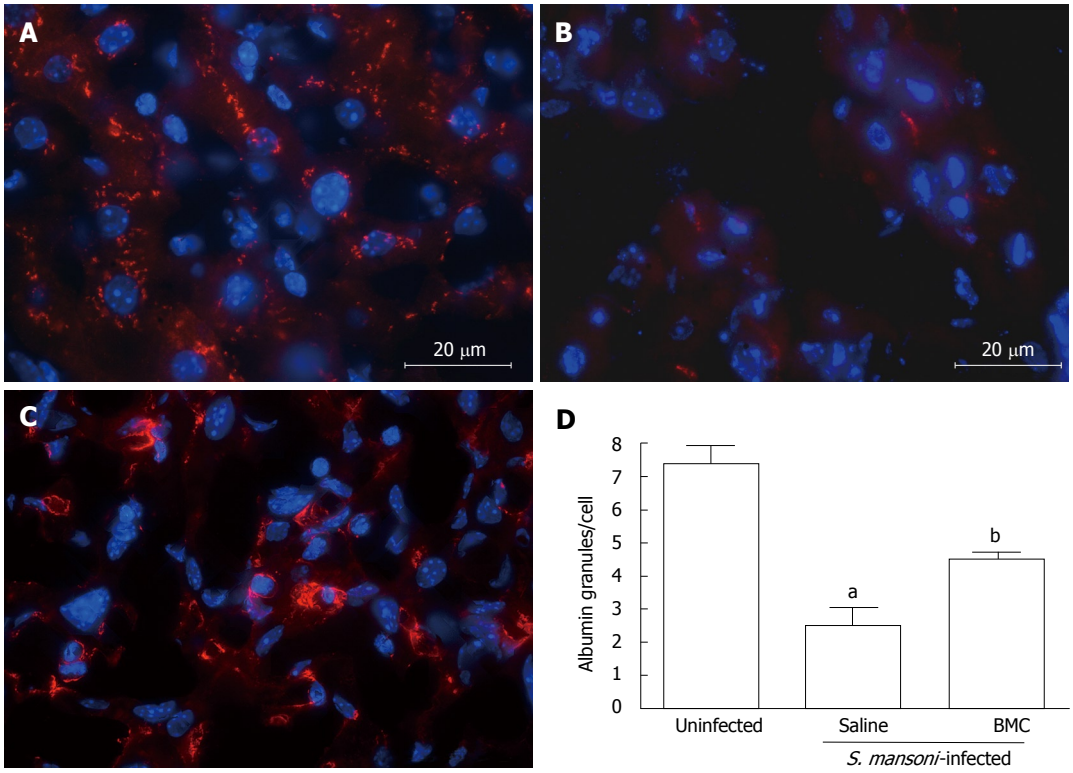


Figure 7 Increased albumin expression after BMC therapy. Albumin (red) and nuclei (blue) staining in sections of uninfected (**A**), saline-treated (**B**) or BMC-treated (**C**), *S.mansoni*-infected mice, 2 mo after treatment by iv route. **D**: Albumin$^+$ granules were quantified by morphometry in liver sections of uninfected, saline-treated or BMC-treated *S.mansoni*-infected mice 2 mo after cellular infusion by iv route. Data represents the mean ± SE of 7 animals per group. $^aP < 0.05$ compared to uninfected group; $^bP < 0.001$ compared to saline-treated group.

cytokine production and albumin synthesis observed in our study.

Fibrosis is a common feature of chronic liver diseases. The increased deposition of extracellular matrix causes structural alterations in the liver and in its function, and portal hypertension due to the obstruction of vessels and focal ischemic lesions. Although spontaneous regression of fibrosis occurs when the stimulus for hepatic liver damage is removed, in diseases such as viral hepatitis the stimulus cannot be completely removed, in addition to being a slow process[24]. Therefore, means to decrease liver fibrosis are extremely relevant to improve liver function and to decrease complications related to chronic hepatopathies. In this regard, schistosomiasis is an interesting model of chronic fibrotic liver disease. In our study, we observed a significant decrease of liver fibrosis after cellular therapy, as demonstrated by morphometrical analysis of granuloma area and total liver fibrosis. This was achieved both by intralobular administration as well as by iv injection, indicating that fibrosis does not hamper the influx of cells to the liver of *S.mansoni*-infected mice. In a recent report, Higashiyama *et al* (2007), demonstrated that bone marrow-derived cells which migrated to

fibrotic livers express matrix metalloproteinases. Thus, the transplanted cells found in the hepatic parenchyma of *S.mansoni*-infected mice may be acting directly to increase the degradation of extracellular matrix components.

One of the main mediators involved on fibrosis deposition during hepatic injury is TGF-β[25]. This cytokine stimulates the transition of stellate cells to myofibroblasts, which secrete high amounts of extracellular matrix and inhibit its degradation[26]. TGF-β levels were lowered after BMC therapy in livers of *S.mansoni*-infected mice, reaching levels close to those found in normal mice. A decrease in TGF-β production was also observed by Fang *et al*[14] after therapy using bone marrow-derived mesenchymal stem cells in mice with liver injury caused by CCl_4 administration.

Oval cells are hepatic precursors of hepatocytes and bile duct cells[27]. An increase in oval cell numbers was found in liver sections of *S.mansoni*-infected mice, compared to those of normal mice. Transplantation of BMC, however, caused a 3-4 fold increase in oval cell numbers in infected mice. This may be explained by the fact that oval cells can be originated from BMCs[4]. Alternatively, the effects of BMC injection on oval cell numbers may be due to their migration to and action in other organs, such as the bone marrow. It is possible that the increase in oval cells contribute to the replacement of areas where fibrosis degradation occurs with functional parenchymatous cells after BMC therapy.

Although alterations in albumin levels in *S.mansoni*-infected individuals are rare, this can be observed in some cases. A study by Cook *et al*[28] reported hypoalbuminemia in *S.mansoni*-infected children, which suggests that this biochemical alterations associated with undernourishment or to effects of repeated digestive hemorrhages. In contrast, chronic infection with *S.mansoni* causes hypoalbuminemia in mice. This phenomenon occurs simultaneously to the increase in collagen deposition and could be associated with the decrease in albumin mRNA found in *S.mansoni*-infected mice[29]. In our study, we also observed a marked alteration of albumin expression in hepatocytes of infected mice, compared to normal controls. BMC therapy significantly increased the expression of albumin in livers of *S.mansoni*-infected mice, although the pattern was still altered in comparison to that of normal livers. An increase in albumin levels was also found after cell therapy in a model of CCl_4-induced lesion in rats[30].

In conclusion, transplantation of BMCs in mice with chronic liver disease caused by *S.mansoni* infection decreased liver fibrosis and contributed to an increase in precursor cells as well as to the generation of new hepatocytes and/or to the improvement of the function of resident hepatocytes. Although there are still many unanswered questions regarding the mechanisms of action of transplanted cells in hepatic lesions, our results reinforce the use of cell-based therapies for patients with chronic liver diseases.

ACKNOWLEDGMENTS

The authors thank Carine Machado Azevedo and Juliana F. Vasconcelos for technical assistance.

COMMENTS

Background

Liver fibrosis occurs in the setting of chronic injury caused by different etiologies, constituting a serious worldwide public health problem. Chronic infection by *S.mansoni* is one of the experimental models of hepatic fibrosis used to elucidate the mechanisms involved in the fibrogenic processes.

Research frontiers

New therapeutic strategies aimed at minimizing damage caused by hepatic fibrogenesis in chronic liver diseases are of great interest. Adult bone marrow contains pluripotent stem cells with the ability to differentiate into diverse cell types, including hepatocytes, in situations under tissue stress in mice and human.

Innovations and breakthroughs

A number of reports have demonstrated that transplantation of bone marrow cells (BMCs) improves liver function, decreases fibrosis and contributes to parenchyma regeneration. In this report, described for the first time, are the effects of BMC therapy in a model of chronic liver disease caused by a parasitic infection.

Applications

Fibrosis is a common feature of chronic liver diseases. The increased deposition of extracellular matrix causes structural alterations in the liver and causes portal hypertension due to the obstruction of vessels and focal ischemic lesions. Therefore, means to decrease liver fibrosis are extremely relevant to improve liver function and to decrease complications related to chronic hepatopathies. In this study, the authors observed a significant decrease in liver fibrosis, an increase in precursor cells, and the generation of new hepatocytes after therapy with BMCs.

Peer review

Authors investigated the putative role in hepatic regenerative medicine by bone marrow mononuclear cells (BM-MC) in the experimental model of *S.mansoni* chronic infection. The experimental animal model used is appropriate and the study is in principle of interest.

REFERENCES

1 **Andrade ZA**, Cheever AW. Characterization of the murine model of schistosomal hepatic periportal fibrosis ('pipestem' fibrosis). *Int J Exp Pathol* 1993; **74**: 195-202
2 **Bataller R**, Brenner DA. Liver fibrosis. *J Clin Invest* 2005; **115**: 209-218
3 **Krause DS**, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R, Neutzel S, Sharkis SJ. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* 2001; **105**: 369-377
4 **Petersen BE**, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, Boggs SS, Greenberger JS, Goff JP. Bone marrow as a potential source of hepatic oval cells. *Science* 1999; **284**: 1168-1170
5 **Alison MR**, Poulsom R, Jeffery R, Dhillon AP, Quaglia A, Jacob J, Novelli M, Prentice G, Williamson J, Wright NA. Hepatocytes from non-hepatic adult stem cells. *Nature* 2000; **406**: 257
6 **Braun KM**, Sandgren EP. Cellular origin of regenerating parenchyma in a mouse model of severe hepatic injury. *Am J Pathol* 2000; **157**: 561-569
7 **Lagasse E**, Connors H, Al-Dhalimy M, Reitsma M, Dohse M, Osborne L, Wang X, Finegold M, Weissman IL, Grompe M. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. *Nat Med* 2000; **6**: 1229-1234
8 **Andrade ZA**, Sadigursky M. [A comparative study of the Feira de Santana (Bahia) and Porto Rico strains of Schistosoma mansoni in experimental infection of mice] *Mem Inst Oswaldo Cruz* 1985; **80**: 37-40

9 **Junqueira LC**, Bignolas G, Brentani RR. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochem J* 1979; **11**: 447-455

10 **Jang YY**, Collector MI, Baylin SB, Diehl AM, Sharkis SJ. Hematopoietic stem cells convert into liver cells within days without fusion. *Nat Cell Biol* 2004; **6**: 532-539

11 **Sakaida I**, Terai S, Yamamoto N, Aoyama K, Ishikawa T, Nishina H, Okita K. Transplantation of bone marrow cells reduces CCl4-induced liver fibrosis in mice. *Hepatology* 2004; **40**: 1304-1311

12 **Fujii H**, Hirose T, Oe S, Yasuchika K, Azuma H, Fujikawa T, Nagao M, Yamaoka Y. Contribution of bone marrow cells to liver regeneration after partial hepatectomy in mice. *J Hepatol* 2002; **36**: 653-659

13 **Kallis YN**, Alison MR, Forbes SJ. Bone marrow stem cells and liver disease. *Gut* 2007; **56**: 716-724

14 **Fang B**, Shi M, Liao L, Yang S, Liu Y, Zhao RC. Systemic infusion of FLK1(+) mesenchymal stem cells ameliorate carbon tetrachloride-induced liver fibrosis in mice. *Transplantation* 2004; **78**: 83-88

15 **Zhao DC**, Lei JX, Chen R, Yu WH, Zhang XM, Li SN, Xiang P. Bone marrow-derived mesenchymal stem cells protect against experimental liver fibrosis in rats. *World J Gastroenterol* 2005; **11**: 3431-3440

16 **de Freitas LA**, Chevallier M, Louis D, Grimaud JA. Human extrahepatic biliary atresia: portal connective tissue activation related to ductular proliferation. *Liver* 1986; **6**: 253-261

17 **Grimaud JA**, Borojevic R. Portal fibrosis: intrahepatic portal vein pathology in chronic human schistosomiasis mansoni. *J Submicrosc Cytol* 1986; **18**: 783-793

18 **de Freitas LA**, Grimaud JA, Chevallier M, Andrade ZA. Morphological aspects of early and late collagen degradation in granulation tissue. *Exp Toxicol Pathol* 1992; **44**: 128-133

19 **Forbes SJ**, Russo FP, Rey V, Burra P, Rugge M, Wright NA, Alison MR. A significant proportion of myofibroblasts are of bone marrow origin in human liver fibrosis. *Gastroenterology* 2004; **126**: 955-963

20 **Kashofer K**, Siapati EK, Bonnet D. In vivo formation of unstable heterokaryons after liver damage and hematopoietic stem cell/progenitor transplantation. *Stem Cells* 2006; **24**: 1104-1112

21 **Higashiyama R**, Inagaki Y, Hong YY, Kushida M, Nakao S, Niioka M, Watanabe T, Okano H, Matsuzaki Y, Shiota G, Okazaki I. Bone marrow-derived cells express matrix metalloproteinases and contribute to regression of liver fibrosis in mice. *Hepatology* 2007; **45**: 213-222

22 **Yan L**, Han Y, Wang J, Liu J, Hong L, Fan D. Peripheral blood monocytes from patients with HBV related decompensated liver cirrhosis can differentiate into functional hepatocytes. *Am J Hematol* 2007; **82**: 949-954

23 **Chen X**, Armstrong MA, Li G. Mesenchymal stem cells in immunoregulation. *Immunol Cell Biol* 2006; **84**: 413-421

24 **Kumar M**, Sarin SK. Is cirrhosis of the liver reversible? *Indian J Pediatr* 2007; **74**: 393-399

25 **Gressner AM**, Weiskirchen R, Breitkopf K, Dooley S. Roles of TGF-beta in hepatic fibrosis. *Front Biosci* 2002; **7**: d793-d807

26 **Gressner AM**. Transdifferentiation of hepatic stellate cells (Ito cells) to myofibroblasts: a key event in hepatic fibrogenesis. *Kidney Int Suppl* 1996; **54**: S39-S45

27 **Fausto N**, Campbell JS. The role of hepatocytes and oval cells in liver regeneration and repopulation. *Mech Dev* 2003; **120**: 117-130

28 **Cook JA**, Baker ST, Warren KS, Jordan P. A controlled study of morbidity of schistosomiasis mansoni in St. Lucian children, based on quantitative egg excretion. *Am J Trop Med Hyg* 1974; **23**: 625-633

29 **Saber MA**, Shafritz DA, Zern MA. Changes in collagen and albumin mRNA in liver tissue of mice infected with Schistosoma mansoni as determined by in situ hybridization. *J Cell Biol* 1983; **97**: 986-992

30 **Oyagi S**, Hirose M, Kojima M, Okuyama M, Kawase M, Nakamura T, Ohgushi H, Yagi K. Therapeutic effect of transplanting HGF-treated bone marrow mesenchymal cells into CCl4-injured rats. *J Hepatol* 2006; **44**: 742-748

S- Editor Li DL **L- Editor** Rippe RA **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5851

World J Gastroenterol 2008 October 14; 14(38): 5851-5856
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

FR167653, a p38 mitogen-activated protein kinase inhibitor, aggravates experimental colitis in mice

Takashi Nishimura, Akira Andoh, Atsushi Nishida, Makoto Shioya, Yuhsuke Koizumi, Tomoyuki Tsujikawa, Yoshihide Fujiyama

Takashi Nishimura, Akira Andoh, Atsushi Nishida, Makoto Shioya, Yuhsuke Koizumi, Tomoyuki Tsujikawa, Yoshihide Fujiyama, Department of Medicine, Shiga University of Medical Science, Seta-Tsukinowa, Otsu 520-2192, Japan
Author contributions: Nishimura T performed research; Andoh A wrote the paper; Nishida A, Shioya M, Koizumi Y, Tsujikawa T, and Fujiyama Y designed research.
Correspondence to: Akira Andoh, MD, Department of Medicine, Shiga University of Medical Science, Seta-Tsukinowa, Otsu 520-2192, Japan. andoh@belle.shiga-med.ac.jp
Telephone: +81-77-5482217 Fax: +81-77-5482219
Received: April 21, 2008 Revised: July 5, 2008
Accepted: July 12, 2008
Published online: October 14, 2008

Key words: p38; Inflammatory bowel disease; Cytokine; Experimental colitis; Tumor necrosis factor-α

Peer reviewer: Peter Mannon, MD, NIH, 10-CRC/6-3742, 9000 Rockville Pike, Bethesda 20892, United States

Nishimura T, Andoh A, Nishida A, Shioya M, Koizumi Y, Tsujikawa T, Fujiyama Y. FR167653, a p38 mitogen-activated protein kinase inhibitor, aggravates experimental colitis in mice. *World J Gastroenterol* 2008; 14(38): 5851-5856 Available from: URL: http://www.wjgnet.com/1007-9327/14/5851.asp DOI: http://dx.doi.org/10.3748/wjg.14.5851

Abstract

AIM: To investigate the effects of FR167653 on the development of dextran sulfate sodium (DSS)-induced colitis in mice.

METHODS: BALB/c mice were fed rodent chow containing 3.5% (wt/wt) DSS. The recipient mice underwent intra-peritoneal injection of vehicles or FR167653 (30 mg/kg per day). The mice were sacrificed on day 14, and the degree of colitis was assessed. Immunohistochemical analyses for $CD4^+$ T cell and $F4/80^+$ macrophage infiltration were also performed. Mucosal cytokine expression was analyzed by RT-PCR.

RESULTS: The body weight loss was more apparent in the FR167653-treated DSS mice than in the vehicle-treated DSS mice. The colon length was shorter in the FR167653-treated DSS mice than in the vehicle-treated DSS mice. Disease activity index and histological colitis score were significantly higher in FR167653- than in vehicle-treated DSS animals. Microscopically, mucosal edema, cellular infiltration (CD4 T cells and F4/80 macrophages), and the disruption of the epithelium were much more severe in FR167653-treated mice than in controls. Mucosal mRNA expression for interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) were found to be markedly reduced in FR167653-treated DSS mice.

CONCLUSION: Treatment with FR167653 aggravated DSS colitis in mice. This effect was accompanied by a reduction of mucosal IL-1β and TNF-α expression, suggesting a role of p38 mitogen-activated protein kinase (MAPK)-mediated proinflammatory cytokine induction in host defense mechanisms.

INTRODUCTION

Inflammatory bowel diseases (IBD), such as ulcerative colitis (UC) and Crohn's disease (CD), are associated with chronic relapsing inflammation of the intestinal tract of unknown etiology. The most widely held hypothesis on the pathogenesis of IBD is that the mucosal immune system shows an aberrant response towards luminal antigens such as dietary factors and/or commensal bacteria in genetically susceptible individuals[1-3]. Histologically, mucosal accumulation of leukocytes is a characteristic feature of IBD, and the activation of T cells and monocytes/macrophages has been regarded as a crucial factor in its pathogenesis[1,2].

Previous studies have clearly demonstrated that bacterial lipopolysaccharides (LPS) and pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), activate the p38 mitogen-activated protein kinase (MAPK) pathway[4-6] and suggested that the p38 MAPK family play important roles in pathophysiology of IBD[1,7-9]. The p38 is a member of the MAPK family, which is ubiquitously expressed. Serine-threonine kinases are playing important roles in various signal transduction pathways in mammalian cells[4]. It has been demonstrated that p38 mediates phosphorylation of transcription factors, thereby regulating gene expression and the induction of cytokine production[6]. p38 is activated by dual phosphorylation on Thr180 and Tyr182 by the upstream MAPKs MKK3 and MKK6. MKK3 is especially

important for TNF-α-induced p38 activation[10] and for the p38-mediated synthesis of IL-12 and IFN-γ[11]. Thus, p38 is a promising candidate for targeted inhibition in acute and chronic inflammation.

FR167653 is a well-characterized inhibitor of p38 MAPK[12-16]. FR167653 specifically inhibits p38 *via* its ability to compete with adenosine triphosphate at adenosine triphosphate binding sites on p38 kinase[17,18], and such pharmacological actions resemble those of pyridinyl imidazole inhibitors of p38 MAPK such as SB 203580 and RWJ6. This compound dramatically and selectively attenuates the activity of p38, but does not significantly modulate JNK and ERK-1/2 activity[19]. FR167653 has been demonstrated to specifically inhibit p38 function and eventual IL-1β and TNF-α protein translation[13,20], but the expression of IL-6 and transforming growth factor (TGF)-β does not appear to be affected[21]. At present, FR167653 has not been studied in any animal models for IBD, and our knowledge of its action in chronic intestinal injury models is limited. In this study, we investigated the effects of FR167653 on the development of dextran sulfate sodium (DSS)-induced colitis in mice.

MATERIALS AND METHODS

Induction of colitis

Six- to eight-week-old male BALB/c mice were purchased from Charles River Japan (Kanagawa, Japan). They were acclimatized for 1 wk before the experiment, and were housed individually in a room maintained at 22℃ under a 12-h day/night cycle throughout the experiments. Mice were fed 3.5% (wt/wt) DSS (molecular weight 5000; Wako Pure Chemical Industries, Ltd, Osaka, Japan) mixed with normal chow (MF; Oriental Yeast Co., Ltd, Tokyo, Japan) and water *ad libitum*. The study protocol was approved by the Animal Care and Use Committee of the Shiga University of Medical Science (Otsu, Japan).

FR167653 was kindly provided by Fujisawa Pharmaceutical (Osaka, Japan). The recipient mice underwent intra-peritoneal injection of vehicles or FR167653 at a dose of 30 mg/kg per day dissolved in 200 μL phosphate-buffered saline (PBS) every day, starting on the first day of DSS administration. The dose of FR167653 was chosen with reference to publications showing *in vivo* effects of the drug[12,19].

Assessment of inflammation in DSS-induced colitis

Daily clinical assessment of DSS-induced colitis was performed, including measurements of food intake and body weight, an evaluation of stool consistency, and the presence of blood in the stools by a guaiac paper test. A validated clinical disease activity index ranging from 0 to 4 was calculated using the following parameters: stool consistency, presence of fecal blood, and changes in body weight[22]. The mice were sacrificed at day 14, and the length and weight of the colons were measured.

Histology

A histological examination was performed on three samples of the distal colon from each animal. The samples were fixed in 10% buffered formalin, dehydrated in ethanol, and then embedded in paraffin. Four micron-thick sections were then prepared and stained with hematoxylin and eosin. All histologic evaluation was performed in a blinded fashion using a validated scoring system[23].

For immunohistochemical staining, freshly isolated tissue from the distal portion of the colon was frozen in dry ice using OCT compound (Sakura Finetek, Tokyo, Japan). Acetone-fixed frozen sections (6 μm) were blocked with DAKO blocking reagent (#X0909, DAKO Japan, Kyoto, Japan) followed by incubation with the primary antibodies [anti-mouse CD4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-mouse F4/80 (BD Pharmingen, San Diego, CA)], diluted 1:100 in PBS containing 5% skim milk overnight at 4℃ in a humidified chamber. After incubation with the primary antibody, the sections were reacted with 0.1% H_2O_2 in 0.1 mol/L PBS, and treated with biotin-conjugated second antibodies (diluted 1:50 in PBS containing 1% skim milk; Vector, Burlingame, CA) for 60 min at room temperature and followed by avidin-biotin-peroxidase complexes (ABC, Vector). The peroxidase activity was visualized using diaminobenzidine.

Reverse transcription (RT-) PCR

Cytokine mRNA expression in the mucosa was evaluated by RT-PCR. Total cellular RNA was isolated by the acid guanidium thiocyanate-phenol-chloroform (AGPC) method[24]. For each sample, the first-strand cDNA was synthesized using 0.5 μg of total cellular RNA with oligo (dT) primer and Superscript reverse transcriptase (GIBCO BRL, Rockville, MD). One μL of the cDNA sample was amplified in a 25 μL reaction containing 10 × Taq buffer (Perkin Elmer Cetus Corp., Norwalk, CT), 1.5 mmol/L $MgCl_2$, 0.1 μmol/L of each 5' and 3' primers, and 1 U of *TaqGold* polymerase (Perkin-Elmer Cetus). The PCR was performed in a thermal cycler (GeneAmp Model 2400; Perkin-Elmer Cetus) for 25 cycles (94℃ for 30 s, 55℃ for 30 s, and 72℃ for 40 s), followed by a 8 min extension at 72℃. Five μL of the PCR products were subjected to electrophoresis on 1.5% agarose gels and stained with 0.5 μg/mL ethidium bromide. A 100-bp DNA ladder (GIBCO BRL) was used as marker. Primers specific for the mouse IL-1β were purchased from BioSorce International, Inc. (Camarillo, CA). Specific primers for mouse TNF-α were constructed according to published sequence data[25] (5'-GCGACGTGGAACTGGCAGAAG-3', and 5'-GGTACAACCCATCGGCTGGCA-3').

Statistical analysis

Statistical analysis was performed using one-way ANOVA with Scheffe's post hoc test or the Kruskal-Wallis test when appropriate. Two-way ANOVA for

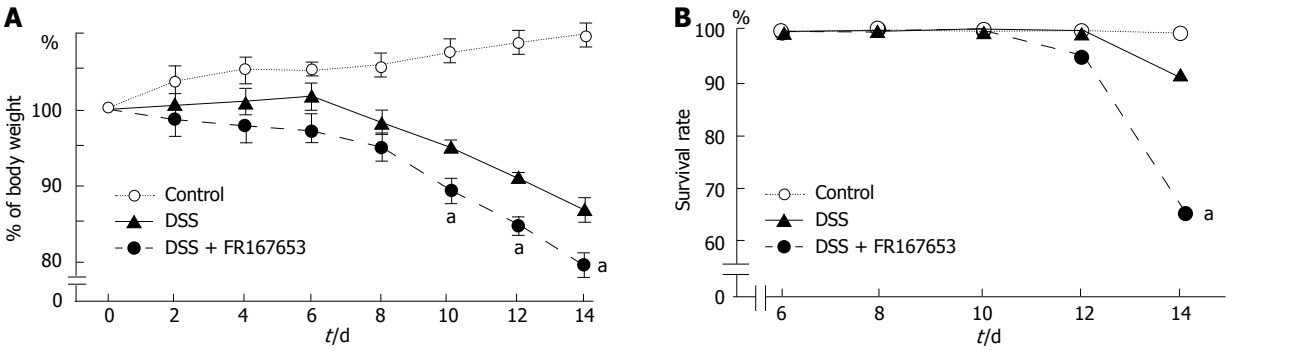


Figure 1 Changes in body weight (**A**) and survival rate (**B**). Mice were fed DSS and injected intraperitoneally with vehicle (PBS) or FR167653 (30 mg/kg per day) daily starting at d 0. The weight of individual mice was followed daily. Data represent mean ± SE (n = 8 mice/group). [a]$P < 0.05$, FR167653 *vs* vehicle (PBS).

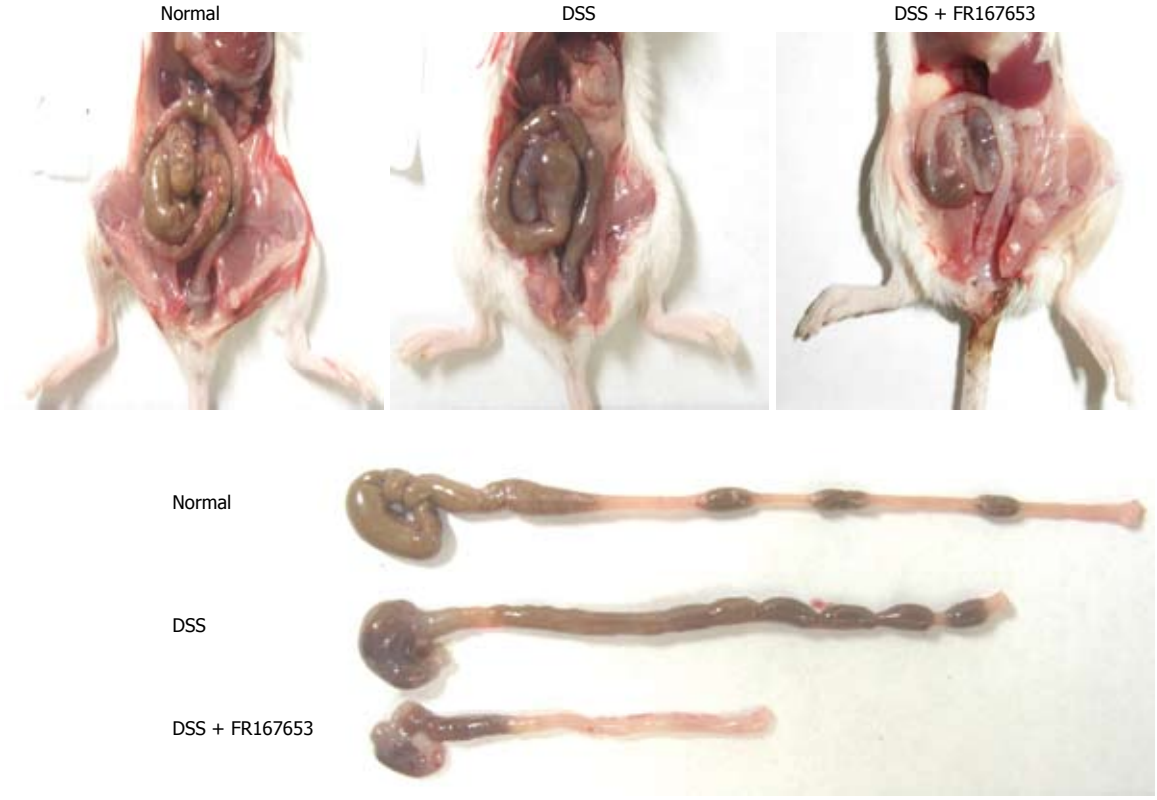


Figure 2 Pictures of laparotomy (upper part) and resected colon (lower part). On day 14, in the FR167653-treated DSS mice stool softening and colon shortening were remarkable, as compared to the vehicle (PBS)-treated DSS mice.

repeated measures was used to test for group and time effects on clinical data (*e.g.* disease activity index) over 7 successive days of clinical observation. $P < 0.05$ was considered to be statistically significant.

RESULTS

To evaluate the effect of FR167653 on DSS colitis, the administration of FR167653 was started at the time of DSS exposure. The administration of FR167653 and vehicle (PBS) was repeated every 24 h. As shown in Figure 1A, on day 10, day 12, and day 14 after the initiation of DSS-induced colitis, the body weight was significantly lower in the FR167653-treated than in the PBS-treated mice. On day 14, survival rate was significantly lower in FR167653- than in the PBS-treated DSS animals (Figure 1B). As shown in Figure 2, colon shortening and stool softening were apparent in the FR167653-treated DSS mice (Figure 2). The total colon length was significantly smaller in the FR167653-treated than in the PBS-treated DSS mice (Figure 3A).

A macroscopic examination of the colon revealed that hyperemia, erosions, and occasional tiny blood coagula occurred mainly in the rectum in both DSS-treated groups. Disease parameters such as the fecal blood score, diarrhea score, and disease activity index were significantly higher in the FR167653-treated than the PBS-treated DSS mice (Figure 3B).

DSS colitis is characterized by histological findings such as edema, infiltration of inflammatory cells into the mucosa and submucosa, ulceration, and mucosal thickening. Our histological analysis indicated that

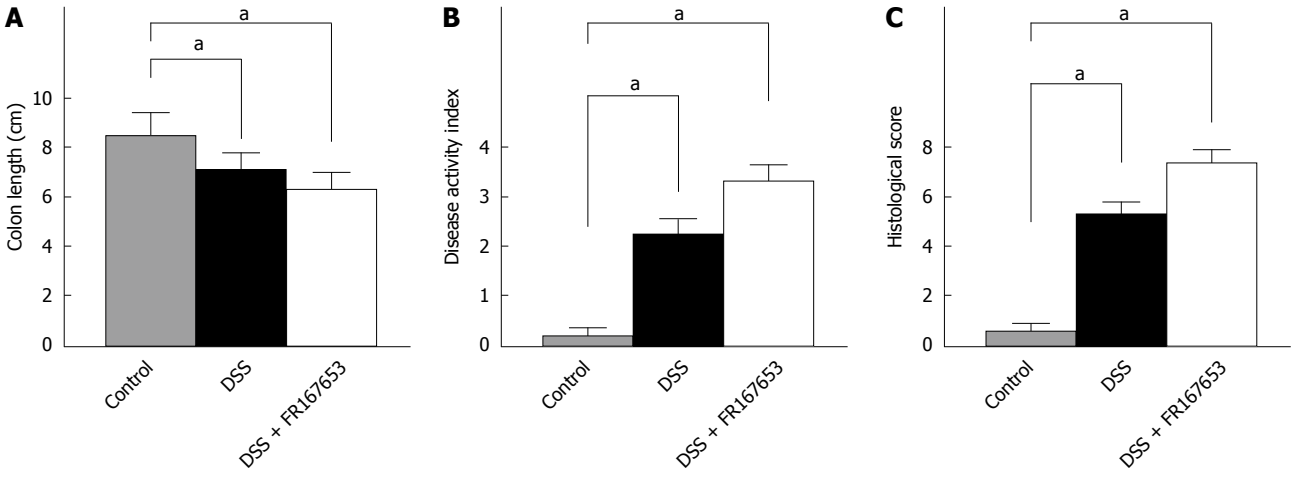


Figure 3 Effects of FR167653 on colon length (**A**), disease activity index (**B**), and histological score (**C**) at day 14. The criteria of scoring are described in Materials and Methods. Data represent mean ± SE (n = 8 mice/group), $^aP < 0.05$.

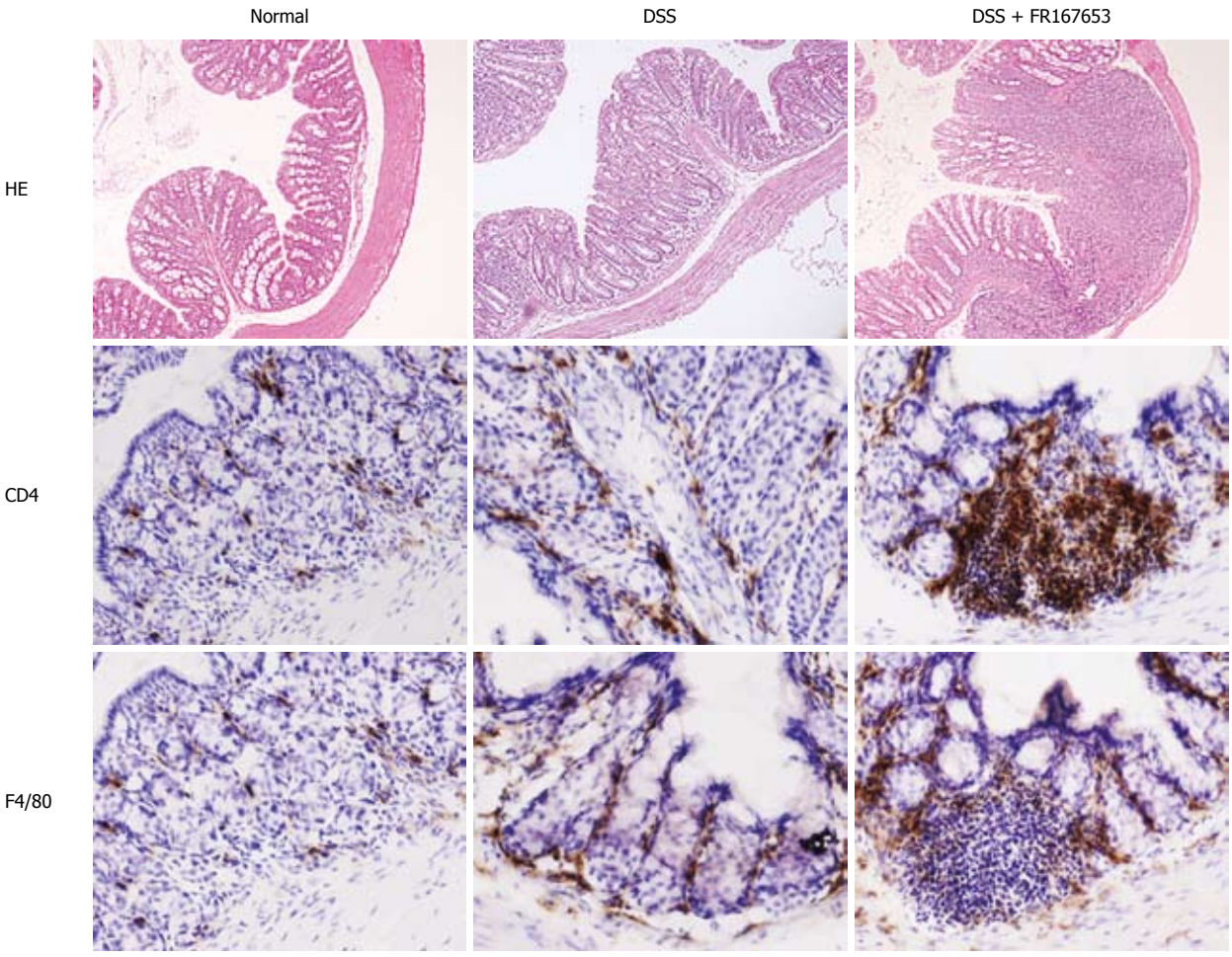


Figure 4 Histological analyses for FR167653 effects on DSS colitis. Colons were excised after 14 d of DSS treatment and stained with HE, anti-CD4 antibody, and anti-F4/80 antibody.

administration of FR167653 markedly enhanced the severity of the colitis as compared to the PBS-treated mice (Figure 3C).

An increase in the number of infiltrating cells and an enhancement of mucosal injury were observed in the PBS-treated DSS mice (Figure 4 upper part). These changes were enhanced in the FR167653-treated DSS mice. The immunohistochemical analysis indicated that the mucosal infiltration of CD4-positive T cells and F4/80-positive monocytes/macrophages was increased in the FR167643-treated DSS mice, compared to the PBS-treated DSS mice (Figure 4 middle and lower parts).

Inhibitory effects of FR167653 on IL-1β and TNF-α expression have been reported[26,27]. To confirm these

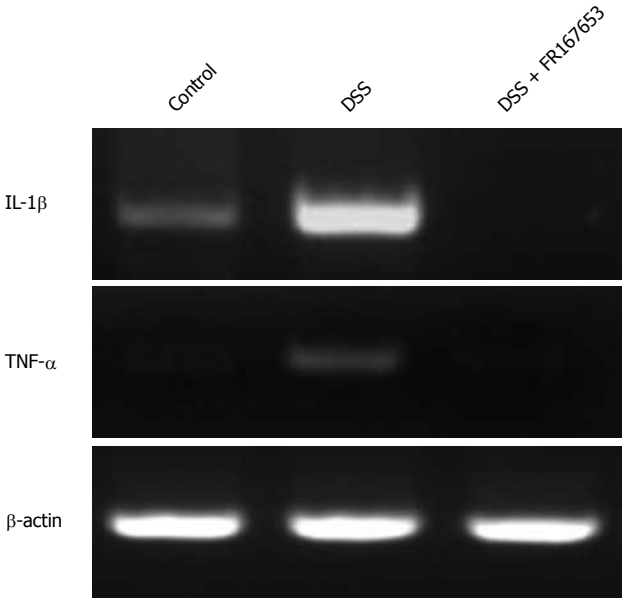


Figure 5 RT-PCR analysis of mucosal cytokine expression in DSS colitis. Representative results of 5 independent experiments are presented.

responses in our model, mucosal IL-1β and TNF-α mRNA expression was evaluated by semi-quantitative RT-PCR. Representative results are shown in Figure 5. DSS mice showed increased expression of IL-1β and TNF-α. The administration of FR167653 markedly reduced the expression of IL-1β and TNF-α mRNAs, although severity of colitis was enhanced. Similar results were observed in 5 different experiments.

DISCUSSION

The aim of this study was to analyze the impact of FR167653 upon inflammation in a mouse model of IBD. DSS-induced colitis in BALB/c mice is used as a model system for the respective human IBD. FR167653 was first discovered to be a potent inhibitor of IL-1 and TNF-α production in LPS-stimulated human monocytes and activated lymphocytes[17]. Recent studies suggest that FR167653 inhibits IL-1β and TNF-α production *via* specific inhibition of p38 MAPK activity[14,19,21]. While we initially expected that inhibition of p38 by FR167653 would block the development of DSS colitis, administration of FR167653 aggravated DSS colitis. Because mucosal IL-1β and TNF-α expression was markedly reduced in the FR167653-treated mice, aggravation of colitis might not be mediated by enhanced proinflammatory responses.

Previous findings of p38 activation in the pathophysiology of IBD are controversial. Waetzig *et al*[7] demonstrated that p38 and JNK1/2 were significantly activated in the inflamed colonic mucosa of IBD patients while the protein and mRNA expression of p38 and JNK were not significantly different between patients and controls. They suggested the role of p38 in the TNF-α signaling regulation loop in active CD patients. Hommes *et al*[8] showed an enhancement of phospho-p38 and phospho-JNK expression in neutrophils, epithelial cells, and lamina propria mononuclear cells in six patients with active CD compared to healthy controls. In contrast, Malamut *et al*[28] could not confirm p38 activation in patients with IBD, by measuring the protein levels and activity of p38. Moreover, a significant decrease in p38 activity and phosphorylated p38 levels was actually observed in the trinitrobenzene sulphonic acid (TNBS) colitis model.

There are two controversial reports of p38-inhibitor effects on experimental colitis. Ten Hove *et al*[29] observed a dichotomous effect of a specific p38 MAPK inhibitor, SB 203580, in TNBS-induced colitis in mice. In SB 203580-treated TNBS mice, weight loss was significantly worse and colon weight was significantly increased. However, activated lymph node cells of SB 203580 treated mice showed decreased IFN-γ but an increased TNF-α production. On the other hand, Hollenbach *et al*[30] showed that SB 203580 improved DSS colitis as reflected in the disease activity and histological disease score. Improvement of colitis was associated with down-regulation of NF-κB signaling and cytokine production.

The precise mechanisms underlying FR167653-induced aggravation of DSS colitis are unclear. Despite of aggravation of proinflammatory cell infiltration, FR167653 strongly reduced mucosal IL-1β and TNF-α expression. It may be that activation of p38 MAPK occurs downstream of the disease perpetuating signal transduction elements and that blockade at this level does not affect disease severity. Alternatively, cells playing roles in mucosal repair or anti-inflammatory process may be dependent on p38 MAPK. For example, it has been reported that p38 MAPK play an important role in epithelial restitution, a process aimed at re-epithelializing the wounded areas[31]. A recent study showed that production of IL-10, a representative anti-inflammatory cytokine, is dependent on p38 MAPK activation[32]. However, observed effects may be specific for models used in this study, and further experiments with other colitis models are needed.

In conclusion, treatment with the p38 MAPK inhibitor FR167653 induced an aggravation of DSS colitis although it reduced mucosal IL-1β and TNF-α production. This indicates that p38 MAPK is not only responsible for the production of proinflammatory cytokines but may also be involved in critical responses in host defense.

COMMENTS

Background

FR167653 is a well-characterized inhibitor of p38 mitogen-activated protein kinase (MAPK). At present, FR167653 has not been studied in any animal models for inflammatory bowel disease (IBD), and knowledge of its action in chronic colitis models is limited.

Research frontiers

Demonstration of the effects of a novel p38 MAPK inhibitor on dextran sulfate sodium (DSS) colitis.

Innovations and breakthroughs

Previous studies suggested that p38 MAPK acts as an enhancer of experimental colitis. Our study demonstrated that FR167653, a p38 MAPK inhibitor, aggravated DSS colitis, suggesting a protective role of p38 MAPK.

Applications
Clinical application of FR167653 to IBD may be possible.

Terminology
FR167653 is a well-characterized inhibitor of p38 MAPK. p38 MAPK mediates a major signal transduction pathway in pro- and anti-inflammatory responses.

Peer review
This is a carefully performed study detailing the effect of co-administration of a parenteral p38 MAPK inhibitor (FR167653) on the induction of DSS colitis in BALB/c mice. The use of this agent in various inflammatory conditions is not novel, nor is targeting p38 in IBD. What is novel is the finding that inhibiting p38 results in more severe DSS colitis.

REFERENCES

1 **Mizoguchi A**, Mizoguchi E. Inflammatory bowel disease, past, present and future: lessons from animal models. *J Gastroenterol* 2008; **43**: 1-17
2 **Sands BE**. Inflammatory bowel disease: past, present, and future. *J Gastroenterol* 2007; **42**: 16-25
3 **Sartor RB**, Muehlbauer M. Microbial host interactions in IBD: implications for pathogenesis and therapy. *Curr Gastroenterol Rep* 2007; **9**: 497-507
4 **Chang L**, Karin M. Mammalian MAP kinase signalling cascades. *Nature* 2001; **410**: 37-40
5 **Cuenda A**, Rousseau S. p38 MAP-kinases pathway regulation, function and role in human diseases. *Biochim Biophys Acta* 2007; **1773**: 1358-1375
6 **Cook R**, Wu CC, Kang YJ, Han J. The role of the p38 pathway in adaptive immunity. *Cell Mol Immunol* 2007; **4**: 253-259
7 **Waetzig GH**, Seegert D, Rosenstiel P, Nikolaus S, Schreiber S. p38 mitogen-activated protein kinase is activated and linked to TNF-alpha signaling in inflammatory bowel disease. *J Immunol* 2002; **168**: 5342-5351
8 **Hommes D**, van den Blink B, Plasse T, Bartelsman J, Xu C, Macpherson B, Tytgat G, Peppelenbosch M, Van Deventer S. Inhibition of stress-activated MAP kinases induces clinical improvement in moderate to severe Crohn's disease. *Gastroenterology* 2002; **122**: 7-14
9 **Waetzig GH**, Schreiber S. Review article: mitogen-activated protein kinases in chronic intestinal inflammation - targeting ancient pathways to treat modern diseases. *Aliment Pharmacol Ther* 2003; **18**: 17-32
10 **Wysk M**, Yang DD, Lu HT, Flavell RA, Davis RJ. Requirement of mitogen-activated protein kinase kinase 3 (MKK3) for tumor necrosis factor-induced cytokine expression. *Proc Natl Acad Sci USA* 1999; **96**: 3763-3768
11 **Lu HT**, Yang DD, Wysk M, Gatti E, Mellman I, Davis RJ, Flavell RA. Defective IL-12 production in mitogen-activated protein (MAP) kinase kinase 3 (Mkk3)-deficient mice. *EMBO J* 1999; **18**: 1845-1857
12 **Yoshino O**, Osuga Y, Koga K, Hirota Y, Hirata T, Ruimeng X, Na L, Yano T, Tsutsumi O, Taketani Y. FR 167653, a p38 mitogen-activated protein kinase inhibitor, suppresses the development of endometriosis in a murine model. *J Reprod Immunol* 2006; **72**: 85-93
13 **Chan KL**, Guo WH, Chan KW, Tam PK. FR167653 ameliorates intestinal allograft ischemic injury. *Transplant Proc* 2000; **32**: 1643-1644
14 **Aleshin A**, Sawa Y, Ono M, Funatsu T, Miyagawa S, Matsuda H. Myocardial protective effect of FR167653; a novel cytokine inhibitor in ischemic-reperfused rat heart. *Eur J Cardiothorac Surg* 2004; **26**: 974-980
15 **Yao HW**, Li J, Chen JQ. FR167653 attenuates murine immunological liver injury. *World J Gastroenterol* 2004; **10**: 2267-2271
16 **Wei YH**, Li Y, Qiang CJ. Effects and mechanisms of FR167653, a dual inhibitor of interleukin-1 and tumor necrosis factor, on adjuvant arthritis in rats. *Int Immunopharmacol* 2004; **4**: 1625-1632
17 **Yamamoto N**, Sakai F, Yamazaki H, Sato N, Nakahara K, Okuhara M. FR167653, a dual inhibitor of interleukin-1 and tumor necrosis factor-alpha, ameliorates endotoxin-induced shock. *Eur J Pharmacol* 1997; **327**: 169-174
18 **Yamamoto N**, Sakai F, Yamazaki H, Nakahara K, Okuhara M. Effect of FR167653, a cytokine suppressive agent, on endotoxin-induced disseminated intravascular coagulation. *Eur J Pharmacol* 1996; **314**: 137-142
19 **Takahashi S**, Keto Y, Fujita T, Uchiyama T, Yamamoto A. FR167653, a p38 mitogen-activated protein kinase inhibitor, prevents Helicobacter pylori-induced gastritis in Mongolian gerbils. *J Pharmacol Exp Ther* 2001; **296**: 48-56
20 **Hato S**, Urakami A, Yamano T, Uemura T, Ota T, Hirai R, Shimizu N. Attenuation of liver and lung injury after hepatic ischemia and reperfusion by a cytokine-suppressive agent, FR167653. *Eur Surg Res* 2001; **33**: 202-209
21 **Nishikori T**, Irie K, Suganuma T, Ozaki M, Yoshioka T. Anti-inflammatory potency of FR167653, a p38 mitogen-activated protein kinase inhibitor, in mouse models of acute inflammation. *Eur J Pharmacol* 2002; **451**: 327-333
22 **Cooper HS**, Murthy SN, Shah RS, Sedergran DJ. Clinicopathologic study of dextran sulfate sodium experimental murine colitis. *Lab Invest* 1993; **69**: 238-249
23 **Rath HC**, Herfarth HH, Ikeda JS, Grenther WB, Hamm TE Jr, Balish E, Taurog JD, Hammer RE, Wilson KH, Sartor RB. Normal luminal bacteria, especially Bacteroides species, mediate chronic colitis, gastritis, and arthritis in HLA-B27/human beta2 microglobulin transgenic rats. *J Clin Invest* 1996; **98**: 945-953
24 **Chomczynski P**, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**: 156-159
25 **Murphy E**, Hieny S, Sher A, O'Garra A. Detection of in vivo expression of interleukin-10 using a semi-quantitative polymerase chain reaction method in Schistosoma mansoni infected mice. *J Immunol Methods* 1993; **162**: 211-223
26 **Yao HW**, Yue L. Effect and mechanisms of FR167653, a dual inhibitor of TNF-alpha and IL-1, on BCG plus LPS induced-liver injury. *Inflamm Res* 2005; **54**: 471-477
27 **Takeyoshi I**, Yoshinari D, Kobayashi M, Kurabayashi M, Morishita Y. A dual inhibitor of TNF-alpha and IL-1 mitigates liver and kidney dysfunction and improves survival in rat endotoxemia. *Hepatogastroenterology* 2005; **52**: 1507-1510
28 **Malamut G**, Cabane C, Dubuquoy L, Malapel M, Derijard B, Gay J, Tamboli C, Colombel JF, Desreumaux P. No evidence for an involvement of the p38 and JNK mitogen-activated protein in inflammatory bowel diseases. *Dig Dis Sci* 2006; **51**: 1443-1453
29 **ten Hove T**, van den Blink B, Pronk I, Drillenburg P, Peppelenbosch MP, van Deventer SJ. Dichotomal role of inhibition of p38 MAPK with SB 203580 in experimental colitis. *Gut* 2002; **50**: 507-512
30 **Hollenbach E**, Neumann M, Vieth M, Roessner A, Malfertheiner P, Naumann M. Inhibition of p38 MAP kinase- and RICK/NF-kappaB-signaling suppresses inflammatory bowel disease. *FASEB J* 2004; **18**: 1550-1552
31 **Karrasch T**, Steinbrecher KA, Allard B, Baldwin AS, Jobin C. Wound-induced p38MAPK-dependent histone H3 phosphorylation correlates with increased COX-2 expression in enterocytes. *J Cell Physiol* 2006; **207**: 809-815
32 **Leghmari K**, Bennasser Y, Tkaczuk J, Bahraoui E. HIV-1 Tat protein induces IL-10 production by an alternative TNF-alpha-independent pathway in monocytes: role of PKC-delta and p38 MAP kinase. *Cell Immunol* 2008; **253**: 45-53

S- Editor Li DL **L- Editor** Mihm S **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5857

World J Gastroenterol 2008 October 14; 14(38): 5857-5867
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Oxidative stress disturbs energy metabolism of mitochondria in ethanol-induced gastric mucosa injury

Jin-Shui Pan, Shao-Zhen He, Hong-Zhi Xu, Xiao-Juan Zhan, Xiao-Ning Yang, Hong-Min Xiao, Hua-Xiu Shi, Jian-Lin Ren

Jin-Shui Pan, Shao-Zhen He, Hong-Zhi Xu, Xiao-Juan Zhan, Xiao-Ning Yang, Hong-Min Xiao, Hua-Xiu Shi, Jian-Lin Ren, Division of Gastroenterology, Zhongshan Hospital, Xiamen University, Xiamen 361004, Fujian Province, China
Jin-Shui Pan, Hong-Zhi Xu, Xiao-Juan Zhan, Xiao-Ning Yang, Hong-Min Xiao, Hua-Xiu Shi, Jian-Lin Ren, Gastroenterology Institute of Xiamen University, Xiamen 361004, Fujian Province, China
Jin-Shui Pan, Hong-Zhi Xu, Xiao-Juan Zhan, Xiao-Ning Yang, Hong-Min Xiao, Hua-Xiu Shi, Jian-Lin Ren, Gastroenterology Center of Xiamen, Xiamen 361004, Fujian Province, China
Shao-Zhen He, Intensive Care Unit, the First Hospital of Zhangzhou, Zhangzhou 363000, Fujian Province, China
Author contributions: Pan JS and Ren JL designed the research; Pan JS, He SZ, Xu HZ, Zhan XJ, Yang XN, Xiao HM and Shi HX performed the research; He SZ, Zhan XJ and Yang XN analyzed the data; Pan JS and Ren JL wrote the paper.
Supported by National Natural Science Foundation of China, No. 30750013; Natural Science Foundation of Fujian Province, No. X0650091
Correspondence to: Jian-Lin Ren, PhD, MD, Professor, Division of Gastroenterology, Zhongshan Hospital, Xiamen University; Gastroenterology Institute of Xiamen University; Gastroenterology Center of Xiamen, Xiamen 361004, Fujian Province, China. jianlinr@msn.com
Telephone: +86-0592-2993170 Fax: +86-0592-2993170
Received: August 2, 2008 Revised: September 20, 2008
Accepted: September 27, 2008
Published online: October 14, 2008

Abstract

AIM: To study the role of mitochondrial energy disorder in the pathogenesis of ethanol-induced gastric mucosa injury.

METHODS: Wistar rats were used in this study. A gastric mucosal injury model was established by giving the rats alcohol. Gross and microscopic appearance of gastric mucosa and ultrastructure of mitochondria were evaluated. Malondiadehyde (MDA) in gastric mucosa was measured with thiobarbituric acid. Expression of ATP synthase (ATPase) subunits 6 and 8 in mitochondrial DNA (mtDNA) was determined by reverse transcription polymerase chain reaction (RT-PCR).

RESULTS: The gastric mucosal lesion index was correlated with the MDA content in gastric mucosa. As the concentration of ethanol was elevated and the exposure time to ethanol was extended, the content of MDA in gastric mucosa increased and the extent of damage aggravated. The ultrastructure of mitochondria was positively related to the ethanol concentration and exposure time. The expression of mtDNA ATPase subunits 6 and 8 mRNA declined with the increasing MDA content in gastric mucosa after gavage with ethanol.

CONCLUSION: Ethanol-induced gastric mucosa injury is related to oxidative stress, which disturbs energy metabolism of mitochondria and plays a critical role in the pathogenesis of ethanol-induced gastric mucosa injury.

Key words: Animal model; Ethanol; Gastric mucosa; Oxygen free radical; Energy metabolism

Peer reviewer: Cintia Siqueira, Professor, Institute of Molecular Medicine, Center of Gastroenterology, Av. Egas Moniz, isboa, 1649-028, Portugal

Pan JS, He SZ, Xu HZ, Zhan XJ, Yang XN, Xiao HM, Shi HX, Ren JL. Oxidative stress disturbs energy metabolism of mitochondria in ethanol-induced gastric mucosa injury. *World J Gastroenterol* 2008; 14(38): 5857-5867 Available from: URL: http://www.wjgnet.com/1007-9327/14/5857.asp DOI: http://dx.doi.org/10.3748/wjg.14.5857

INTRODUCTION

Many people all over the world indulge themselves in drinking, which is correlated to a wide spectrum of medical, psychological, behavioral, and social problems. Ethanol is the major component of drinkable wine and alcoholic beverages. After drinking, alcohol is absorbed rapidly into the blood stream from the stomach and intestinal tract. High-concentration ethanol erodes directly the gastric mucosa and causes acute gastritis, leading to hyperemia, edema, hemorrhage, ulcer, *etc*. It is well known that chronic alcohol abuse may induce gastrointestinal dysfunction, chronic atrophic gastritis and is closely related with gastric carcinoma. However, the detailed mechanism by which ethanol affects the gastrointestinal mucosa remains to be elucidated.

Thorough research on how ethanol affects gastric mucosa will benefit the protection of gastric mucosa.

The effect of ethanol on gastric mucosa is a complicated and multifaceted process. It may be associated with disturbance of the balance between gastric mucosal defense and offensive factors. Gastric mucosa contains gastric acid, pepsin, stimulant, *etc*, while the offensive factors contain gastric slime layer, mucosal blood flow, HCO_3^-, prostaglandins (PGs), epidermal growth factor (EGF) and epithelial cell renewing. Ethanol induces vascular endothelium injury of gastric mucosa, disorder of microcirculation and ischemia as a result of more production of oxygen free radicals (OFR). *In vivo*, ethanol metabolism also produces OFR. Alarcon *et al*[1] found that perfusion of OFR induces gastric mucosal injury in rats. However, perfusion of superoxide dismutase (SOD) reduces gastric mucosal injury. Itoh and Guth[2] reported that SOD and catalase (CAT) significantly diminish gastric mucosal injury after ischemia reperfusion. OFR play an crucial role in acute gastric mucosal injury. Similar to other tissue inflammation, numerous inflammatory factors which cause infiltration of neutrophils are released when gastric mucosa is attacked by various insult factors. These neutrophils produce a lot of OFR by respiratory burst. OFR, mostly the superoxide radical anion ($O_2^{\cdot-}$), are metabolized through two endocellular pathways. One is peroxidation of cytomembrane lipid which produces lipoperoxide, malondiadehyde (MDA) and 4-hydroxynonena (4-HNE) after β-oxidation. MDA, a reaction product between OFR and cells, indicates the intracellular OFR. In the other path, OFR reacting with OFR scavengers (*e.g.* SOD) generate hydrogen peroxide (H_2O_2), which will be further oxidized by glutathione peroxidase (GPx) or CAT to H_2O and CO_2. Both of which are closely related to enzyme complexes of respiratory chain locating on the mitochondrial membrane.

Gastric parietal cells are rich in mitochondria[3-5]. Mitochondria provide energy for cells by oxidative phosphorylation (OXPHOS), which is critical to maintain the proper morphology and function of gastric mucosa. F_0F_1-ATP synthase, also known as cytochrome C oxidase (complex Ⅳ), is critical to oxidative phosphorylation and mediates generation of ATP[6]. The complex Ⅳ phosphorylates ADP to ATP at the advantage of transmembrane electrochemical proton gradient generated by respiratory electron-transport chain[7]. Mitochondrial ATP synthase (ATPase) subunits 6 and 8, the components of the F_0 part of this enzyme, which is encoded by mitochondrial DNA (mtDNA) independently, control the synthesis of ATP by mediating the proton transfer. Mitochondrion is an easily injured organelle and mtDNA is the major target of intracellular oxidative stress associated with ethanol[8]. However, the relation between OFR and alternation of mitochondrial ultra microstructure as well as how OFR interferes with gastric mucosal energy metabolism are obscure. This study was focused on the ultra microstructure alteration of mitochondria in response to the injury induced by and the role of disturbance of mitochondrial energy metabolism in ethanol-related gastropathy.

In this research, we established an acute and chronic animal model of gastric mucosa injury by ethanol gavage. The related parameters include index of gastric mucosa injury, pathological damage, alteration of mitochondria ultra microstructure, content of MDA and expression of mtDNA of ATPase subunits 6 and 8 in rat gastric mucosa. According to the above parameters, we aimed to find the relation between OFR and mediating factors of energy metabolism as well as the role of ethanol in ethanol-induced gastric mucosal injury.

MATERIALS AND METHODS

Rats

Healthy male Wistar rats of clean grade, purchased from Shanghai Slac Laboratory Animal Company, were bred in Anticancer Research Center of Xiamen University. All animal experiments were performed under the animal experimental rules of PRC.

Reagents

Medical anhydrous ethanol, purchased from the Shanghai Chemical Reagent Corporation, was diluted to the desired concentration with distilled water before experiment. Ten percents (W/W) formaldehyde fixatives were provided by Pathology Department of Zhongshan Hospital, Xiamen University. MDA kits were purchased from the General Hospital of People's Liberation Army. TRIzol reagent (Cat No. 15596-026) came from Invitrogen Corporation. RT-PCR kits (Cat No. A3500), Taq DNA polymerase, and 100 bp DNA ladder were bought from the Promega Corporation.

Equipments

Gavage needles and a set of animal anatomic equipments were provided by Anticancer Research Center and Zhongshan Hospital of Xiamen University, respectively. PCR was performed on Gene Amp2700 (Applied Biosystems, USA). Gel analysis was performed by GAS7100X (USA), and transmission electron microscope was made in Japan (Hitachi-Hu-12A, Hitachi, Japan).

Establishing animal model of ethanol-induced acute gastric mucosal injury

Thirty-two healthy male Wistar rats of clean grade, weighing 200-220 g, were randomly divided into normal control group (n = 8) and experimental group (n = 24). The rats were abstained from any food but water 24 h before experiment with free movement. Two hours before experiment, the rats were deprived of water. Rats in the control group were gavaged with 1 mL normal saline. Rats in the experimental group were divided into three subgroups (n = 8). Rats in each subgroup were gavaged respectively with 1 mL of anhydrous ethanol, 70% (W/W) ethanol and 40% (W/W) ethanol, respectively. Two hours later, all the animals were

anesthetized with ether, and their abdominal cavity was dissected along the inferior border of xiphoid and their stomach was mobilized, then the gastric cavity was dissected along the greater curvature and the gastric mucosa was spread. After the mucosa was poached three times with physiologic saline, degree of the gastric mucosal injury was determined according to the Guth law and biopsy was performed.

Establishing animal model of ethanol-induced chronic gastric mucosal injury

Ninety healthy male Wistar rats of clean grade, weighing 160-180 g, were randomly divided into 3 normal control groups and 6 experimental groups (n = 10) after feeding for a week. The 3 normal control groups were gavaged with normal saline, while the 6 experimental groups were gavaged two times with 25% (W/W) ethanol and 45% (W/W) ethanol daily. The volume of gavage solution was calculated according to 9 mL/kg per day. Rats in the control and experimental groups were sacrificed on days 3, 6, and 9, respectively, and dissected in the same way as described in establishing animal model of ethanol-induced acute gastric mucosal injury.

Determination of gross injury index for gastric mucosa

Gastric mucosal injury indices were calculated according to the Guth standard[9]: 1 point for the punctate hemorrhage, 2 points for the injury focus with its length shorter than 1 mm, 3 points for the injury focus with its length ranging 1-2 mm, 4 points for the injury focus with its length ranging 2-4 mm, 5 points for the injury focus with its length longer than 4 mm, respectively, for the streak-like hemorrhage. The score was doubled if the streak was wider than 2 mm. Injury index of the whole gastric mucosa for each rat was the summation of injury indices for each lesion.

Determination of pathological injury integral for gastric mucosa

A 0.5 cm × 1.0 cm tissue was taken from gastric gland, fixed with a 10% (W/W) formaldehyde solution and made into a paraffin block. The tissue was cut into 5-μm thick sections which were stained with hematoxylin and eosin. Pathological changes in the gastric mucosa were observed under optical microscope. Integrals were calculated according to the scoring standard reported by Masuda *et al*[10], namely, 0 point for normal tissue, 1 point for epithelial damage of surface layer, 2 points for hyperemia or edema of the upper stratum, 3 points for hyperemia, edema or even hemorrhage of stratum medium or under layer. Upper mucosal glands with structural disorders or necrosis had a score of 4 points, and the tissue with deep ulcer or necrosis had a score of 5 points. Integral of each section should not exceed 15 points.

Preparation of specimens for transmission electron microscopy (TEM)

All specimens were fixed with 3% (W/W) glutaraldehyde and 1.5% (W/W) paraformaldehyde at 4℃ for several days or hours, then postfixed for 1.5 h in 1% (W/W) osmium tetroxide and 1.5% (W/W) potassium ferrocyanide, and finally rinsed with PBS. The paraffin tissue block was stained in a solution buffer containing 70% (W/W) ethnal and uranyl acetate, then gradually dehydrated with alcohol-acetone and embedded in Epon 618. The tissue block was cut into ultra-thin (80 nm) sections, which were double stained with uranyl acetate and lead citrate for 5 min and examined under a Hitachi Hu-12A electron microscope.

Determination of MDA content in gastric mucosa with thiobarbituric acid (TBA) method

Mucosal specimens were taken from gastric glands and weighed, then immersed into 0.02 mmol/L Tris-HCl (pH 7.4) at the ratio of 1:10 (mg/μL). Tissue homogenate was centrifuged at 13000 r/min for 15 min at 4℃ and precipitates were discarded. Supernatant (0.15 mL) was transferred to a testing tube. A standard solution (0.15 mL) was added to each standard testing tube, and 0.15 mL of distilled water was added to another blank testing tube, 2.5 mL thiobarbituric acid (TBA) was added into each of these two tubes, which were agitated several times, and then incubated at 100℃ for 1 h. After that, each specimen was cooled to room temperature and centrifuged at 3000 r/min for 15 min. Finally, supernatant in each tube underwent colorimetric assay at 532 nm. MDA content (nmol/mL) was calculated according to the following formula: (absorbance of testing tube/absorbance of standard tube) × 2.5.

Detection of mRNA expression in mtDNA ATPase subunits 6 and 8 by RT-PCR

Stomach of each rat was split completely and washed at least three times with 0.1% (W/W) diethyl-pyrocarbonate (DEPC). Gastric mucosa was peeled carefully, and put into a frozen-storage pipe. Finally, each specimen was kept in liquid nitrogen. The anatomic equipments were degermed at a high-pressure and toasted at 200℃ for no less than 5 h beforehand. Meanwhile, frozen pipes were soaked in 0.1% DEPC water overnight and degermed at a high-pressure to remove all vestigial DEPC. Total RNA was extracted with TrizolTM (Invitrogen) according to its manufacturer's instructions. Reverse transcription (RT) of mRNA was performed with an AMV reverse transcription kit (Promega), following the manufacturer's instructions. RT reaction conditions were as follows: at 42℃ for 60 min, at 95℃ for 5 min, at 4℃ for 5 min. Reacting systems are listed in Table 1. Four uL of RT products (cDNA) was used as the template for PCR amplification. Primers were designed according to the sequences of mitochondrial ATPase subunits 6 and 8 for amplifying. For comparison, negative and positive controls were set up. *β-actin* gene was taken as a positive control. PCR primers were synthesized by Invitrogen Corporation. PCR products were assayed by 1.35% (W/W) agarose gel electrophoresis and stained with ethidium bromide to confirm the expected product size. Expression of ATPase subunits 6 and 8 was normalized to that of the corresponding *β-actin*. Analysis

Table 1 Reacting system of RT

Component	Amount
25 mmol/L $MgCl_2$	4.00 μL
Reverse transcription 10 × buffer	2.00 μL
10 mmol/L dNTP	2.00 μL
Recombinant ribonuclease inhibitor	0.50 μL
AMV reverse transcriptase	15.00 U
Oligo (dT) 15 primer	0.50 μg
Total RNA	1.00 μg
Nuclease-rree water	Proper volume
Reacting volume	20.00 μL

Table 2 Reacting system of PCR

Component	Amount
cDNA	4.00 μL
10 × PCR buffer solution	1.96 μL
$MgCl_2$ (25 mmol/L)	2.40 μL
Upper primer (20 pmol/L)	1.00 μL
Down primer (20 pmol/L)	1.00 μL
dNTP (10 mmol/L)	0.36 μL
Taq DNA polymerase (5 IU/μL)	0.10 μL
Deionized water	10.08 μL
Reacting volume	20.00 μL

Table 3 Primers used for PCR amplification

Primers	GenBank (No.)	Sequence
β-actin	NM_031144 (nt 1-1296)	Up: 5′-TCACCCACACTGTGCCCATCTATGA-3′ Down: 5′-CATCGGAACCGCTCATTGCCGATAG-3′
ATPase subunit6	X14848 (nt 7904-8584)	Up: 5′-ACA CCAAAAGGACGAACCTG-3′ Down: 5′-CGGTGAGAAGTGGGCTAAAG-3′
ATPase subunit8	X14848 (nt 7743-7946)	Up: 5′-TGCCACAACTAGACACATCCA-3′ Down: 5′-TGTGGGGGTAATGAAAGAGG-3′

of PCR products was performed on GAS7100X. PCR reacting system, information about primers, amplifying conditions were listed in Tables 2-4.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 for Windows. $P < 0.05$ was considered statistically significant.

RESULTS

Changes in morphology of gastric mucosa with ethanol-induced acute injury

Gastric mucosa was injured in all experimental groups. A positive correlation was observed between injury and ethanol concentrations. Gastric mucosa from rats in the control group that received saline was smooth, complete, and pink, without congestion, edema, and erosion or bleeding. The mucosa was normal under microscope. With the increasing ethanol concentration, edema and congestion became obvious. In the rats that administrated 40% (W/W) ethanol, gastric mucosa became edematous and congestive. Furthermore, point and scattered bleeding lesions, focal hemorrhage, necrosis occurred. The necrosis was limited to the superficial 1/3 layer of mucosa. In the rats that administrated 70% (W/W) ethanol, congestion and edema aggravated. Superficial vessels of gastric mucosa became obviously dilated. Linear ulcer and bleeding lesions occurred. The common length of lesions was more than 4 mm, and there was significant infiltration of neutrophils and eosinophile granulocytes. Mucosa necrosis expanded to more than half of the whole layer. Rare normal mucosa was observed in the rats that administrated 100% (W/W) ethanol. Giant and deep ulcers were visible (Figures 1 and 2).

Table 4 PCR amplification conditions (mean ± SD)

Target sequence	Amplification conditions
β-actin	94.0℃ 30 s, 59.0℃ 30 s, 72.0℃ 30 s, 35cycles
ATPase subunit 6	94.0℃ 30 s, 53.0℃ 30 s, 72.0℃ 30 s, 33cycles
ATPase subunit 8	94.0℃ 30 s, 52.0℃ 30 s, 72.0℃ 30 s, 32cycles

Table 5 LI and MDA content in acute gastric mucosa injury treated with different concentrations of ethanol (mean ± SD)

Group	LI	MDA (nmol/mL)
Control	0.000 ± 0.000	0.968 ± 0.136
40% (W/W) ethanol	38.375 ± 6.368[a]	1.363 ± 0.152[a]
70% (W/W) ethanol	70.000 ± 8.018[a,b]	1.703 ± 0.124[a,b]
100% (W/W) ethanol	118.750 ± 14.820[a,b,c]	2.083 ± 0.153[a,b,c]

[a]$P < 0.01$ *vs* control group; [b]$P < 0.01$ *vs* 40% (W/W) ethanol group; [c]$P < 0.01$ *vs* 70% (W/W) ethanol group.

Integral index of acute injury in gastric mucosa

After administration of different concentrations of ethanol, the integral index was different for rats in different groups. The injury index was positively correlated with the ethanol concentration ($P < 0.01$, Table 5).

Changes in morphology of gastric mucosa with ethanol-induced chronic injury

Gastric mucosa from the control group was smooth and intact. In the rats that administrated with 25% (W/W) or 45% (W/W) ethanol, injuries exacerbated with the increasing gavage time. No significant change was observed in gastric mucosa of rats that received 25% (W/W) ethanol after three weeks. Occasionally, congestion and edema could be observed under microscope. After six weeks, congestion and edema became obvious but no obvious bleeding was found. After nine weeks, congestion and edema exaggerated and some superficial bleeding lesions were noted. Disorder of glands could be observed under microscope. Gastric mucosa of rats that received 45% (W/W) ethanol became dark red after three weeks. Congestion, edema and even point erosion

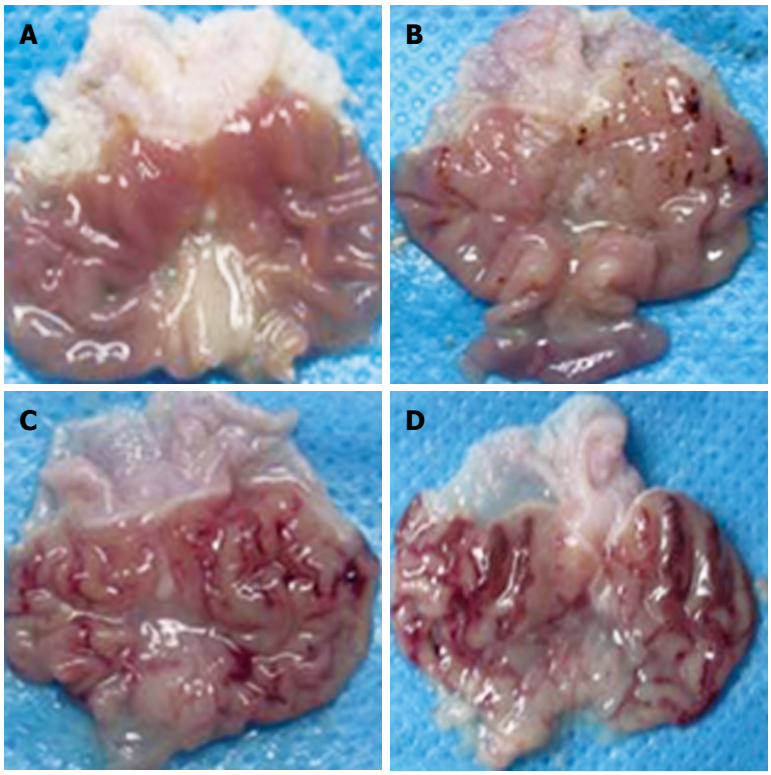


Figure 1 Gross appearance of gastric mucosa in rats with ethanol-induced acute gastric mucosa injury 2 h after administration of normal sodium (**A**), 40% (W/W) ethanol (**B**), 70% (W/W) ethanol (**C**), and 100% (W/W) ethanol (**D**), respectively.

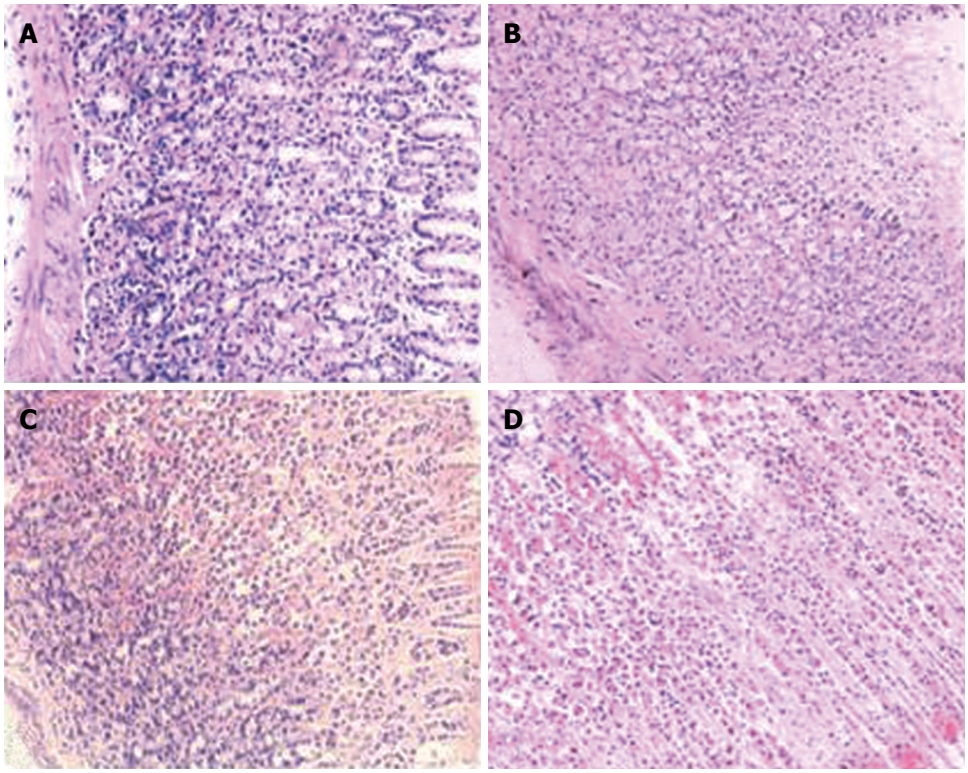


Figure 2 Histological changes in gastric mucosa of rats with ethanol-induced acute gastric mucosa injury (x 100) 2 h after administration of normal sodium (**A**), 40% (W/W) ethanol (**B**), 70% (W/W) ethanol (**C**), and 100% (W/W) ethanol (**D**), respectively.

could be observed. There was infiltration of neutrophils and eosinophile granulocytes but no disturbed glandular structure. After six weeks, the number of scattered hemorrhage lesions increased. There was infiltration of neutrophils, lymphocytes and eosinophile granulocytes. Principal cells and parietal cells became swollen. The upper structure of glandular mucosa was damaged. After nine weeks, gastric mucosa mainly displayed a dark red or red and white performance. Congestion and edema became obviously. Infiltration of neutrophils, lymphocytes and eosinophile granulocytes exacerbated. Principal cells and parietal cells became swollen and diminished. The upper glandular structure was disturbed and the nuclei of glandular epithelium in the lower part were deeply stained. Atypical hyperplasia occurred (Figures 3 and 4).

Integral index of chronic injury in gastric mucosa

Integral indices were different for all groups and

Table 6 Integral indices and pathological integrals for chronic gastric mucosa injury (mean ± SD)

Group	3 wk		6 wk		9 wk	
	Integral indices	Pathological integrals	Integral indices	Pathological integrals	Integral indices	Pathological integrals
Normal sodium	0.000 ± 0.000	0.200 ± 0.422	0.000 ± 0.000	0.500 ± 0.707	0.000 ± 0.000	0.600 ± 0.699
25% (W/W) alcohol	0.200 ± 0.422	0.700 ± 0.675	1.100 ± 0.568	2.400 ± 0.516	1.700 ± 0.483	4.500 ± 1.080
45% (W/W) alcohol	0.600 ± 0.699	1.500 ± 0.527	3.300 ± 1.059	6.000 ± 0.817	8.900 ± 0.994	11.400 ± 0.966

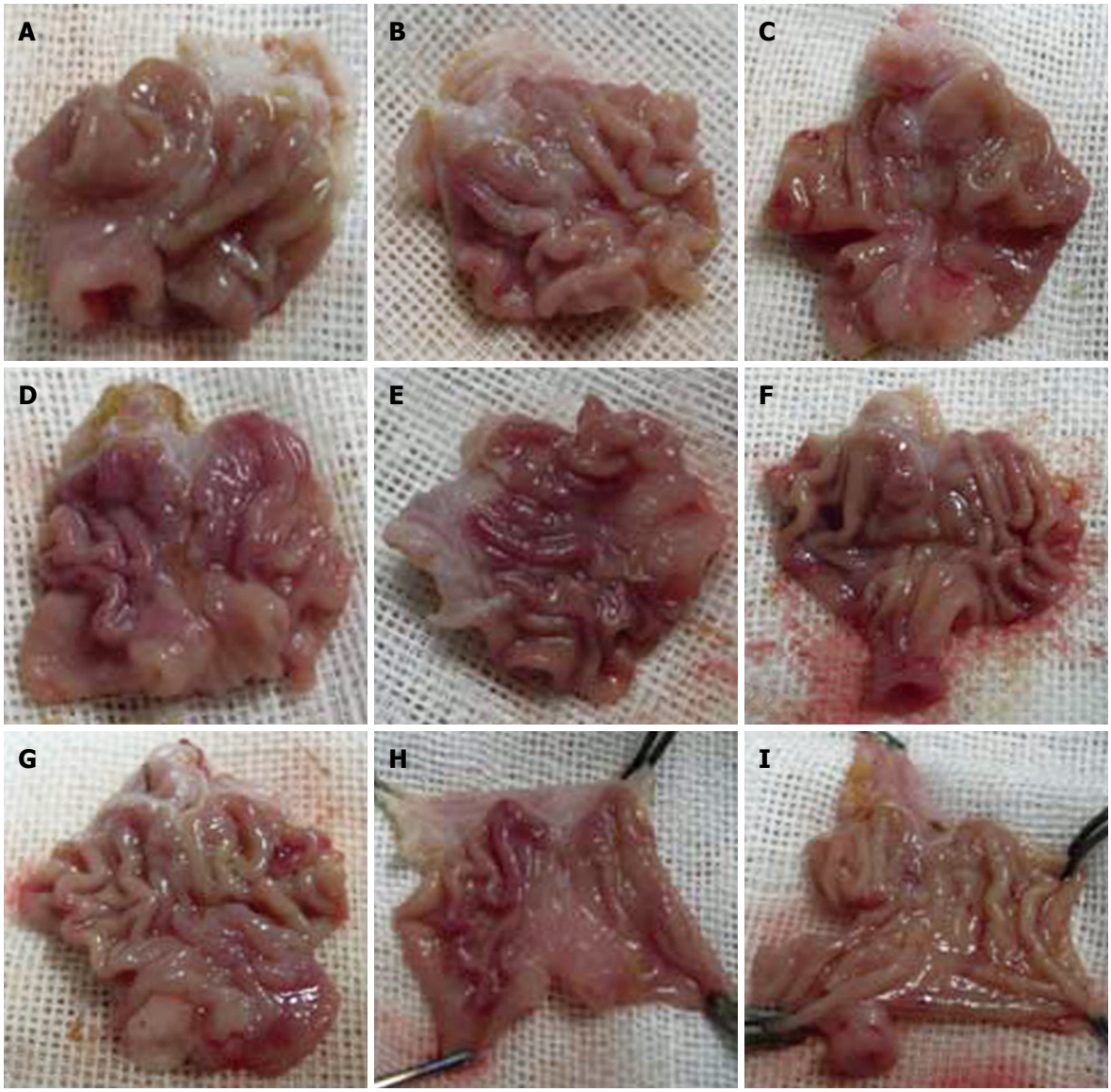


Figure 3 Gross changes in gastric mucosa of rats with ethanol-induced chronic gastric mucosa injury 3 wk after administration of normal sodium (**A**), 25% (W/W) ethanol (**B**), and 45% (W/W) ethanol (**C**), respectively; 6 wk after administration of normal sodium (**D**), 25% (W/W) ethanol (**E**), and 45% (W/W) ethanol (**F**), respectively; 9 wk after administration of normal sodium (**G**), 25% (W/W) ethanol (**H**), and 45% (W/W) ethanol (**I**), respectively.

increased with the increasing ethanol concentration ($P < 0.01$) and the gavage time ($P < 0.05$, Table 6).

Pathological integral of severity in chronic injury of gastric mucosa

Pathological integrals of all groups were different. Integral index increased with the increasing ethanol concentration ($P < 0.01$) and the gavage time ($P < 0.01$, Table 6).

Ultrastructure of mitochondria

Swollen mitochondria were observed in parietal cells under transmission electron microscope and collapsed 2 h after 100% (W/W) ethanol gavage. Mitochondria became homogeneous and vacuolar. Mitochondrial cristae diminished. However, no significant change occurred in mitochondria 2 h after 40% (W/W) ethanol or 70% (W/W) ethanol gavage. The structure of mitochondria was intact and mitochondrial cristae were clear. The number of mitochondria decreased with the increasing ethanol concentration. This was especially true in rats that received 100% (W/W) ethanol. In chronic alcohol damage experiment, abnormal

Table 7 Mitochondria, ATPase subunits 6 and 8 mRNA in ethanol-induced acute gastric mucosal injury (mean ± SD)

Group	Mitochondria (*n*)	ATPase subunit 6 mRNA	ATPase subunit 8 mRNA
Normal sodium	74.800 ± 15.399	0.935 ± 0.162	0.768 ± 0.145
40% (W/W) ethanol	69.200 ± 16.262	1.113 ± 0.135[a]	0.945 ± 0.139[a]
70% (W/W) ethanol	63.500 ± 14.246	0.868 ± 0.089[b]	0.677 ± 0.106[b]
100% (W/W) ethanol	51.500 ± 18.739	0.691 ± 0.053[a,b,c]	0.433 ± 0.055[a,b,c]

[a]$P < 0.01$ *vs* control group; [b]$P < 0.01$ *vs* 40% (W/W) ethanol group; [c]$P < 0.01$ *vs* 70% (W/W) ethanol group.

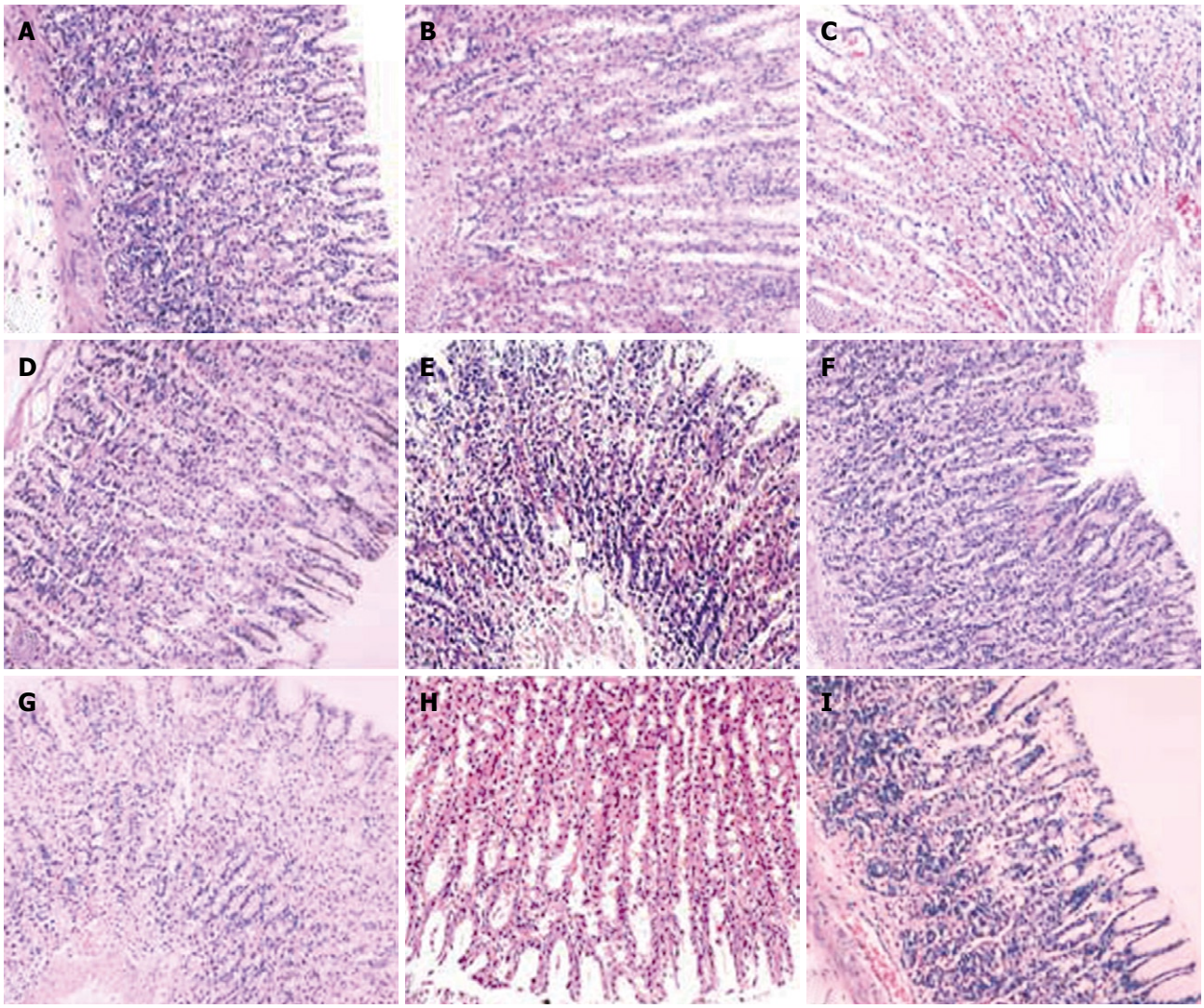


Figure 4 Histological changes in gastric mucosa of rats with ethanol-induced chronic gastric mucosa injury (HE stain) 3 wk after administration of normal sodium (**A**), 25% (W/W) ethanol (**B**), and 45% (W/W) ethanol (**C**), respectively; 6 wk after administration of normal sodium (**D**), 25% (W/W) ethanol (**E**), and 45% (W/W) ethanol (**F**), respectively; 9 wk after administration of normal sodium (**G**), 25% (W/W) ethanol (**H**), and 45% (W/W) ethanol (**I**), respectively.

mitochondrial changes were observed only in rats that received 40% (W/W) ethanol. Mitochondria became sparse and swollen. The structure was blurred (Tables 7 and 8, Figures 5 and 6).

MDA content in gastric mucosa with ethanol-induced acute injury

MDA content was higher in rats with ethanol-induced acute injury than in those of the control group. MDA content was positively related with ethanol concentrations ($P < 0.01$, Table 5).

MDA content in gastric mucosa with ethanol-induced chronic injury

MDA content was higher in rates with ethanol-induced chronic injury than in those of the control group. MDA content increased with the increasing ethanol concentration and gavage time ($P < 0.01$, Table 8).

Expression of mtDNA ATPase subunits 6 and 8 mRNA in gastric mucosa with ethanol-induced acute injury

The expression of mtDNA ATPase subunits 6 and 8 mRNA in ethanol-induced acute injury decreased with

Table 8 Mitochondria and MDA content in ethanol-induced chronic gastric mucosa injury (mean ± SD)

Group	3 wk		6 wk		9 wk	
	Mitochondria (*n*)	MDA content	Mitochondria (*n*)	MDA content	Mitochondria (*n*)	MDA content
Normal sodium	123.000 ± 23.613	0.717 ± 0.057	109.400 ± 30.303	0.757 ± 0.064	104.600 ± 20.571	0.728 ± 0.044
25% (W/W) alcohol	111.000 ± 18.514	0.820 ± 0.051	106.500 ± 17.031	1.029 ± 0.102	86.300 ± 26.833	1.156 ± 0.058
45% (W/W) alcohol	101.100 ± 13.186	0.969 ± 0.126	75.700 ± 17.639	1.225 ± 0.053	59.400 ± 21.767	1.477 ± 0.072

Table 9 Expression of mtDNA ATPase subunits 6 and 8 mRNA in ethanol-induced chronic gastric mucosa injury[1] (mean ± SD)

Group	3 wk		6 wk		9 wk	
	mtDNA ATPase 6 mRNA	mtDNA ATPase 8 mRNA	mtDNA ATPase 6 mRNA	mtDNA ATPase 8 mRNA	mtDNA ATPase 6 mRNA	mtDNA ATPase 8 mRNA
Normal sodium	0.907 ± 0.079	0.678 ± 0.033	0.876 ± 0.045	0.671 ± 0.038	0.894 ± 0.059	0.659 ± 0.037
25% (W/W) alcohol	0.857 ± 0.062	0.655 ± 0.029	0.737 ± 0.060	0.557 ± 0.028	0.664 ± 0.039	0.512 ± 0.043
45% (W/W) alcohol	0.756 ± 0.051	0.617 ± 0.026	0.607 ± 0.039	0.474 ± 0.031	0.501 ± 0.043	0.366 ± 0.028

[1]Magnitude relative to *β-actin* from respective specimens.

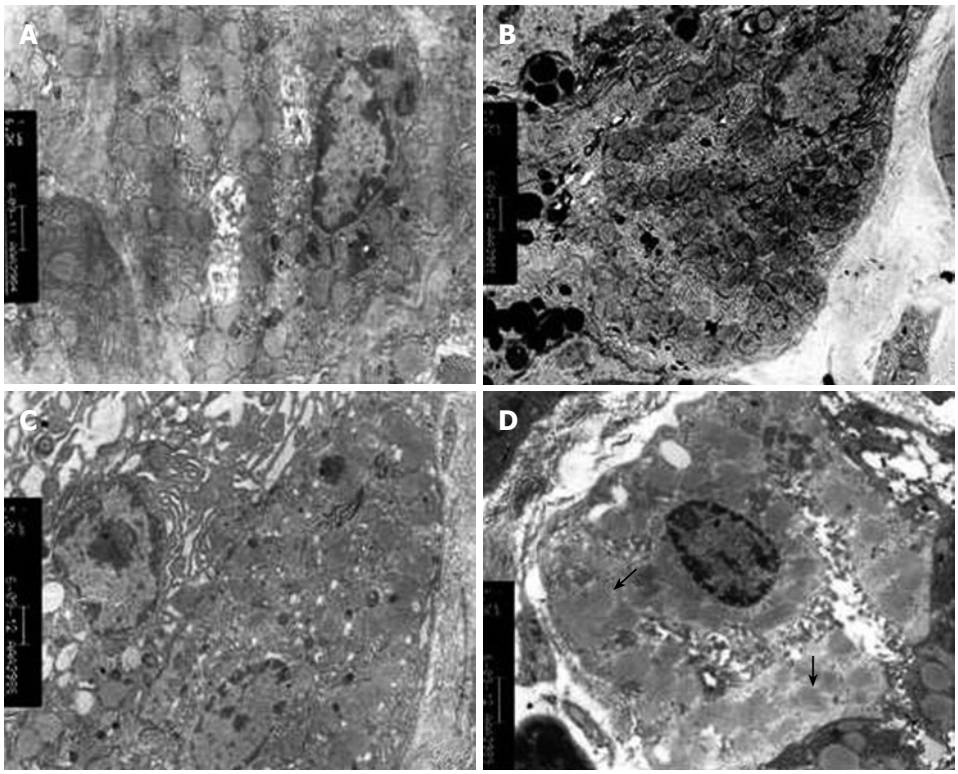


Figure 5 Changes in mitochondrial ultra-structure of rats with ethanol-induced acute gastric mucosa injury 2 h after administration of normal sodium (**A**), 40% (W/W) ethanol (**B**) 70% (W/W) ethanol (**C**), and 100% (W/W) ethanol (**D**), respectively. Arrows indicate swollen and collapsed mitochondria and dissolved and diminished mitochondrial cristae.

the increasing ethanol concentration. Compared with the control group, the expression of mtDNA ATPase subunits 6 and 8 mRNA increased in rats that received 40% (W/W) ethanol. However, the expression of mtDNA ATPase subunits 6 and 8 mRNA decreased in rats that received 100% (W/W) ethanol compared with rats in the control group. A same tendency was observed in rats that received 70% (W/W) ethanol (Table 7, Figure 7).

Expression of mtDNA ATPase subunits 6 and 8 mRNA in gastric mucosa with ethanol-induced chronic injury

The expression of mtDNA ATPase subunits 6 and 8 mRNA was significantly different in rats with alcohol-induced chronic injury ($P < 0.01$). However, it was negatively related with both of ethanol concentration and gavage time (Table 9, Figures 8 and 9).

Correlation analysis of mucosal injury, MDA, ATPase subunits 6 and 8

MDA and LI were positively correlated with ethanol-induced acute and chronic injury. However, ATPase subunits 6 and 8 were negatively correlated with L1 and MDA.

DISCUSSION

We successfully established an acute and chronic gastric mucosa injury model by oral administration of ethanol. MDA, a product of interaction between OFR and cell membrane, indirectly represents the OFR content in

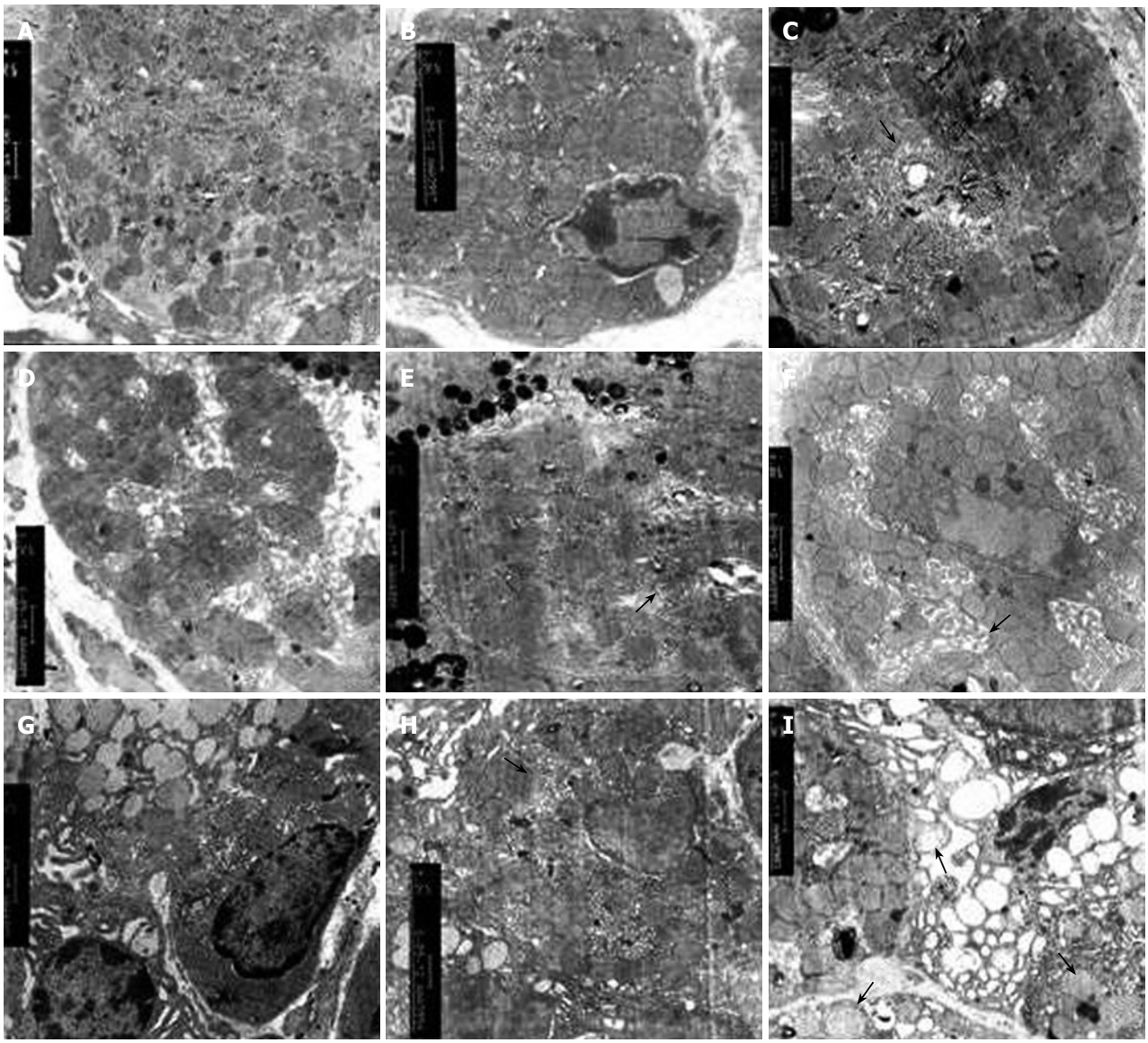


Figure 6 Changes in mitochondrial ultra-structure of rats with ethanol-induced chronic gastric mucosa injury 3 wk after administration of normal sodium (**A**), 25% (W/W) ethanol (**B**), and 45% (W/W) ethanol (**C**), respectively; 6 wk after administration of normal sodium (**D**), 25% (W/W) ethanol (**E**), and 45% (W/W) ethanol (**F**), respectively; 9 wk after administration normal sodium (**G**), 25% (W/W) ethanol (**H**), and 45% (W/W) ethanol (**I**), respectively.

cells. The MDA content was significantly higher in rats with ethanol-induced acute and chronic injury than in those of the normal control group. Furthermore, MDA increased with the increasing ethanol concentrations indicating that MDA is positively correlated with ethanol concentration and that ethanol metabolism in the stomach produces a large quantity of OFR in a dose-dependent manner. In the acute and chronic gastric mucosa injury model, the gastric mucosal damage aggravated with the increasing content of gastric MDA. OFR tended to react with unsaturated fatty acids in cell membrane, indicating that lipid peroxidation chain reaction decreases unsaturated fatty acid content and finally affects the fluidity and permeability of cell membrane[11,12]. Gastric mucosa is rich in protein sulfhydryl groups, which may be the target of OFR. Oxidized protein sulfhydryl groups lead to protein denaturation or enzyme inactivation, and receptor damage or modification on cell membrane, thus contributing to mucosal injury[13,14]. Gastric parietal cells are abundant in mitochondria, which are critical for maintaining the normal morphology and function of gastric mucosa since they afford usable energy through oxidative phosphorylation. Two hours after administration of 100% (W/W) ethanol, mitochondrial structure was significantly damaged in the acute gastric mucosal injury model, mitochondria became swollen and disaggregated, and mitochondrial cristae were dissolved and disappeared. Nine weeks after administration of 45% (W/W) ethanol, obscuring mitochondrial structure and swollen mitochondrial cristae were found in the chronic gastric mucosal injury model. However, no significant changes occurred in other experimental groups, which may be due to the following factors, such as a small sample size. The number of mitochondria in the gastric parietal cells reduced with the increasing ethanol concentration and time, and the number of mitochondria was negatively correlated with MDA in

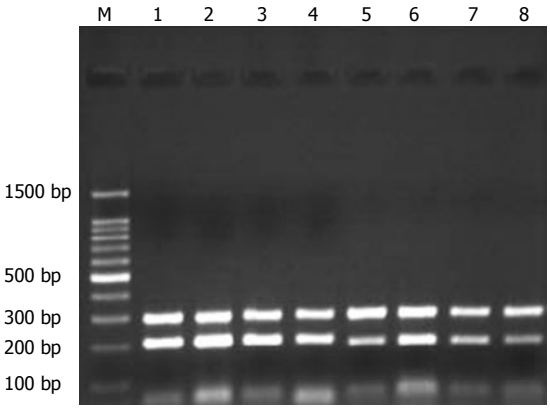


Figure 7 RT-PCR for expression of mtDNA ATPase subunits 6 and 8 mRNA in rates with ethanol-induced acute gastric mucosa injury. M: 100 bp DNA ladder was a RT-PCR product of *β-actin* mRNA (upper row, 300 bp); lanes 1-4: RT-PCR products of mtDNA ATPase subunit 6 mRNA after treatment with normal sodium, 40% (W/W) ethanol, 70% (W/W) ethanol, and 100% (W/W) ethanol, respectively; lanes 5-8: RT-PCR products of mtDNA ATPase subunit 8 mRNA after treatment with normal sodium, 40% (W/W) ethanol, 70% (W/W) ethanol, and 100% (W/W) ethanol, respectively.

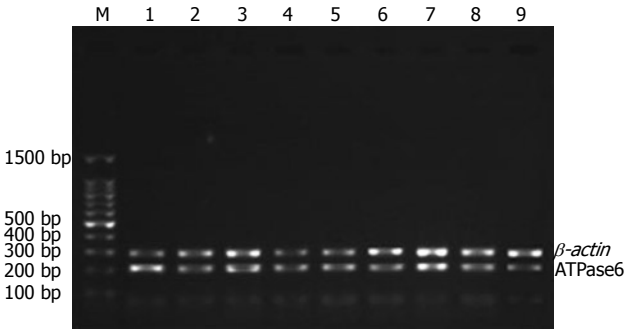


Figure 8 RT-PCR for expression of mtDNA ATPase subunit 6 mRNA in rats with ethanol-induced chronic gastric mucosa injury. M: 100 bp DNA ladder of RT-PCR products of *β-actin* mRNA (upper row, 300 bp); lanes 1-9: RT-PCR products of mtDNA ATPase subunit 6 mRNA 3 wk after treatment with normal sodium, 25% (W/W) ethanol, and 45% (W/W) ethanol, respectively; 6 wk after treatment with normal sodium, 25% (W/W) ethanol, and 45% (W/W) ethanol, respectively; 9 wk after treatment with normal sodium, 25% (W/W) ethanol, and 45% (W/W) ethanol, respectively.

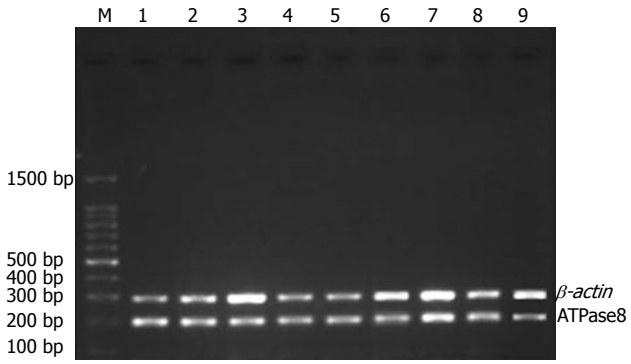


Figure 9 RT-PCR for expression of mtDNA ATPase subunit 8 mRNA in rats with ethanol-induced chronic gastric mucosa injury. M: 100 bp DNA ladder of RT-PCR products of *β-actin* mRNA (upper row, 300 bp); lanes 1-9: RT-PCR products of mtDNA ATPase subunit 8 mRNA 3 wk after treatment with normal sodium, 25% (W/W) ethanol, and 45% (W/W) ethanol, respectively; 6 wk after treatment with normal sodium, 25% (W/W) ethanol, and 45% (W/W) ethanol, respectively; 9 wk after treatment with normal sodium, 25% (W/W) ethanol, and 45% (W/W) ethanol, respectively.

the acute and chronic gastric mucosal injury model, suggesting that OFR can damage mitochondria.

mtDNA and expression of encoding genes have direct effects on mitochondrial respiration and ATP synthesis. mtDNA ATPase subunits 6 and 8, which are independently encoded by mtDNA, are critical parts of F_0 in the respiratory enzyme, F_0F_1-ATPase and involved in cellular energy metabolism, acting as a proton channel in the synthesis of ATP and controlling ATP synthesis by regulating the proton transfer. mtDNA is susceptible to the attack of OFR[15], which leads to the alteration of gene expression[16]. The following characteristics of mtDNA are believed to be the main cause of this phenomenon[17-19]. mtDNA lacks the protection of histone and effective repair systems against DNA damage and is near to the inner mitochondrial membrane where OFR, a highly mutagenic product of oxidative phosphorylation, generates. mtDNA has tightly aligned genes containing few intron sequences so that random mutation may significantly change the encoding sequence of mtDNA. In the field of ethanol-induced gastric injury, few reports are available on whether OFR affects the expression of mtDNA ATPase. In the present study, the mRNA expression of mtDNA ATPase subunits 6 and 8 in acute and chronic gastric injury model decreased with the increasing ethanol concentration and was negatively correlated with the increasing MDA content, showing that OFR is negatively related with the expression of ATPase subunits 6 and 8 and that ethanol-induced gastric mucosa injury may be achieved through the detrimental effect of OFR on cellular energy metabolism. Decreasing the mRNA expression of ATPase subunits 6 and 8 can reduce the synthesis of ATPase subunit 6 and 8, the encumbrance of proton channel F_0 and the activity of ATPase. Thus, the synthesis of ATP may be hindered and bring about the scarcity of provided energy. However, injured cells have an urgent demand of energy to fulfill the repair process. The contradiction between energy supply and demand eventually aggravates gastric mucosa injury, falling into a vicious cycle. The reduction in or lack of ATP synthesis may damage the gastric mucosa by inducing apoptosis and hampering the reconstruction of gastric epithelial injury. One important reaction to the epithelial damage is the rapid repair or epithelia rebuilding to recover the continuity and barrier function of mucosal epithelia. As reconstruction of gastric epithelial injury is a highly ATP-dependent process, lack of ATP may hinder this process and oxidative phosphorylation[20,21]. Lack of ATP may lead to metabolic acidosis, cellular edema, intracellular calcium overload, and further damage to gastric mucosa cells[22]. Therefore, it sounds reasonable that OFR plays an important role in ethanol-induced gastric mucosa injury. Damage may eventually affect, at least in part, cellular energy metabolism. The expression of ATPase subunits 6 and 8 in rats that received 40% (W/W) ethanol

was higher than that in those of the control group, suggesting that injured gastric mucosa can protect itself against a certain extent of oxidative stress.

Our study systematically described gastric mucosa injury from three levels, namely gross, microscopic appearance and ultrastructure. We also discussed the relationship between disturbed mitochondrial structure and oxidative stress caused by ethanol. We conclude that disorder of mitochondrial energy metabolism may play an important, role in the pathogenesis of ethanol-related gastropathy. However, changes in ATPase subunits 6 and 8 at protein level and alteration of mitochondrial membrane potential remain to be elucidated.

REFERENCES

1 **Alarcon JM**, Quevedo L, Reyes P. Inhibitory action of hydrogen peroxide on a high-resistance epithelium. *Pharmacology* 1995; **50**: 111-118

2 **Itoh M**, Guth PH. Role of oxygen-derived free radicals in hemorrhagic shock-induced gastric lesions in the rat. *Gastroenterology* 1985; **88**: 1162-1167

3 **Duman JG**, Pathak NJ, Ladinsky MS, McDonald KL, Forte JG. Three-dimensional reconstruction of cytoplasmic membrane networks in parietal cells. *J Cell Sci* 2002; **115**: 1251-1258

4 **Shimokawa T**, Yamagiwa D, Hondo E, Nishiwaki S, Kiso Y, Makita T. Histological observation of the proper gastric gland in Minke whale, Balaenoptera acutorostrata. *J Vet Med Sci* 2003; **65**: 423-426

5 **Yin GY**, Zhang WN, Shen XJ, Chen Y, He XF. Ultrastructure and molecular biological changes of chronic gastritis, gastric cancer and gastric precancerous lesions: a comparative study. *World J Gastroenterol* 2003; **9**: 851-857

6 **Baracca A**, Barogi S, Carelli V, Lenaz G, Solaini G. Catalytic activities of mitochondrial ATP synthase in patients with mitochondrial DNA T8993G mutation in the ATPase 6 gene encoding subunit a. *J Biol Chem* 2000; **275**: 4177-4182

7 **Li HS**, Zhang JY, Thompson BS, Deng XY, Ford ME, Wood PG, Stolz DB, Eagon PK, Whitcomb DC. Rat mitochondrial ATP synthase ATP5G3: cloning and upregulation in pancreas after chronic ethanol feeding. *Physiol Genomics* 2001; **6**: 91-98

8 **Hoek JB**, Cahill A, Pastorino JG. Alcohol and mitochondria: a dysfunctional relationship. *Gastroenterology* 2002; **122**: 2049-2063

9 **Guth PH**, Aures D, Paulsen G. Topical aspirin plus HCl gastric lesions in the rat. Cytoprotective effect of prostaglandin, cimetidine, and probanthine. *Gastroenterology* 1979; **76**: 88-93

10 **Masuda E**, Kawano S, Nagano K, Tsuji S, Takei Y, Hayashi N, Tsujii M, Oshita M, Michida T, Kobayashi I. Role of endogenous endothelin in pathogenesis of ethanol-induced gastric mucosal injury in rats. *Am J Physiol* 1993; **265**: G474-G481

11 **Bagchi D**, Carryl OR, Tran MX, Krohn RL, Bagchi DJ, Garg A, Bagchi M, Mitra S, Stohs SJ. Stress, diet and alcohol-induced oxidative gastrointestinal mucosal injury in rats and protection by bismuth subsalicylate. *J Appl Toxicol* 1998; **18**: 3-13

12 **Repetto M**, Maria A, Guzman J, Giordano O, Llesuy S. Protective effect of Artemisia douglasiana Besser extracts in gastric mucosal injury. *J Pharm Pharmacol* 2003; **55**: 551-557

13 **Bilici D**, Suleyman H, Banoglu ZN, Kiziltunc A, Avci B, Ciftcioglu A, Bilici S. Melatonin prevents ethanol-induced gastric mucosal damage possibly due to its antioxidant effect. *Dig Dis Sci* 2002; **47**: 856-861

14 **La Casa C**, Villegas I, Alarcon de la Lastra C, Motilva V, Martin Calero MJ. Evidence for protective and antioxidant properties of rutin, a natural flavone, against ethanol induced gastric lesions. *J Ethnopharmacol* 2000; **71**: 45-53

15 **Penta JS**, Johnson FM, Wachsman JT, Copeland WC. Mitochondrial DNA in human malignancy. *Mutat Res* 2001; **488**: 119-133

16 **Ballinger SW**, Patterson C, Yan CN, Doan R, Burow DL, Young CG, Yakes FM, Van Houten B, Ballinger CA, Freeman BA, Runge MS. Hydrogen peroxide- and peroxynitrite-induced mitochondrial DNA damage and dysfunction in vascular endothelial and smooth muscle cells. *Circ Res* 2000; **86**: 960-966

17 **Bai RK**, Wong LJ. Detection and quantification of heteroplasmic mutant mitochondrial DNA by real-time amplification refractory mutation system quantitative PCR analysis: a single-step approach. *Clin Chem* 2004; **50**: 996-1001

18 **Zeviani M**, Antozzi C. Mitochondrial disorders. *Mol Hum Reprod* 1997; **3**: 133-148

19 **Shukla A**, Jung M, Stern M, Fukagawa NK, Taatjes DJ, Sawyer D, Van Houten B, Mossman BT. Asbestos induces mitochondrial DNA damage and dysfunction linked to the development of apoptosis. *Am J Physiol Lung Cell Mol Physiol* 2003; **285**: L1018-L1025

20 **Scholte HR**. The biochemical basis of mitochondrial diseases. *J Bioenerg Biomembr* 1988; **20**: 161-191

21 **Scholte HR**, Busch HF, Luyt-Houwen IE, Vaandrager-Verduin MH, Przyrembel H, Arts WF. Defects in oxidative phosphorylation. Biochemical investigations in skeletal muscle and expression of the lesion in other cells. *J Inherit Metab Dis* 1987; **10** Suppl 1: 81-97

22 **Rong Q**, Utevskaya O, Ramilo M, Chow DC, Forte JG. Nucleotide metabolism by gastric glands and H(+)-K(+)-ATPase-enriched membranes. *Am J Physiol* 1998; **274**: G103-G110

S- Editor Xiao LL **L- Editor** Wang XL **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5868

World J Gastroenterol 2008 October 14; 14(38): 5868-5875
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Effects of cardiopulmonary bypass on tight junction protein expressions in intestinal mucosa of rats

Ying-Jie Sun, Wei-Min Chen, Tie-Zheng Zhang, Hui-Juan Cao, Jin Zhou

Ying-Jie Sun, Wei-Min Chen, Department of Anesthesiology, ShengJing Hospital, China Medical University, Shenyang 110004, Liaoning Province, China
Ying-Jie Sun, Tie-Zheng Zhang, Hui-Juan Cao, Jin Zhou, Department of Anesthesiology, the Northern Hospital of China, Shenyang 110016, Liaoning Province, China
Author contributions: Chen WM, Zhang TZ and Sun YJ designed the research; Chen WM, Zhang TZ, Sun YJ, Cao HJ and Zhou J performed the research; Sun YJ and Cao HJ offered new reagents/analytic tools; Sun YJ, Cao HJ and Zhou J analyzed data; and Chen WM, Zhang TZ and Sun YJ wrote the paper.
Correspondence to: Dr. Wei-min Chen, Department of Anesthesiology, ShengJing Hospital, China Medical University, Shenyang 110004, Liaoning Province, China. chenwm2h@cmu.com
Telephone: +86-24-83955043 Fax: +86-24-23268873
Received: March 6, 2008 Revised: July 13, 2008
Accepted: July 20, 2008
Published online: October 14, 2008

Abstract

AIM: To investigate the tight junction protein expressions of intestinal mucosa in an experimental model of cardiopulmonary bypass (CPB) in rats.

METHODS: Thirty anesthetized rats were randomly divided into two groups: Group S (n = 10) served as sham operation and group C (n = 20) served as CPB which underwent CPB for 1 h. Expression of occludin and zonula occludens-1 (ZO-1) were determined by Western blotting and immunocytochemistry, respectively. Plasma levels of diamine oxidase (DAO) and d-lactate were determined using an enzymatic spectrophotometry.

RESULTS: Immunohistochemical localization of occludin and ZO-1 showed disruption of the tight junctions in enterocytes lining villi at the end of CPB and 2 h after CPB. The intensities of the occludin and ZO-1 at the end of CPB were lower than those of control group (76.4% ± 22.5% *vs* 96.5% ± 28.5% and 62.4% ± 10.1% *vs* 85.5% ± 25.6%, $P < 0.05$) and were further lower at 2 h after CPB (50.5% ± 10.5% and 45.3% ± 9.5%, $P < 0.05$). Plasma d-lactate and DAO levels increased significantly (8.688 ± 0.704 *vs* 5.745 ± 0.364 and 0.898 ± 0.062 *vs* 0.562 ± 0.035, $P < 0.05$) at the end of CPB compared with control group and were significantly higher at 2 h after CPB than those at the end of CPB (9.377 ± 0.769 and 1.038 ± 0.252, $P < 0.05$). There were significant negative correlations between occludin or ZO-1 expression and DAO ($r^2 = 0.5629$, $r^2 = 0.5424$, $P < 0.05$) or d-lactate levels ($r^2 = 0.6512$, $r^2 = 0.7073$, $P < 0.05$) both at the end of CPB and 2 h after CPB.

CONCLUSION: CPB markedly down-regulates the expression of occludin and ZO-1 proteins in intestinal mucosa of rats. The close correlation between expression of tight junctions (TJs) and plasma levels of DAO or d-lactate supports the hypothesis that intestinal permeability increases during and after CPB because of decreases in the expressions of TJs.

Key words: Cardiopulmonary bypass; Occludin; ZO-1; Tight junction; Diamine oxidase; d-lactate

Peer reviewer: Jordi Camps, PhD, Centre de Recerca Biomèdica, Hospital Universitari de Sant Joan, C. Sant Joan s/n, Reus 43201, Spain

Sun YJ, Chen WM, Zhang TZ, Cao HJ, Zhou J. Effects of cardiopulmonary bypass on tight junction protein expressions in intestinal mucosa of rats. *World J Gastroenterol* 2008; 14(38): 5868-5875 Available from: URL: http://www.wjgnet.com/1007-9327/14/5868.asp DOI: http://dx.doi.org/10.3748/wjg.14.5868

INTRODUCTION

Intestinal mucosa serves as a major anatomic and functional barrier separating potentially harmful intraluminal elements such as bacteria and endotoxins from extraintestinal tissues and the systemic circulation[1]. Some studies have shown that cardiopulmonary bypass (CPB) predisposes the intestines to inadequate perfusion, hypoxic injury, and increases in gut permeability[2,3]. During CPB, endotoxin which may originate from the gut has been found to rise in the circulation and may contribute to the cytokine and complement activation caused by the exposure of blood to artificial surfaces of the oxygenator. The resulting proinflammatory reaction can cause fever and high oxygen consumption in the direct postoperative period, *i.e.*, the so-called post-perfusion (or "post-pump") syndrome[4-6].

Tight junctions (TJs) are located at the apical part

of lateral membranes of polar epithelial cells, forming a barrier that regulates the permeability of ions, macromolecules, and cells through the paracellular pathway[7,8]. Three groups of macromolecules are considered as bona fide integral components of the TJ: occludin(s), claudins and junction adhesion molecule[9]. Zonula occludens (ZO)-1, -2 and -3 are the major TJ-plaque proteins that bind to the intracellular domain of occludins. The interaction between occludin and ZO-1 plays a crucial role in maintaining the structure of TJ and epithelial barrier function[10]. TJ structure and function present a highly dynamic nature, subjected to regulation by a variety of intracellular and extracellular signals, both in normal and pathologic conditions[7,8]. Intracellular events that may influence TJs' stability are related to energy depletion and extracellular events include cytokine activation, oxidative stress, attachment of enteropathogens on epithelial surface and neutrophil transmigration[7,11-14]. Most of these events have been demonstrated to play an important role during CPB[15-17].

According to our knowledge, there are no previous studies investigating whether cardiopulmonary bypass disrupts intestinal mucosal barrier by altering TJs' formation and function. The present study was initiated to address two important questions in the field of CPB-induced gut barrier dysfunction and the molecular mechanisms involved: first, to determine any change in occludin and ZO-1 expressions in the intestinal epithelium and, second, to determine if there is any correlation between occludin or ZO-1 expressions and intestinal mucosal damage during CPB, as reflected by plasma levels of diamine oxidase (DAO) and *d*-lactate[18,19].

MATERIALS AND METHODS

Materials

Adult male Sprague-Dawley (SD) rats (provided by the department of experimental animals, China Medical University) weighing 300-400 g were used in the present study. They were housed in stainless steel cages, three rats per cage, under controlled temperature (23℃) and humidity conditions, with 12-h dark/light cycles, and maintained on standard laboratory diet with tap water ad libitum throughout the experiment, except for an overnight fast before surgery.

The experiments were carried out according to the guidelines set forth by the Ethics Committee of China Medical University, Shenyang, China.

Methods

Experimental protocol: Thirty rats were randomly divided into two groups: Group S (n = 10) served as sham-operation and group C (n = 20) served as CPB. The rats of group C underwent 60 min of CPB and were sacrificed at the end of CPB (n = 10) or at 2 h after CPB (n = 10) randomly.

Surgical procedure for CPB[20-22]: Rats were anaesthetized with intraperitoneal administration of chloral hydrate (350 mg/kg). Once the anesthesia was achieved for the surgical level, the rats were placed in supine position. A 14G catheter was inserted into the trachea and the lungs were mechanically ventilated with a small animal ventilator (DH2800, Zhejiang University Medical Apparatus Co, China). The tidal volume was approximately 10 mL/kg, the respiratory rate was 60/min, peak airway pressure was 20-30 mmH_2O and the respiration concentration of O_2 was 100%. Anesthesia was maintained throughout the experiment with additional doses of chloral hydrate (100-150 mg/kg). All subsequent procedures were performed under aseptic conditions.

The left femoral artery was cannulated with a 24-gauge catheter to monitor systemic arterial pressure (SAP) and to collect arterial blood (0.2 mL) for arterial blood gas analysis before CPB, 30 min, 1 h and 2 h after CPB (blood gasanalyzer, GEM Premier 3000, USA). The homolateral femoral vein was cannulated with a 20-gauge catheter for fluid replacement (0.9% NS 0.5-1.0 mL/h). Following administration of heparin (300 U/kg), a 16-gauge catheter, modified to a multiside-orifices cannula in the forepart, was inserted into the right jugular vein and advanced to the right atrium for blood drained. A 22-gauge catheter was cannulated to the right carotid artery which served as the arterial infusion line for the CPB circuit. The electrocardiogram (ECG) was performed and rectal temperature (RT) was monitored.

The minute CPB circuit comprised a venous reservoir, a specially designed membrane oxygenator (Ke Wei Co. Ltd, Guandong, China), a roller pump(Longer Precision Pump Co. Ltd, Baoding, China), and sterile tubing with an inner diameter of 4 mm for the venous line and of 1.6 mm for the arterial line. The main priming fluid of the circuit constituted Hespander (synthetic colloid, 7 mL), Ringer's lactate solution (7 mL), 15% mannitol (1 mL) and 8% sodium bicarbonate (1 mL). After careful check, the clamps were released and the bypass was started. With an inspired oxygen fraction of 100%, 50 mL/min gas flow was sufficient to achieve adequate oxygenation and to maintain the $PaCO_2$ within 35-45 mmHg. At the initiation of perfusion, the flow-rate was gradually adjusted to a level that would sustain the arterial pressure near 60 mmHg. At this point, mechanical ventilation was terminated and CPB was stably performed at 90-100 mL/kg per min throughout the experiment. Body central temperature was monitored with a rectal probe and kept at about 33℃. After 60 min, the bypass was terminated when the temperature gradually elevated to 37℃ by a heat lamp placed above the animal and the infusion warmer (BIGGER, CBW68, USA). The mechanical ventilation was resumed until the rats gained steady active breath. The remaining priming solution was infused gradually after the termination of CPB and dopamine (3 μg/kg per min) was infused when the main arterial pressure was less than 60 mmHg.

The anesthetic and surgical procedure performed in the sham-group (n = 10) were the same as in the CPB group. Stable Mbp was maintained, but without CPB, for the same duration of surgery and anesthesia as in the CPB 1 h or 2 h group.

Sample collection: The rats from each group were sacrificed by decapitation. The arterial blood (0.2 mL) was sampled before CPB, at the end of CPB and 2 h after CPB for determination of serum DAO and *d*-lactate levels. At end of CPB and 2 h after CPB, intestinal (ileum) tissue samples were collected for immunohisto-chemical and Western blotting analysis. The animals in the sham-group were sacrificed at the end of the time period equivalent to the CPB-2 h group. All samples were determined in a blinded fashion; results were not available until completion of the study.

Plasma *d*-lactate measurement: Plasma *d*-lactate levels were measured by an enzymatic spectrophotometric assay using a centrifugal analyzer at 30℃ (Hoffmann-LaRoche, Basle, Switzerland) as described earlier[23]. *d*-lactate, *d*-lactate dehydro-genase and NAD^+ were purchased from Sigma Chemical Company (Milan, Italy).

Plasma DAO measurement: Plasma DAO activities were determined using an enzymatic spectrophotometry as described earlier[24]. O-dianisidine, Gadaverine Dihydrochloride and DAO were purchased from Sigma Chemical Company (Milan, Italy).

Immunohistochemistry for occludin and ZO-1: Four to 5 μm thick histologic sections, mounted on gelatinized glass slides, were dewaxed in xylene and hydrated through a series of graded concentrations of alcohol. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide in methanol for 15 min. Sections were then processed in a microwave oven twice, 5 min each time at high power for antigen retrieval. Subsequently, a standard streptavidin-biotin-peroxidase technique was applied to detect occludin and ZO-1. After incubation with 1% bovine serum albumin to block nonspecific binding, sections were incubated with rabbit polyclonal antioccludin or antiZO-1 antibody (1:100, Zymed Laboratories, San Francisco, CA) for 1 h, and with biotinylated antirabbit immunoglobulin-G for 30 min. Color was developed with diaminobenzidine (Sigma Fast DAB tablets, D-4293) and counterstained with Harris hematoxylin. All procedures took place at room temperature and sections were washed in tris-buffered saline. The specimens were examined under microscope (Olympus BX51, Japan). The integrated optical density of ZO-1 or occludin in intestinal sections was analyzed using an image analysis system (UIC, MetaMorph, USA).

Western blotting analysis: After rats were killed as described above, terminal ileums were isolated, excised, flushed with ice-cold PBS to remove the fecal debris, and then opened longitudinally. Intestinal mucosa was harvested with a razor blade. Tissues were homogenized by mechanical disruption in lysis buffer (50 mmol/L Tris-Cl, pH 8.0, 150 mmmol/L NaCl, 0.1% dodecyl sodium sulfate, 1% Nonidet P-40, 0.5% sodium deoxycholate, 100 mg/mL phenylmethylsulfonyl fluoride, 1 mg/mL Aprotinin) and incubated on ice for 30 min. Homogenate was then pelleted at 6000 *g* for 10 min and supernatant was collected. Using Lowry method, the quantity of total protein was determined in the supernatant. The protein extracts were incubated with primary antisera (rabbit anti-occludin and anti-ZO-1, 1:500; Zymed Laboratories, San Francisco, CA) at 4℃ overnight and then the mixture was incubated with secondary antibody (horseradish peroxidase conjugated goat anti-rabbit IgG, 1:1000; Beijing Zhongshan Biotech,Co.) at 4℃ for 6-8 h. Immunoprecipitates were subjected to SDS-PAGE and electrotransferred to nitrocellulose membrane. After a final wash in TBST, using the enhanced chemiluminescence (ECL) kit (Shang hai Pufei Biotech, Co.), chemluminescent signals were collected on autoradiographic film. The quantity of band intensity was carried out using electrophoresis Gel Imaging Analysis System (ChemiImager 5500, ALPHA INNOTECH, USA). Results were expressed as ratio of relative intensity of target protein to that of the internal standard, β-actin.

Electron microscopy: Samples were immediately fixed in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4) for 2 h at 22℃, rinsed for 18 h (4℃) with 0.05 Tris buffer (pH 7.6), and washed three times, for 5 min each time. An H-2000 transmission electron microscope was used for the examination.

Statistical analysis

Data were analyzed using a sigma stat statistical software (SPSS, Chicago, IL) and expressed as mean ± SD. Statistical significance of differences between groups were determined using one way analysis of variance (ANOVA) followed by LDS (for multiple comparisons. Linear regression analysis was used to assess associations between TJ protein relative expression levels and DAO or *d*-lactate levels at each period. $P < 0.05$ was considered as significant in difference.

RESULTS

Rat profile

All experiments progressed without incident and all animals survived from the operative process. Table 1 displays the physiologic data from the CPB and Sham rats over the study period. In the CPB group, CPB and post-CPB MAP, PaO_2 values were significantly lower than baseline ($P < 0.05$), whereas $PaCO_2$ remained stable. In the Sham group, there were no changes in MAP, PaO_2 and $PaCO_2$during the whole experiment. Hematocrit (HCT) had a significant decrease in the CPB group ($P < 0.05$) during the perfusion process but had no change in the Sham group. The pH values in the CPB group decreased and this group showed a tendency to develop a metabolic acidosis.

DAO activity (U/L)

There were no significantly differences between CPB

Table 1 Physiological data over the study period

Variables	Groups	Pre-CPB	30 min after CPB	60 min after CPB	2 h after CPB
MAP (mmHg)	CPB	84.50 ± 7.05	62.14 ± 15.23[a,c]	67.08 ± 19.12[a,c]	72.18 ± 17.39[a,c]
	Sham	86.33 ± 16.82	84.26 ± 14.55	85.45 ± 10.36	83.63 ± 11.24
HR (/min)	CPB	315 ± 30	286 ± 28	305 ± 42	310 ± 37
	Sham	325 ± 34	320 ± 25	315 ± 20	324 ± 15
pH	CPB	7.41 ± 0.03	7.34 ± 0.05[a,c]	7.33 ± 0.06[a,c]	7.39 ± 0.02
	Sham	7.40 ± 0.02	7.41 ± 0.03	7.39 ± 0.02	7.40 ± 0.02
PaO_2 (mmHg)	CPB	399 ± 23	209 ± 94[a,c]	125 ± 40[a,c]	366 ± 40
	Sham	402 ± 22	396 ± 17	396 ± 14	402 ± 20
BE	CPB	-1.86 ± 0.35	-3.36 ± 1.22[a,c]	-4.30 ± 1.84[a,c]	-2.30 ± 1.50
	Sham	-1.96 ± 0.45	-2.36 ± 0.75	-1.54 ± 0.85	-1.95 ± 0.54
Hct (%)	CPB	41.80 ± 6.73	26.45 ± 4.24[a,c]	21.54 ± 3.71[a,c]	27.82 ± 3.66[a,c]
	Sham	41.10 ± 1.85	40.20 ± 1.53	40.60 ± 1.46	39.80 ± 1.35
RT (℃)	CPB	36.17 ± 1.85	33.17 ± 1.83[ab]	35.12 ± 2.05	36.21 ± 1.24
	Sham	36.25 ± 1.42	37.35 ± 1.65	36.42 ± 1.05	36.85 ± 1.06

MAP: Blood pressure; Hct: Hematocrit; RT: Rectal temperature; BE: Buffer excess. [a]$P < 0.05$ *vs* baseline; [c]$P < 0.05$ *vs* Sham group.

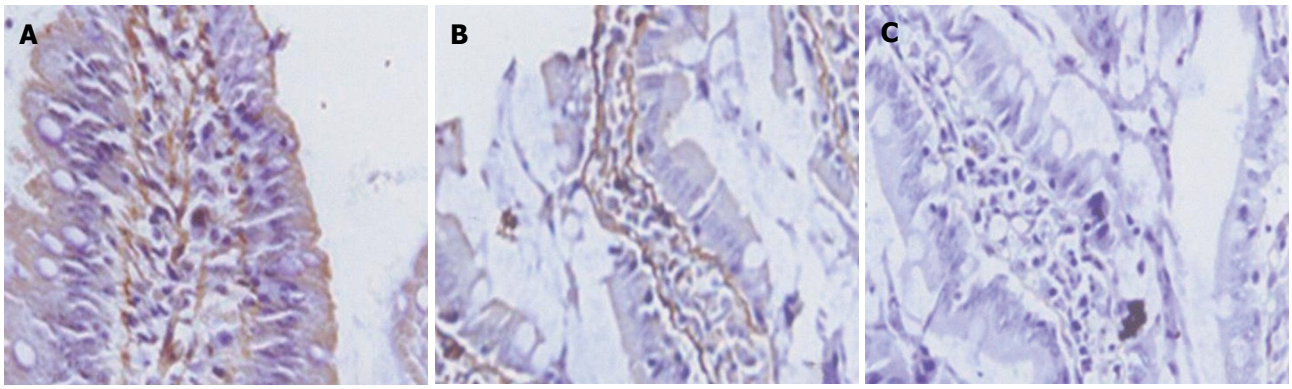


Figure 1 Immunohistochemical expression of occludin in the rat terminal ileum (x 40). **A**: Control, sham-operated, the total of epithelia cell lining villi express occludin; **B**: End of CPB, loss of occludin expression in about 50% enterocytes at the villi; **C**: 2 h after CPB, loss of occludin expression in about 85% enterocytes at the villi.

Table 2 Levels of plasma DAO and *d*-lactate (n = 10, mean ± SD)

Variables	Groups	Pre-CPB	60 min after CPB	2 h after CPB
DAO (U/L)	CPB	0.543 ± 0.061	0.898 ± 0.062[a,c]	1.038 ± 0.252[a,c,e]
	Sham	0.562 ± 0.035	0.603 ± 0.045	0.625 ± 0.054
d-lactate	CPB	5.697 ± 0.272	8.688 ± 0.704[a,c]	9.377±0.769[a,c,e]
(mg/L)	Sham	5.745 ± 0.364	5.983 ± 0.632	6.136 ± 0.847

[a]$P < 0.05$ *vs* baseline; [c]$P < 0.05$ *vs* Sham group; [e]$P < 0.05$ *vs* CPB 60 min.

groups and Sham group at baseline ($P > 0.05$, Table 2). In the CPB group, CPB and post-CPB DAO activity was significantly higher than baseline ($P < 0.05$), and DAO activity at 2 h after CPB was significantly higher than that at the end of CPB ($P < 0.05$). In the Sham group, there were no changes in plasma DAO activity during the whole experiment.

d-lactate evels (mg/L)

There were no significantly differences between CPB groups and Sham group at baseline ($P > 0.05$, Table 2). Plasma *d*-lactate levels increased significantly ($P < 0.05$) at the end of CPB compared with baseline or control group and remained higher at 2 h after CPB. In the Sham group, there were no changes in plasma *d*-lactate levels during the experiment.

Evaluation of ZO-1 and occludin immunostaining

All of the epithelial cells lining villi exhibited positive immunostaining for ZO-1 and occludin in control group (Figures 1-2A). In group C, there was loss of occludin and ZO-1 expressions in about 50% enterocytes lining villi (Figures 1-2B); this effect was more profound at 2 h after CPB and loss was about 80% (Figures 1 and 2).

Western blot analysis

Western blot analyses demonstrated alterations in expression of tight junctional proteins after CPB. Figure 3A shows the integral protein occludin with dual bands at 65-kDa .The 65-kDa band showed a significant ($P < 0.05$) decrease in group C compared with control(76.4% ± 22.5% *vs* 96.5% ± 28.5%) and reached lower value at 2 h after CPB (50.5% ± 10.5%). Figure 3B shows that ZO-1 expression significantly ($P < 0.05$) decreased after CPB compared with control (62.4% ± 10.1% *vs* 85.5% ± 25.6%) and became lower at 2 h after CPB (45.3% ± 9.5%).

Relationship between occludin or ZO-1 expression and DAO or d-lactate levels

To assess the relationship between changes of occludin

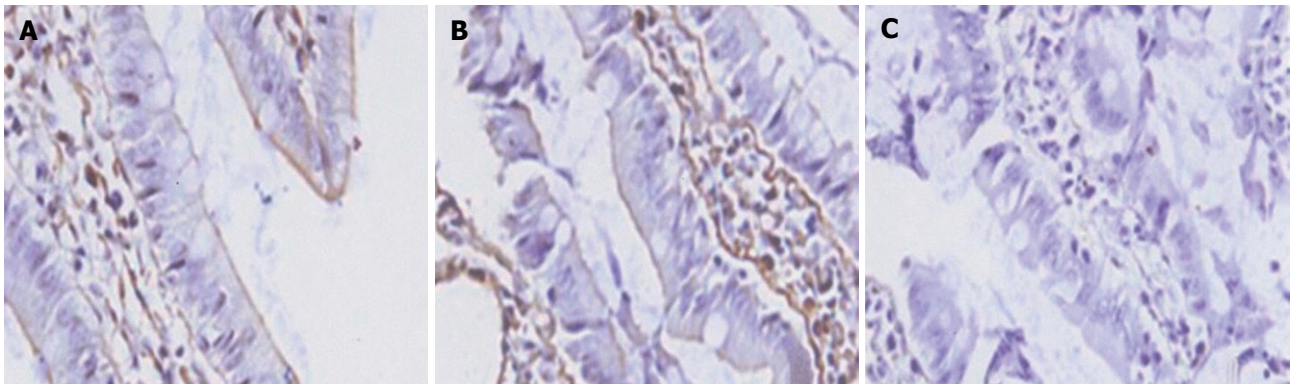


Figure 2 Immunohistochemical expression of ZO-1 in the rat terminal ileum (x 40). **A**: Control, shame-operated: the total of epithelia cell lining villi express ZO-1; **B**: End of CPB: loss of ZO-1 expression in about 50% enterocytes at the villi; **C**: 2 h after CPB: loss of ZO-1 expression in about 80% enterocytes at the villi.

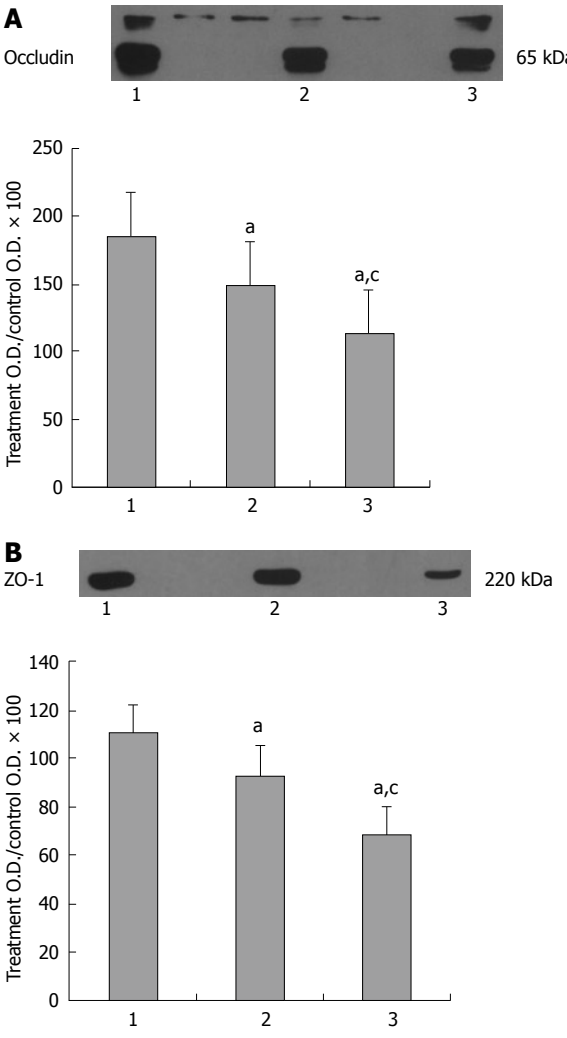


Figure 3 Western blot analysis of occludin and ZO-1 in terminal ileums. Lane 1: Control group; Lane 2: End of CPB; Lane 3: 2 h after CPB. **A**: Oclludin protein expression; **B**: ZO-1 protein expression. Total protein was extracted from rat terminal ileums. Band densities were normalized to the mean density of saline bands and expressed as % relative content. Gel staining was used as a loading control. Bars are mean % relative content ± SE, n = 10 for each group.

and ZO-1 expressions and the damages of intestinal mucosa after CPB, we used regression analysis to assess associations between TJ protein relative expression levels, DAO and *d*-lactate levels at each period. Significant negative correlations were observed between occludin or ZO-1 expression and DAO (r^2 = 0.5629, r^2 = 0.5424, $P < 0.05$) or *d*-lactate levels (r^2 = 0.6512, r^2 = 0.7073, $P < 0.05$) both at the end of CPB and at 2 h after CPB.

Ultrastructural examination of intestinal mucosa

To assess the relationship between changes of occludin and ZO-1 expressions and alterations of intestinal mucosal ultrastructure after CPB, we performed a morphological analysis of TJ (Figure 4), as occludin is an important component in TJ. In control group, regularly-aligned microvilli in intestinal epithelium, integral mitochondria and rough endoplasmic reticulum (RER) and distinct junction complex were observed. In experimental group, the amounts of microvilli were decreased with irregular length and arrangement. The mitochondria were swollen with cracked and vacuolated cristae. Some structures of the RER were destroyed. Intercellular space between epithelial cells was widened. The structure of TJ became short, and the dotted crystal structures were obscured or disappeared; other structures were disrupted and even disappeared. The damage was more severe at 2 h after CPB.

DISCUSSION

Although CPB is essential for some procedures in cardiovascular surgery, it causes peripheral hypoperfusion because of nonpulsatile flow, low blood pressure, hemodilution, and other nonphysiologic conditions. Furthermore, an increase in intestinal permeability and bacterial translocation (BT) has been demonstrated not only in animal models but also in patients during CPB[3,25,26]. Therefore, gastrointestinal integrity during the perioperative period is now recognized as an important factor for outcome of cardiac surgical procedures. In general, changes in mucosal permeability and morphology during CPB are used to reflect the degree of damage of the barrier. But studies on the molecular mechanism of the increased permeability of intestinal mucosa during CPB are rare.

The purpose of this study was to investigate the

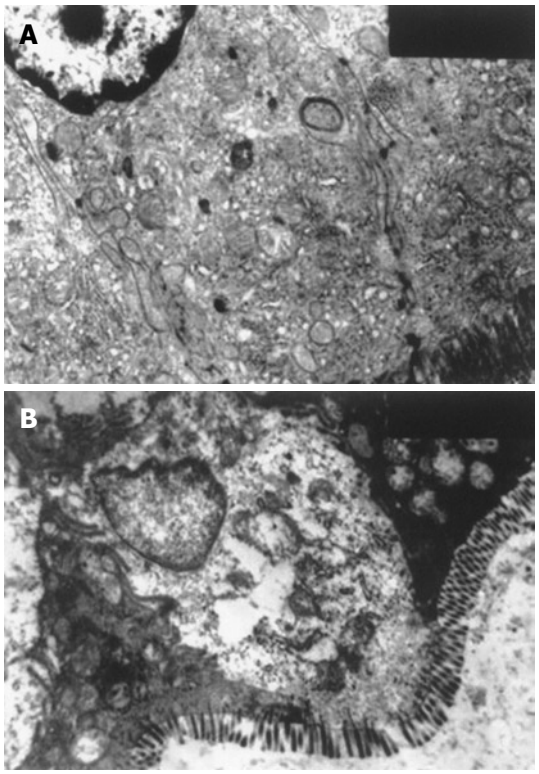


Figure 4 Effect of CPB on the ileum epithelia cells (x 7200). **A**: CPB group, epithelia damage was demonstrated by swollen mitochondria and loss of cristae, tight junction was disrupted; **B**: Control group, regularly-aligned microvilli in intestinal epithelium, integral mitochondria and RER and distinct junction complex were observed.

TJ protein expressions of intestinal mucosa in an experimental model of CPB. We chose the rat model because of the anatomical and physiologic similarity of the digestive tract and cardiovascular systems in rats and humans[27]. Using immunoblotting and immunohistochemistry, we found that CPB markedly down-regulated the expression of occludin and ZO-1 proteins in intestinal mucosa. The result demonstrated that all of epithelial cells lining villi exhibited positive immunostaining for ZO-1 and occludin in control group. In group C there was loss of occludin and ZO-1 expressions in about 50% enterocytes lining villi; this effect was more profound at 2 h after CPB. The intensity of occludin and ZO-1 at the end of CPB was weakened, and more weakened 2 h after CPB. These changes of TJs' proteins are positively associated with alterations of intestinal mucosal ultrastructure. After CPB, the mitochondria was swollen with cracked and vacuolated cristae. The structure of TJ became short, and the dotted crystal structures were obscured or disappeared; other structures were disrupted and even disappeared.

The TJ of epithelial cells forms the most apical component of the junction component of the junction complex, and appears to be a network of continuous and anastomosing filaments between adjacent epithelial cells, which consists of a continuous permeability barrier. It prevents both bacteria and toxin in the intestinal lumen from the paracellular space getting into deep tissues.

To elucidate the relationships between occludin or ZO-1 expression and intestinal mucosal damage during CPB, we measured the plasma levels of DAO and *d*-lactate used as indices of intestinal mucosal injury. DAO is localized mainly in the small intestine and reported to be elevated in intestinal ischemia[18]. Tsunooka demonstrated simultaneous increases in serum DAO activity and peptidoglycan concentration during clinical CPB, suggesting occurrence of small intestinal mucosal ischemia and BT[28,29]. In the present study, we got a similar result. During CPB, plasma DAO activity increased significantly and remained high at 2 h after CPB.

d-lactate is the dextrorotatory form of lactate commonly tested in the intensive care unit. Mammals only have one type of enzyme: *l*-lactate dehydrogenase. *l*-Lactate is a marker of cell hypoxemia, and its level correlates with survival of patients with septic shock[30-32]. Microorganisms, particularly bacteria, are equipped with *d*-lactate dehydrogenase and produce *d*-lactate during fermentation. *d*-lactate is therefore a marker of bacterial infection. *d*-lactate has also recently been proposed as a sensitive, specific, and early marker for translocation in gut ischemia[19]. In our rat model of CPB, plasma *d*-lactate levels increased significantly at the end of CPB compared with control group. It was significantly higher at 2 h after CPB.

We found significant negative correlations between occludin or ZO-1 expression and the plasma levels of DAO or *d*-lactate. These data support the hypothesis that CPB decreases occludin and ZO-1 expressions and then disruptes the intestinal integrity. Therefore, the plasma DAO activity and *d*-lactate level of the rats significantly increased. This further showed an increase of the intestinal permeability during CPB and remained higher 2 h after CPB. But the strong correlation obviously does not prove the cause and effect on intestinal permeability.

There are limitations to this complex model using rats subjected to CPB. The most important limitation of our study is that the CPB groups of animals (as compared with the Sham/control group) were exposed to severe hemodilution with much lower hematocrit, moderate hypotension, lower PaO_2, and probably lower systemic perfusion, and that these differences (*i.e.*, reduced oxygen delivery and tissue perfusion) may have caused or explained the abnormalities we observed in the CPB groups. Another limitation of the current study is that the model was non-transthoracic CPB not followed by deep hypothermic circulatory arrest. An additional limitation for this study may be the limited time that the animals were monitored after CPB. Although the postoperative period was only 2 h, numerous studies indicate that cytokine changes and neutrophil infiltration occur shortly after CPB in animals[33] and children[34].

CONCLUSION

To the best of our knowledge, this is the first study directly assessing CPB disrupts intestinal mucosal barrier by altering occludin and formation and function of

ZO-1 in rat model of CPB. Our data imply that CPB markedly down-regulates the expression of occludin and ZO-1 proteins in intestinal mucosa, indicating a possible mechanism for the increased intestinal permeability and BT during CPB. Thus, further studies are required to elucidate the regulation of the TJ of intestinal epithelium.

ACKNOWLEDGMENTS

We thank Professor Davy Cheng for his technical assistance and critically reading the manuscript. This work has been presented in a poster form at the 14th World Congress of Anesthesiologists in Cape Town, South Africa, March 5, 2008.

COMMENTS

Background

Some studies have shown that cardiopulmonary bypass (CPB) predisposes the intestines to inadequate perfusion, hypoxic injury, and increases in gut permeability. Tight junctions (TJs) are located at the apical part of lateral membranes of polar epithelial cells, forming a barrier that regulates the permeability of ions, macromolecules, and cells through the paracellular pathway. There are no previous studies investigating whether CPB disrupts intestinal mucosal barrier by altering the formation and function of TJs.

Research frontiers

In general, changes in mucosal permeability and morphology during CPB are used to reflect the degree of damage of the barrier function. But studies on the molecular mechanism of the increased permeability of intestinal mucosa during CPB are rare.

Innovations and breakthroughs

In this study, the effects of CPB on occludin and ZO-1 expressions in the intestinal epithelium of rats were investigated. Authors chose the rat model because of the anatomical and physiologic similarity of the digestive tract and cardiovascular systems in rats and humans.

Applications

The results show for the first time that CPB induced gut barrier dysfunction is associated, at the cellular level, with regional loss of occludin and ZO-1 expressions in the intestinal epithelium. These findings offer further insight in the pathophysiology of gut barrier failure during and after CPB. Understanding the pathophysiology of barrier alterations during CPB at the molecular level will help find novel and more effective treatment approaches.

Peer review

This is an interesting paper and it deals with a relevant clinical problem. The study appears well performed and the hypothesis is clear.

REFERENCES

1 **Deitch EA**. The role of intestinal barrier failure and bacterial translocation in the development of systemic infection and multiple organ failure. *Arch Surg* 1990; **125**: 403-404
2 **Ohri SK**, Bjarnason I, Pathi V, Somasundaram S, Bowles CT, Keogh BE, Khaghani A, Menzies I, Yacoub MH, Taylor KM. Cardiopulmonary bypass impairs small intestinal transport and increases gut permeability. *Ann Thorac Surg* 1993; **55**: 1080-1086
3 **Riddington DW**, Venkatesh B, Boivin CM, Bonser RS, Elliott TS, Marshall T, Mountford PJ, Bion JF. Intestinal permeability, gastric intramucosal pH, and systemic endotoxemia in patients undergoing cardiopulmonary bypass. *JAMA* 1996; **275**: 1007-1012
4 **Oudemans-van Straaten HM**, Jansen PG, Hoek FJ, van Deventer SJ, Sturk A, Stoutenbeek CP, Tytgat GN, Wildevuur CR, Eysman L. Intestinal permeability, circulating endotoxin, and postoperative systemic responses in cardiac surgery patients. *J Cardiothorac Vasc Anesth* 1996; **10**: 187-194
5 **Oudemans-van Straaten HM**, Jansen PG, te Velthuis H, Beenakkers IC, Stoutenbeek CP, van Deventer SJ, Sturk A, Eysman L, Wildevuur CR. Increased oxygen consumption after cardiac surgery is associated with the inflammatory response to endotoxemia. *Intensive Care Med* 1996; **22**: 294-300
6 **Hamilton-Davies C**, Barclay GR, Cardigan RA, McDonald SJ, Purdy G, Machin SJ, Webb AR. Relationship between preoperative endotoxin immune status, gut perfusion, and outcome from cardiac valve replacement surgery. *Chest* 1997; **112**: 1189-1196
7 **Madara JL**. Warner-Lambert/Parke-Davis Award lecture. Pathobiology of the intestinal epithelial barrier. *Am J Pathol* 1990; **137**: 1273-1281
8 **Anderson JM**, Van Itallie CM. Tight junctions and the molecular basis for regulation of paracellular permeability. *Am J Physiol* 1995; **269**: G467-G475
9 **Fanning AS**, Mitic LL, Anderson JM. Transmembrane proteins in the tight junction barrier. *J Am Soc Nephrol* 1999; **10**: 1337-1345
10 **Wittchen ES**, Haskins J, Stevenson BR. Protein interactions at the tight junction. Actin has multiple binding partners, and ZO-1 forms independent complexes with ZO-2 and ZO-3. *J Biol Chem* 1999; **274**: 35179-35185
11 **Schmitz H**, Fromm M, Bentzel CJ, Scholz P, Detjen K, Mankertz J, Bode H, Epple HJ, Riecken EO, Schulzke JD. Tumor necrosis factoralpha (TNF-a) regulates the epithelial barrier in the human intestinal cell line HT-29/B6. *J Cell Sci* 1999; **112**: 137-146
12 **Rao RK**, Basuroy S, Rao VU, Karnaky Jr KJ, Gupta A. Tyrosine phosphorylation and dissociation of occludin-ZO-1 and E-cadherin-beta-catenin complexes from the cytoskeleton by oxidative stress. *Biochem J* 2002; **368**: 471-481
13 **Simonovic I**, Rosenberg J, Koutsouris A, Hecht G. Enteropathogenic Escherichia coli dephosphorylates and dissociates occludin from intestinal epithelial tight junctions. *Cell Microbiol* 2000; **2**: 305-315
14 **Kucharzik T**, Walsh SV, Chen J, Parkos CA, Nusrat A. Neutrophil transmigration in inflammatory bowel disease is associated with differential expression of epithelial intercellular junction proteins. *Am J Pathol* 2001; **159**: 2001-2009
15 **Wehberg KE**, Foster AH, Wise RM, McLaughlin JS, Brunner MJ. Nitric oxide mediates fluid accumulation during cardiopulmonary bypass. *J Thorac Cardiovasc Surg* 1996; **112**: 168-174
16 **Hayashi Y**, Sawa Y, Nishimura M, Tojo SJ, Fukuyama N, Nakazawa H, Matsuda H. P-selectin participates in cardiopulmonary bypass-induced inflammatory response in association with nitric oxide and peroxynitrite production. *J Thorac Cardiovasc Surg* 2000; **120**: 558-565
17 **Hayashi Y**, Sawa Y, Fukuyama N, Nakazawa H, Matsuda H. Inducible nitric oxide production is an adaptation to cardiopulmonary bypass-induced inflammatory response. *Ann Thorac Surg* 2001; **72**: 149-155
18 **Bounous G**, Echave, Vobecky SJ, Navert H, Wollin A. Acute necrosis of the intestinal mucosa with high serum levels of diamine oxidase. *Dig Dis Sci* 1984; **29**: 872-874
19 **Günel E**, Cağlayan O, Cağlayan F. Serum D-lactate levels as a predictor of intestinal ischemia-reperfusion injury. *Pediatr Surg Int* 1998; **14**: 59-61
20 **Gourlay T**, Ballaux PK, Draper ER, Taylor KM. Early experience with a new technique and technology designed for the study of pulsatile cardiopulmonary bypass in the rat. *Perfusion* 2002; **17**: 191-198
21 **Dong GH**, Xu B, Wang CT, Qian JJ, Liu H, Huang G, Jing H. A rat model of cardiopulmonary bypass with excellent survival. *J Surg Res* 2005; **123**: 171-175
22 **Ballaux PK**, Gourlay T, Ratnatunga CP, Taylor KM. A literature review of cardiopulmonary bypass models for rats. *Perfusion* 1999; **14**: 411-417
23 **Fürst W**, Schiesser A. Test for stereospecifity of an automated Dd-lactate assay based on selective removal of

Ll-lactate. *Anal Biochem* 1999; **269**: 214-215
24 **Li JY**, Yu Y, Hao J, Jin H,Xu HJ. Determination of diamine oxdase activity in intestinal tissue and blood using spectrophotometry. *Amino Acids & Biotic Resources* 1996; **18**: 28-30
25 **Aydin NB**, Gercekoglu H, Aksu B, Ozkul V, Sener T, Kiygil I, Turkoglu T, Cimen S, Babacan F, Demirtas M. Endotoxemia in coronary artery bypass surgery: a comparison of the off-pump technique and conventional cardiopulmonary bypass. *J Thorac Cardiovasc Surg* 2003; **125**: 843-848
26 **Watarida S**, Mori A, Onoe M, Tabata R, Shiraishi S, Sugita T, Nojima T, Nakajima Y, Matsuno S. A clinical study on the effects of pulsatile cardiopulmonary bypass on the blood endotoxin levels. *J Thorac Cardiovasc Surg* 1994; **108**: 620-625
27 **Welsby IJ**, Jones WL, Arepally G, De Lange F, Yoshitani K, Phillips-Bute B, Grocott HP, Becker R, Mackensen GB. Effect of combined anticoagulation using heparin and bivalirudin on the hemostatic and inflammatory responses to cardiopulmonary bypass in the rat. *Anesthesiology* 2007; **106**: 295-301
28 **Tsunooka N**, Hamada Y, Imagawa H, Nakamura Y, Shiozaki T, Suzuki H, Kikkawa H, Miyauchi K, Watanabe Y, Kawachi K. Ischemia of the intestinal mucosa during cardiopulmonary bypass. *J Artif Organs* 2003; **6**: 149-151
29 **Tsunooka N**, Maeyama K, Hamada Y, Imagawa H, Takano S, Watanabe Y, Kawachi K. Bacterial translocation secondary to small intestinal mucosal ischemia during cardiopulmonary bypass. Measurement by diamine oxidase and peptidoglycan. *Eur J Cardiothorac Surg* 2004; **25**: 275-280
30 **Bakker J**, Coffernils M, Leon M, Gris P, Vincent JL. Blood lactate levels are superior to oxygen-derived variables in predicting outcome in human septic shock. *Chest* 1991; **99**: 956-962
31 **Bakker J**, Gris P, Coffernils M, Kahn RJ, Vincent JL. Serial blood lactate levels can predict the development of multiple organ failure following septic shock. *Am J Surg* 1996; **171**: 221-226
32 **Bernardin G**, Pradier C, Tiger F, Deloffre P, Mattei M. Blood pressure and arterial lactate level are early indicators of short-term survival in human septic shock. *Intensive Care Med* 1996; **22**: 17-25
33 **Brix-Christensen V**, Petersen TK, Ravn HB, Hjortdal VE, Andersen NT, Tønnesen E. Cardiopulmonary bypass elicits a pro- and anti-inflammatory cytokine response and impaired neutrophil chemotaxis in neonatal pigs. *Acta Anaesthesiol Scand* 2001; **45**: 407-413
34 **Journois D**, Israel-Biet D, Pouard P, Rolland B, Silvester W, Vouhé P, Safran D. High-volume, zero-balanced hemofiltration to reduce delayed inflammatory response to cardiopulmonary bypass in children. *Anesthesiology* 1996; **85**: 965-976

S- Editor Li DL **L- Editor** Ma JY **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5876

World J Gastroenterol 2008 October 14; 14(38): 5876-5879
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

High frequency of the c.3207C>A (p.H1069Q) mutation in *ATP7B* gene of Lithuanian patients with hepatic presentation of Wilson's disease

Laimutis Kucinskas, Jolanta Jeroch, Astra Vitkauskiene, Raimundas Sakalauskas, Vitalija Petrenkiene, Vaidutis Kucinskas, Rima Naginiene, Hartmut Schmidt, Limas Kupcinskas

Laimutis Kucinskas, Institute of Biology of Kaunas University of Medicine, Mickevičiaus 9, Kaunas LT-44307, Lithuania
Jolanta Jeroch, Rima Naginiene, Institute of Biomedical Research of Kaunas University of Medicine, Eiveniu 4, Kaunas LT-50009, Lithuania
Astra Vitkauskiene, Laboratory of Immunology and Genetics, Kaunas University of Medicine, Eiveniu 2, Kaunas LT-50009, Lithuania
Raimundas Sakalauskas, Department of Pulmonology and Immunology, Kaunas University of Medicine, Eiveniu 2, Kaunas LT-50009, Lithuania
Vitalija Petrenkiene, Limas Kupcinskas, Department of Gastroenterology, Kaunas University of Medicine, Eiveniu 2, Kaunas LT-50009, Lithuania
Vaidutis Kucinskas, Department of Human and Medical Genetics, Faculty of Medicine, Vilnius University, Santariskiu 2, Vilnius LT-08661, Lithuania
Hartmut Schmidt, Department of Hepatotransplanthology, University of Munster, Schlossplatz 2, Munster D-48149, Germany
Author contributions: Kucinskas L and Kupcinskas L contributed equally to this work; Kucinskas L designed research/performed research/wrote paper; Jeroch J wrote paper; Vitkauskiene A wrote paper/contributed new reagents/analytic tools; Sakalauskas R designed research; Petrenkiene V analysed data; Kucinskas V designed research; Naginiene R performed research; Kupcinskas L designed research/wrote paper; Schmidt H performed research/wrote paper.
Supported by The National Science and Education Foundation of Lithuania, No. M-06005
Correspondence to: Dr. Limas Kupcinskas, PhD, Professor, Department of Gastroenterology, Kaunas University of Medicine, Eiveniu 2, Kaunas LT-50009, Lithuania. likup@takas.lt
Telephone: +370-68-640575 Fax: +370-37-3361458
Received: May 30, 2008 Revised: July 19, 2008
Accepted: July 26, 2008
Published online: October 14, 2008

Abstract

AIM: To investigate the prevalence of the *ATP7B* gene mutation in patients with hepatic presentation of Wilson's disease (WD) in Lithuania.

METHODS: Eleven unrelated Lithuanian families, including 13 WD patients were tested. Clinically WD diagnosis was established in accordance to the Leipzig scoring system. Genomic DNA was extracted from whole venous blood using a salt precipitation method. Firstly, the semi-nested polymerase chain reaction (PCR) technique was used to detect the c.3207C>A (p.H1069Q) mutation. Patients not homozygous for the c.3207C>A (p.H1069Q) mutation were further analyzed. The 21 exons of the WD gene were amplified in a thermal cycler (Biometra T3 Thermocycler, Göttingen, Germany). Direct sequencing of the amplified PCR products was performed by cycle sequencing using fluorescent dye terminators in an automatic sequencer (Applied Biosystems, Darmstadt, Germany).

RESULTS: Total of 13 WD patients (mean age 26.4 years; range 17-40; male/female 3/10) presented with hepatic disorders and 16 their first degree relatives (including 12 siblings) were studied. Some of WD patients, in addition to hepatic symptoms, have had extrahepatic disorders (hemolytic anemia 3; Fanconi syndrome 1; neuropsychiatric and behavioural disorder 2). Liver biopsy specimens were available in all of 13 WD patients (8 had cirrhosis; 1-chronic hepatitis; 3-acute liver failure, 1-liver steatosis). Twelve of 13 (92.3%) WD patients had the c.3207C>A (p.H1069Q) mutation, 6 of them in both chromosomes, 6 were presented as compound heterozygotes with additional c.3472-82delGGTTTAACCAT, c.3402delC, c.3121C>T (p.R1041W) or unknown mutations. For one patient with liver cirrhosis and psychiatric disorder (Leipzig score 6), no mutations were found. Out of 16 first degree WD relatives, 11 (68.7%) were heterozygous for the c.3207C>A (p.H1069Q) mutation. Two patients with fulminant WD died from acute liver failure and 11 are in full remission under penicillamine or zinc acetate treatment. Three women with WD successfully delivered healthy babies.

CONCLUSION: The c.3207C>A (p.H1069Q) missense mutation is the most characteristic mutation for Lithuanian patients with WD. Even 92.3% of WD patients with hepatic presentation of the disease are homozygous or compound heterozygotes for the p.H1069Q mutation.

Key words: Wilson disease; *ATP7B* gene; c.3207C>A (p.H1069Q) mutation; Cirrhosis; Urine copper; Copper in liver biopsies

Peer reviewer: Peter Laszlo Lakatos, Professor, 1st Department of Medicine, Semmelweis University, Budapest H1083, Hungary

Kucinskas L, Jeroch J, Vitkauskiene A, Sakalauskas R, Petrenkiene V, Kucinskas V, Naginiene R, Schmidt H, Kupcinskas L. High frequency of the c.3207C>A (p.H1069Q) mutation in *ATP7B* gene of Lithuanian patients with hepatic presentation of Wilson's disease. *World J Gastroenterol* 2008; 14(38): 5876-5879 Available from: URL: http://www.wjgnet.com/1007-9327/14/5876.asp DOI: http://dx.doi.org/10.3748/wjg.14.5876

INTRODUCTION

Wilson's disease (WD) is a rare autosomal recessive disorder of copper metabolism with prevalence of 1/30 000 and a carrier frequency of 1/90[1,2]. The prevalence is as high as one in 10 000 in China, Japan, and Sardinia[3,4]. The disease is characterized by hepatic, neurological or psychiatric disturbances and decreasing biliary copper excretion with consequent copper accumulation in the liver and extrahepatic tissue[5]. The gene currently known to be associated with WD is the *ATP7B* gene, which was identified in 1993[6,7]. The *ATP7B* gene has 21 exons and encodes a copper-transporting *P*-type adenosine triphosphatase. A copper-transporting *P*-type ATPase incorporates copper into the serum ferroxidase, ceruloplasmin, and excretes by biliary tree. More than 360 mutations in the *ATP7B* gene have been identified as summarised in the human gene mutation database of the institute of medical genetics in Cardiff (accession date May 2008; http://www.hgmd.cf.ac.uk/ac/gene.php?gene=ATP7B). *ATP7B* gene mutations are rare and the majority of patients are compound heterozygotes. Consequently, identification of mutations in WD patients often requires sequencing of a significant proportion of the *ATP7B* gene. It is important to know regional distribution of mutations of the *ATP7B* gene to design appropriate diagnostic strategies for the disease[8]. As no data exist about WD genetic characteristics in the Lithuanian population, the purpose of the current study was to carry out a mutation analysis of Lithuanian WD patients.

MATERIALS AND METHODS

Patients

Lithuanian cohort, drawn from 11 unrelated families, consisted of 29 individuals and was comprised of 13 WD patients (mean age at onset of symptoms 26.4 years; range 17-40; male/female 3/10) and 16 of their first degree relatives. Patients were admitted at the Department of Gastroenterology, Hospital of Kaunas University of Medicine, Lithuania. The diagnosis of WD was established using the scoring system developed at the 8th International Meeting on WD, Leipzig/Germany, 2001. The diagnosis was accepted if the WD Leipzig score was $\geqslant 4$[9].

ATP7B gene mutation analysis

Genomic DNA was extracted from whole venous blood using a salt precipitation method[10]. Firstly, rapid, semi-nested PCR technique was used to detect the c.3207C>A (p.H1069Q) mutation as described previously[11]. Patients not homozygous for c.3207C>A (p.H1069Q) mutation were further analyzed. The 21 exons of the WD gene were amplified in a thermal cycler (Biometra T3 Thermocycler, Göttingen, Germany) as described elsewhere[12]. Direct sequencing of the amplified polymerase chain reaction (PCR) products was performed by cycle sequencing using fluorescent dye terminators in an automatic sequencer (Applied Biosystems, Darmstadt, Germany). Mutations were quoted according to the guidelines from http://www.HGVS.org/mutnomen/, using the reference sequence with the GenBank accession number NM_00053.1.

RESULTS

Total of 13 WD patients presented with hepatic disorders and 16 their first degree relatives (including 12 siblings) were studied. All WD patients and their relatives were consulted in Department of Gastroenterology of Kaunas University of Medicine during 1999-2008. WD patient's clinical, laboratory data, and mutation type are summarized in Table 1.

Some of WD patients, in addition to hepatic symptoms, have had extrahepatic disorders (hemolytic anemia 3; Fanconi syndrome 1; neuropsychiatric and behavioural disorder 2). Specimens of liver biopsy (either percutaneous or transjugular) were available in all of 13 WD patients (8 case of cirrhosis; chronic hepatitis-1; fulminant hepatitis-3; steatosis-1). Kayser-Fleischer ring was present in 6 out of 13 patients (46%). Hepatic copper content was measured in 7 WD patients and was above 250 μg/g dry weight in 4 cases.

Twelve of 13 (92.3%) WD patients had the c.3207C>A (p.H1069Q) mutation, 6 of them in both chromosomes, and 6 were presented as compound heterozygotes with additional c.3472-82delGGTTTAACCAT, c.3402delC, c.3121C>T (p.R1041W) or unknown mutation. For 1 patient with liver cirrhosis only mutation (p.H1069Q) in one allele was revealed, and for another patient with cirrhosis and psychiatric disorder, (Leipzig score 6) no mutations were found (Table 1). Out of 16 first degree WD relatives, 11 (68.7%) were heterozygous for the c.3207C>A (p.H1069Q) mutation. Two patients with fulminant WD died from acute liver failure (ALF), and 11 are in full remission under penicillamine or zinc acetate treatment. Three women with WD successfully delivered healthy baby.

DISCUSSION

WD might be a potentially life threatening disorder; however, with early diagnosis and consequent treatment the prognosis of WD is excellent and usually the need for liver transplantation can be prevented[13]. None of

Table 1 Clinical and genetic characteristics of Lithuanian WD patients

Patient	Gender	Age (yr) at disease diagnosis	Clinical presentation	Kayser-Fleischer ring	Ceruloplasmin lower than 0.2 g/L	Urine cooper (μg/24 h)	Cooper in liver biopsy (μg/g)	Mutation	Outcome
1	F	27	Cirrhosis	Yes	Yes	120	264	[p.His1069Gln] + [p.His1069Gln]	Alive
2	F	18	ALF	No	Yes	144	ND	[p.His1069Gln] + [p.His1069Gln]	Alive
3	M	35	Cirrhosis	Yes	Yes	84	ND	[p.His1069Gln] + [p.His1069Gln]	Alive
4	F	34	ALF, HA	No	No	280	536	[p.His1069Gln] + [p.Arg1041Trp]	Death
5	F	26	Asymptomatic CH	No	Yes	38	35	[p.His1069Gln] + [p.Arg1041Trp]	Alive
6	F	19	ALF, HA	No	No	324	326	[p.His1069Gln] + [c.3472-82 del-GGTTTAACCAT]	Death
7	F	17	Cirrhosis	No	Yes	120	126	[p.His1069Gln] + [c.3472-82 del-GGTTTAACCAT]	Alive
8	F	18	Cirrhosis, FS, HA	No	Yes	182	258	[p.His1069Gln] + [c.3402delC]	Alive
9	M	24	Cirrhosis, NPD	Yes	Yes	116	188	NK/NK	Alive
10	F	23	Cirrhosis	Yes	Yes	130	ND	[p.His1069Gln] + [p.His1069Gln]	Alive
11	F	27	Cirrhosis	Yes	Yes	145	ND	[p.His1069Gln] + [p.His1069Gln]	Alive
12	M	40	Liver steatosis, NPD	Yes	Yes	350	ND	[p.His1069Gln] + [p.His1069Gln]	Alive
13	F	36	Cirrhosis	No	Yes	126	ND	[p.His1069Gln] + NK	Alive

Mutational analysis was performed only for presence of p.H1069Q. 4th and 5th patients were siblings; 6th and 7th patients were siblings. ALF: Acute liver failure; CH: Chronic hepatitis; HA: Hemolytic anaemia (Coombs-negative); FS: Fancony syndrome; NPD: Neuropsychiatric disorder; ND: Not determined; NK: Not known.

the commonly used parameters alone allows a certain diagnosis of WD[14]. In our study, only in 6 (46%) of our patients was Kayser-Fleischer ring present and serum ceruloplasmin levels were even higher than 0.3 g/L in 2 patients with ALF. Urine cooper excretion exceeded 100 μg/24 h in 11 (84.6%) of 13 patients and in one asymptomatic sibling with WD it was even lower than 40 μg/24 h. Therefore, urinary cooper excretion and hepatic copper concentration, because of potential errors in evaluation, should always be assessed in the context of other diagnostic criteria[9,15].

WD is found worldwide with different geographical distribution of *ATP7B* gene mutations. The c.3207C>A (p.His1069Gln) missense mutation is the most frequent in Northern, Eastern and Central Europe[2,8,16], the c3400delC mutation is the most common in Brazil[17], and c.2333G>T (p.Arg778Leu) in Asia countries[18-21].

Knowledge of the regional distribution of mutations in the WD gene is important to design appropriate screening strategies[8]. In Central and Eastern Europe, the frequency of the c.3207C>A (p.H1069Q) mutation in exon 14 previously has been reported as the highest in Poland and Eastern Germany and decreases in Western and Southern European countries[8,22]. In Poland 72% of WD patients carry at least one allele with the c.3207C>A (p.H1069Q) mutation[23]. Caca *et al*[2] revealed that in East Germany 39% of WD patients were homozygous and 48% heterozygous for the c.3207C>A (p.H1069Q) mutation. Our study was the first to analyze genetic mutations of WD patients in Lithuania, a country in Baltic Sea area with 3.4 million inhabitants. The prevalence of the c.3207C>A (p.H1069Q) mutation in the *ATP7B* gene in WD patients in Lithuania was 92.3% (with 46.2% homozygotes and 46.2% compound heterozygotes). These findings were even higher than in Poland, a neighbour state, and were considerable higher than in Russia (49%) or Sweden (38%)[24]. In compound heterozygotes Lithuanian WD patients additional c.3472-82delGGTTTAACCAT, c.3402delC, and never mentioned new c.3121C>T (p.R1041W) mutations were detected. Only patients with hepatic or hepatic-neuropsychiatric presentation of WD were included in our study; however, other authors[25] have shown that the c.3207C>A (p.H1069Q) mutation is also associated with a late and neurological presentation of the disease.

In conclusion, the results of our study showed that the c.3207C>A (p.H1069Q) missense mutation is characteristic for Lithuania patients of WD. Even 92.3% of WD patients with hepatic presentation of the disease are homozygous or compound heterozygote for this mutation. Therefore, limited genetic testing of c.3207C>A (p.H1069Q) mutation might be of high value both to confirm WD disease diagnosis and for familial genetic screening in Lithuanian population.

ACKNOWLEDGMENTS

We are grateful to the head Dr. Olegas Abdrachmanovas and all personnel of the Laboratory for Environmental Health Research of the Institute for Biomedical Research for the copper analysis and Birute Sabaniene from Laboratory of Immunology and Genetics of Kaunas University of Medicine for excellent technical support.

COMMENTS

Background

Wilson disease (WD) is a life threatening autosomal recessive genetic disorder with impaired cooper metabolism and associated with hepatic, neurological and psychiatric symptoms. The diagnosis of WD is usually established using clinical signs and biochemical (hepatic copper concentration, ceruloplasmin concentration, copper urine excretion) tests; however, the most sensitive and

specific test is the detection of *ATP7B* gene mutations. The genetic testing is becoming the most important for confirmation of diagnosis and presymptomatic diagnostics in WD patients.

Research frontiers

The research was done to obtain data about the frequency and type of *ATP7B* gene mutations in WD patients in Lithuania.

Innovations and breakthroughs

It is important to know regional distribution of mutations of the *ATP7B* gene to design appropriate diagnostic strategies of WD. We have found an extremely high percentage of Lithuanian WD patients having the c.3207C>A (p.H1069Q) mutation. This is a new and interesting finding, showing that this mutation is typical for Lithuania WD patients, similar as was reported in some other Middle-Eastern European countries.

Applications

The present study indicates that due to high frequency of the c.3207C>A (p.H1069Q) mutation in Lithuania, even limited genetic testing of this mutation might be of high value both to confirm WD diagnosis and for familial genetic screening in Lithuanian population.

Terminology

ATP7B encodes a Cu-transporting P-type ATPase protein common to P-type ATPases. p.H1069Q missense mutations causes an amino acid substitution and disrupts ATP binding.

Peer review

This short report investigates the frequency and type of *ATP7B* gene mutations in Lithuanian patients with WD. The detected frequency (92.3%) of the c.3207C>A (p.H1069Q) mutation is one of the highest reported from Middle-Eastern European countries. This study is interesting.

REFERENCES

1 **Scheinberg IH**, Sternlieb I. Wilson disease and idiopathic copper toxicosis. *Am J Clin Nutr* 1996; **63**: 842S-845S

2 **Caca K**, Ferenci P, Kuhn HJ, Polli C, Willgerodt H, Kunath B, Hermann W, Mossner J, Berr F. High prevalence of the H1069Q mutation in East German patients with Wilson disease: rapid detection of mutations by limited sequencing and phenotype-genotype analysis. *J Hepatol* 2001; **35**: 575-581

3 **Saito T**. An assessment of efficiency in potential screening for Wilson's disease. *J Epidemiol Community Health* 1981; **35**: 274-280

4 **Loudianos G**, Dessi V, Lovicu M, Angius A, Nurchi A, Sturniolo GC, Marcellini M, Zancan L, Bragetti P, Akar N, Yagci R, Vegnente A, Cao A, Pirastu M. Further delineation of the molecular pathology of Wilson disease in the Mediterranean population. *Hum Mutat* 1998; **12**: 89-94

5 **Ala A**, Schilsky ML. Wilson disease: pathophysiology, diagnosis, treatment, and screening. *Clin Liver Dis* 2004; **8**: 787-805, viii

6 **Bull PC**, Thomas GR, Rommens JM, Forbes JR, Cox DW. The Wilson disease gene is a putative copper transporting P-type ATPase similar to the Menkes gene. *Nat Genet* 1993; **5**: 327-337

7 **Tanzi RE**, Petrukhin K, Chernov I, Pellequer JL, Wasco W, Ross B, Romano DM, Parano E, Pavone L, Brzustowicz LM. The Wilson disease gene is a copper transporting ATPase with homology to the Menkes disease gene. *Nat Genet* 1993; **5**: 344-350

8 **Ferenci P**. Regional distribution of mutations of the ATP7B gene in patients with Wilson disease: impact on genetic testing. *Hum Genet* 2006; **120**: 151-159

9 **Ferenci P**, Caca K, Loudianos G, Mieli-Vergani G, Tanner S, Sternlieb I, Schilsky M, Cox D, Berr F. Diagnosis and phenotypic classification of Wilson disease. *Liver Int* 2003; **23**: 139-142

10 **Gustincich S**, Manfioletti G, Del Sal G, Schneider C, Carninci P. A fast method for high-quality genomic DNA extraction from whole human blood. *Biotechniques* 1991; **11**: 298-300, 302

11 **Maier-Dobersberger T**, Ferenci P, Polli C, Balac P, Dienes HP, Kaserer K, Datz C, Vogel W, Gangl A. Detection of the His1069Gln mutation in Wilson disease by rapid polymerase chain reaction. *Ann Intern Med* 1997; **127**: 21-26

12 **Waldenstrom E**, Lagerkvist A, Dahlman T, Westermark K, Landegren U. Efficient detection of mutations in Wilson disease by manifold sequencing. *Genomics* 1996; **37**: 303-309

13 **Gunther P**, Hermann W, Kuhn HJ, Wagner A. [Wilson's disease] *Ther Umsch* 2007; **64**: 57-61

14 **Ferenci P**, Czlonkowska A, Merle U, Ferenc S, Gromadzka G, Yurdaydin C, Vogel W, Bruha R, Schmidt HT, Stremmel W. Late-onset Wilson's disease. *Gastroenterology* 2007; **132**: 1294-1298

15 **Roberts EA**, Schilsky ML. Diagnosis and treatment of Wilson disease: an update. *Hepatology* 2008; **47**: 2089-2111

16 **Firneisz G**, Lakatos PL, Szalay F, Polli C, Glant TT, Ferenci P. Common mutations of ATP7B in Wilson disease patients from Hungary. *Am J Med Genet* 2002; **108**: 23-28

17 **Deguti MM**, Genschel J, Cancado EL, Barbosa ER, Bochow B, Mucenic M, Porta G, Lochs H, Carrilho FJ, Schmidt HH. Wilson disease: novel mutations in the ATP7B gene and clinical correlation in Brazilian patients. *Hum Mutat* 2004; **23**: 398

18 **Nanji MS**, Nguyen VT, Kawasoe JH, Inui K, Endo F, Nakajima T, Anezaki T, Cox DW. Haplotype and mutation analysis in Japanese patients with Wilson disease. *Am J Hum Genet* 1997; **60**: 1423-1429

19 **Okada T**, Shiono Y, Hayashi H, Satoh H, Sawada T, Suzuki A, Takeda Y, Yano M, Michitaka K, Onji M, Mabuchi H. Mutational analysis of ATP7B and genotype-phenotype correlation in Japanese with Wilson's disease. *Hum Mutat* 2000; **15**: 454-462

20 **Ye S**, Gong L, Shui QX, Zhou LF. Wilson disease: identification of two novel mutations and clinical correlation in Eastern Chinese patients. *World J Gastroenterol* 2007; **13**: 5147-5150

21 **Liu XQ**, Zhang YF, Liu TT, Hsiao KJ, Zhang JM, Gu XF, Bao KR, Yu LH, Wang MX. Correlation of ATP7B genotype with phenotype in Chinese patients with Wilson disease. *World J Gastroenterol* 2004; **10**: 590-593

22 **Folhoffer A**, Ferenci P, Csak T, Horvath A, Hegedus D, Firneisz G, Osztovits J, Kosa JP, Willheim-Polli C, Szonyi L, Abonyi M, Lakatos PL, Szalay F. Novel mutations of the ATP7B gene among 109 Hungarian patients with Wilson's disease. *Eur J Gastroenterol Hepatol* 2007; **19**: 105-111

23 **Tarnacka B**, Gromadzka G, Rodo M, Mierzejewski P, Czloonkowska A. Frequency of His1069Gln and Gly1267Lys mutations in Polish Wilson's disease population. *Eur J Neurol* 2000; **7**: 495-498

24 **Shah AB**, Chernov I, Zhang HT, Ross BM, Das K, Lutsenko S, Parano E, Pavone L, Evgrafov O, Ivanova-Smolenskaya IA, Anneren G, Westermark K, Urrutia FH, Penchaszadeh GK, Sternlieb I, Scheinberg IH, Gilliam TC, Petrukhin K. Identification and analysis of mutations in the Wilson disease gene (ATP7B): population frequencies, genotype-phenotype correlation, and functional analyses. *Am J Hum Genet* 1997; **61**: 317-328

25 **Stapelbroek JM**, Bollen CW, van Amstel JK, van Erpecum KJ, van Hattum J, van den Berg LH, Klomp LW, Houwen RH. The H1069Q mutation in ATP7B is associated with late and neurologic presentation in Wilson disease: results of a meta-analysis. *J Hepatol* 2004; **41**: 758-763

S- Editor Li DL **L- Editor** Rippe RA **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5880

World J Gastroenterol 2008 October 14; 14(38): 5880-5886
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Hepatitis C virus enhances incidence of idiopathic pulmonary fibrosis

Yasuji Arase, Fumitaka Suzuki, Yoshiyuki Suzuki, Norio Akuta, Masahiro Kobayashi, Yusuke Kawamura, Hiromi Yatsuji, Hitomi Sezaki, Tetsuya Hosaka, Miharu Hirakawa, Satoshi Saito, Kenji Ikeda, Hiromitsu Kumada

Yasuji Arase, Fumitaka Suzuki, Yoshiyuki Suzuki, Norio Akuta, Masahiro Kobayashi, Yusuke Kawamura, Hiromi Yatsuji, Hitomi Sezaki, Tetsuya Hosaka, Miharu Hirakawa, Satoshi Saito, Kenji Ikeda, Hiromitsu Kumada, Department of Hepatology, Toranomon Hospital, Tokyo 105-8470, Japan
Author contributions: Arase Y contributed to design, data collection, data analysis, manuscript development and oversight; Suzuki F contributed to design, data collection, data analysis, manuscript development; Suzuki Y contributed to data collection; Akuta N contributed to data collection; Kobayashi M contributed to data collection; Kawamura Y contributed to data collection; Yatsuji H contributed to data collection; Sezaki H contributed to data collection; Hosaka T contributed to data collection; Hirakawa M contributed to data collection; Saito S contributed to data collection; Ikeda K contributed to data collection; Kumada H contributed to design, data collection, data analysis, manuscript development and oversight.
Supported by Grants-in-Aid from Okinaka Memorial Institute for Medical Research and the Japanese Ministry of Health, Labour and Welfare
Correspondence to: Yasuji Arase, MD, Department of Hepatology, Toranomon Hospital, 2-2-2, Toranomon, Minato-ku, Tokyo 105-8470, Japan. es9y-ars@asahi-net.or.jp
Telephone: +81-3-3588-1111 Fax: +81-3-3582-7068
Received: May 7, 2008 Revised: August 18, 2008
Accepted: August 25, 2008
Published online: October 14, 2008

Abstract

AIM: To investigate the cumulative development incidence and predictive factors for idiopathic pulmonary fibrosis in hepatitis C virus (HCV) positive patients.

METHODS: We studied 6150 HCV infected patients who were between 40-70 years old (HCV-group). Another 2050 patients with hepatitis B virus (HBV) were selected as control (HBV-group). The mean observation period was 8.0 ± 5.9 years in HCV-group and 6.3 ± 5.5 years in HBV-group. The primary goal is the development of idiopathic pulmonary fibrosis (IPF) in both groups. The cumulative appearance rate of IPF and independent factors associated with the incidence rate of IPF were calculated using the Kaplan-Meier method and the Cox proportional hazard model. All of the studies were performed retrospectively by collecting and analyzing data from the patient records in our hospital.

RESULTS: Fifteen patients in HCV-group developed IPF. On the other hand, none of the patients developed IPF in HBV-group. In HCV-group, the cumulative rates of IPF development were 0.3% at 10th year and 0.9% at 20th year. The IPF development rate in HCV-group was higher than that in HBV-group (P = 0.021). The IPF development rate in patients with HCV or HBV was high with statistical significance in the following cases: (1) patients ⩾ 55 years (P < 0.001); (2) patients who had smoking index (package per day × year) of ⩾ 20 (P = 0.002); (3) patients with liver cirrhosis (P = 0.042).

CONCLUSION: Our results indicate that age, smoking and liver cirrhosis enhance the development of IPF in HCV positive patients.

Key words: Hepatitis B virus; Hepatitis C virus; Idiopathic pulmonary fibrosis; A retrospective cohort study

Peer reviewer: Sammy Saab, PhD, UCLA, 200 Medical Plaza, Suite 214, Los Angeles 90095, United States

Arase Y, Suzuki F, Suzuki Y, Akuta N, Kobayashi M, Kawamura Y, Yatsuji H, Sezaki H, Hosaka T, Hirakawa M, Saito S, Ikeda K, Kumada H. Hepatitis C virus enhances incidence of idiopathic pulmonary fibrosis. *World J Gastroenterol* 2008; 14(38): 5880-5886 Available from: URL: http://www.wjgnet.com/1007-9327/14/5880.asp DOI: http://dx.doi.org/10.3748/wjg.14.5880

INTRODUCTION

Hepatitis C virus (HCV) is one of the more common causes of chronic liver disease in world. Chronic hepatitis C is an insidiously progressive form of liver disease that relentlessly but silently progresses to cirrhosis in 20%-50% of cases over a period of 10-30 years[1-3]. In addition, HCV is a major risk for hepatocellular carcinoma (HCC)[4-8]. Yearly incidence of HCC in patients with HCV-related cirrhosis is estimated at 5%-10%, and it is one of the major causes of death, especially in Japan.

Chronic HCV infection has been associated with a variety of extrahepatic complications such as essential mixed cryoglobulinemia[9-11], porphyria

cutanea tarda[9], membranoproliferative glomerulonep hritis[12,13], autoimmune thyroiditis[14-16], sialadenitis[17], and cardiomyopathy[18]. A few previous studies have presented conflicting results, with some suggesting that an incidence of anti-HCV antibody positivity in patients with idiopathic pulmonary fibrosis (IPF) is significantly higher than that in patients without IPF both in Italy and Japan[19,20]. Others found that incidence of anti-HCV antibody positivity is not high compared to controlled patients[21]. These discrepancies might depend on factors such as geographical differences of race and/or ethnicity or differences in the false positive rate of anti-HCV testing.

In any case, there is little or no information on the yearly cumulative incidence and risk factors on the development rate of IPF in patients with HCV. In our hospital, we evaluate a large number of patients with HCV-related hepatitis, and often find HCC among our patients. Interestingly, we also find a small proportion of patients with HCV-related hepatitis who develop IPF.

With this background in mind, the present retrospective cohort study was initiated to investigate the cumulative incidence and risk factors of IPF among HCV-infected patients.

MATERIALS AND METHODS

Patients

The number of patients who were diagnosed with chronic HCV infection between April 1975 and March 2006 in the Department of Hepatology, Toranomon Hospital, Tokyo, Japan was 11 500. Of these, 6150 patients met the following criteria: (1) age of 40-70 years; (2) features of chronic hepatitis or cirrhosis diagnosed by laparoscopy, liver biopsy, ultrasonography clinical features, and/or laboratory tests; (3) positive for anti-HCV antibody and HCV-RNA; (4) no history of treatment with antiviral agents; (5) negative for hepatitis B surface antigens (HBsAg), antinuclear antibodies, or antimitochondrial antibodies in serum, as determined by radioimmunoassay or spot hybridization; (6) no evidence of HCC nodules as shown by ultrasonography and/or computed tomography; (7) no underlying systemic disease, such as systemic lupus erythmatosus, rheumatic arthritis; (8) no cough or dyspnea after exercising; (9) no history of chronic lung disease. Patients with any of the following criteria were excluded from the study: (1) alpha-fetoprotein of 400 ng/mL or higher; (2) advanced and decompensated stage of cirrhosis with encephalopathy, icterus, or refractory ascites; (3) a short follow-up period of 6 mo or less; (4) development of HCC within 6 mo after the initiation of follow-up. These 6150 patients were regarded as in HCV-group. The 2050 patients that did not have the HCV marker and have the HBsAg marker were selected as a control and they comprised the hepatitis B virus (HBV)-group. The patients in HBV-group meet all of the above criteria but 3. Control patients were matched 1:3 with HCV positive patients for age and sex. We compared the differences of the cumulative development rate of IPF in both the HC-group and the HBV-group. Next, we assessed predictive factors for IPF in patients with hepatitis C. All of the studies were performed retrospectively by collecting and analyzing data from the patient records.

Table 1 Diagnostic criteria of IPF

Number	Criteria
Major criteria 1	Exclusion of other known caused of interstitial lung disease, such as certain drug toxicities, environmental exposures, and connective tissue diseases
Major criteria 2	Abnormal pulmonary function studies that include evidence of restriction (reduced breathing vital capacity) and impaired gas exchange (increased $AaPO_2$ with rest or after exercising or decreased diffusion lung capacity)
Major criteria 3	Bibasilar reticular abnormalities with minimal ground glass opacities on conventional chest radiographs or high-resolution computed tomography scans
Major criteria 4	Histological lung examination or bronchoalveolar lavage showing no features to support an alternative diagnosis
Minor criteria 1	Age > 50 yr
Minor criteria 2	Insidious onset of otherwise unexplained dyspnea on exertion
Minor criteria 3	Duration of illness ⩾ 3 mo
Minor criteria 4	Bibasilar, inspiratory crackles (dry or "Velcro" type in quality)

Definition of IPF

IPF was diagnosed by respiratory specialist based on the presence of at the least three of the following four diagnostic major criteria, as well as all of the following four minor criteria, as shown in Table 1. Diagnostic criteria of IPF were recommended by American Thoracic Society/European Respiratory Society[22]. We excluded hepatopulmonary syndrome by conventional chest radiographs or high-resolution computed tomography scans, electrocardiogram, and/or ultrasonic cardiography.

Viral markers of HCV and HBV

Anti-HCV was detected using a second-generation enzyme-linked immunosorbent assay (ELISA Ⅱ) (Abbott Laboratories, North Chicago, IL). HCV-RNA was determined by the Amplicor method (Cobas Amplicor HCV Monitor Test, v2.0, Roche Molecular Systems, Inc., NJ). HBsAg was tested by radioimmunoassay (Abbott Laboratories, Detroit, MI). The used serum samples were stored -80℃ at the first consultation. Diagnosis of HCV infection was based on detection of serum HCV antibody and positive RNA. The study started in April 1975. At that time, HCV testing was not available. Thus, the patients diagnosed as HCV-infected were tested after HCV testing became available.

Evaluation of liver cirrhosis

Status of liver cirrhosis was mainly determined on the basis of peritoneoscopy and/or liver biopsy. Six thousand eight hundred and twenty six out of 8200 were diagnosed by peritoneoscopy and/or liver biopsy. Liver biopsy specimens were obtained using a modified Vim Silverman

Table 2 Clinical characteristics[1]

	Total	HBV-group	HCV-group	*P*
Number (*n*)	8200	2050	6150	
Age (yr)	51.8 ± 9.0	51.7 ± 8.7	51.8 ± 9.1	1
Sex (male, %)	77.8% (6380)	77.8% (1595)	77.8% (4785)	1
Liver cirrhosis[2]	20.2% (1659)	18.5% (379)	20.8% (1280)	< 0.001
Total alcohol intake of > 200 kg[3]	23.0% (1490/6465)	17.4% (253/1450)	24.7% (1237/5015)	< 0.001
Smoking index of > 20[3]	27.9% (1680/6032)	23.5% (293/1246)	29.0% (1387/4786)	< 0.001
AST (IU/L)	75.9 ± 124.5	82.9 ± 138.2	73.8 ± 120.3	< 0.001
ALT (IU/L)	104.2 ± 107.5	124.4 ± 119.9	98.5 ± 103.6	< 0.001
Total bilirubin (mg/dL)	0.83 ± 0.94	0.98 ± 0.85	0.81 ± 0.77	< 0.001
γGTP (IU/L)	74.0 ± 106.2	77.1 ± 128.7	73.2 ± 99.8	0.951
Platelet count (x 10^4/mm^3)	19.3 ± 18.7	19.1 ± 14.4	19.4 ± 19.7	0.725

[1]Data are percent (number of patients) or mean ± SD; [2]The 1594 (77.8%) out of 2050 in HBV-group and 5232 (85.1%) of 6150 in HCV-group were diagnosed by laparoscopy and/or liver biopsy. The breakdown of histological staging in HCV-group was as follows: stage 1, 2707; stage 2, 1188; stage 3, 300; stage 4 (liver cirrhosis), 1037. The outbreak of histological staging in HBV-group was as follows: stage 1, 705; stage 2, 439; stage 3, 157; stage 4 (liver cirrhosis), 293; [3]Smoking index = (package/d) × yr; Total alcohol intake and smoking index indicate the sum of before and after first consultation.

needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo, Japan), fixed in 10% formalin, and stained with hematoxylin-eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. The size of specimens for examination was more than six portal areas. Baseline liver histology of chronic hepatitis was classified according to the extent of fibrosis, into four stages in progression order: stage 1, periportal expansion; stage 2, portoportal septa; stage 3, portocentral linkage or bridging fibrosis; stage 4, liver cirrhosis[23]. Remaining patients were diagnosed by clinical features, laboratory tests, and ultrasonographic findings. Ultrasonography was performed with a high-resolution, real-time scanner (model SSD-2000; Aloka Co., Ltd, Tokyo Japan. Logic 700 MR; GE-Yokokawa Medical Systems, Tokyo, Japan). The diagnosis of liver cirrhosis was defined as having a score of > 8 in ultrasonographical scoring system based on liver surface, liver parenchyma, hepatic vessel and spleen size as reported by Lin *et al*[24].

Follow-up

Patients were followed-up monthly to tri-monthly after the first medical examination in our hospital. Physical examination and biochemical tests were conducted at each examination together with regular check up using abdominal CT or US imaging in each patient. When a patient had any symptoms in relation to IPF (dry cough, dyspnea), we further explored the possibility of that patient having IPF. Three hundred thirty-four patients were lost to follow-up. Because the appearance of IPF and death was not identified in these 334 patients, they were considered as censored data in statistical analysis[25]. Moreover, patients treated with anti-viral agents were regarded as withdrawals at the time of starting antiviral agents.

Statistical analysis

Nonparametric procedures were employed for the analysis of background features of the patients, including the Mann-Whitney *U* test and χ^2 method. The cumulative appearance rate of IPF was calculated from the period of the first medical examination at our hospital to the appearance of IPF, using the Kaplan-Meier method. Differences in the development of IPF were tested using the log rank test. Independent factors associated with the incidence rate of IPF were analyzed by the Cox proportional hazard model. The following nine variables were analyzed for potential covariates for incidence of IPF at the time of first medical examination at our hospital: age, sex, state of liver disease (chronic hepatitis or liver cirrhosis), smoking index, total alcohol intake, platelet count, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase. $P < 0.05$ in two-tailed test was considered significant. Data analysis was performed using the SPSS computer program package (SPSS 11.5 for Windows, SPSS, Chicago, IL).

RESULTS

Patients' characteristics

Table 2 shows the characteristics of the 8200 patients with HCV or HBV. There were no significant differences between the two groups with regard to sex ratio or age. However, there were significant differences in histopathological stage of the liver, AST, ALT, total bilirubin, total intake of alcohol, and smoking index. The 1594 (77.8%) out of 2050 in HBV-group and 5232 (85.1%) of 6150 in HCV-group were diagnosed by laparoscopy and/or liver biopsy. The breakdown of histological staging in HCV-group was as follows: stage 1, 2707; stage 2, 1188; stage 3, 300; stage 4 (liver cirrhosis), 1037. The outbreak of histological staging in HBV-group was as follows: stage 1, 705; stage 2, 439; stage 3, 157; stage 4 (liver cirrhosis), 293.

On relationship between liver histology and IPF, liver biopsies were done in ten of 15 patients with IPF before diagnosis of IPF. The period before diagnosis of IPF was 10.2 ± 4.1 years. There was no evidence of plasma cells to indicate possible autoimmune hepatitis, which as a systemic disease could be associated with IPF.

Incidence of IPF in patients with HCV or HBV

In the HCV-group, 15 patients developed IPF during a

Table 3 Predictive factors for IPF development[1]

Factor	Univariate analysis				Multivariate analysis[1]			
	Category	Hazard ratio	95% CI	*P*	Category	Hazard ratio	95% CI	*P*
Age (yr)	< 55/≥ 55	1/11.78	3.52-39.37	< 0.001	< 55/≥ 55	1/12.52	3.52-44.59	< 0.001
Smoking index[1]	< 20/≥ 20	1/4.56	1.52-13.61	0.007	< 20/≥ 20	1/5.90	1.95-17.82	0.002
Liver staging (fibrosis)	Non-LC/LC	1/3.67	1.29-10.48	0.015	Non-LC/LC	1/3.00	1.04-8.64	0.042
Sex	Male/Female	1/0.45	0.13-1.62	0.223				
Platelet (x 10^4/mm^3)	< 15/≥ 15	1/0.47	0.10-2.23	0.341				
γGTP (IU/L)	< 110/≥ 110	1/1.95	0.41-9.31	0.405				
Total alcohol intake[1]	< 200/≥ 200	1/1.50	0.50-4.48	0.467				
AST (IU/L)	< 76/≥ 76	1/1.16	0.30-4.53	0.834				
ALT (IU/L)	< 100/≥ 100	1/1.05	0.29-3.74	0.946				

Data are number of patients or mean ± SD. [1]Smoking index = (package/d) × year. Total alcohol intake and smoking index indicate the sum of before and after first consultation

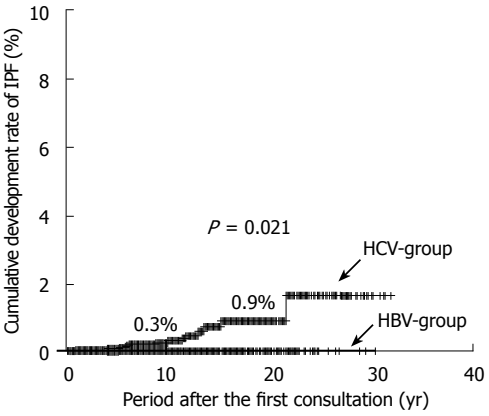


Figure 1 Cumulative rate of the incidence of IPF from the first medical examination at our hospital in patients with HCV or HBV.

mean observation period of 8 years. The cumulative rate of newly diagnosed IPF was 0.3% at the end of the 10th year, and 0.9% at the 20th year (Figure 1). On the other hand, none of the patients developed IPF during a mean observation period of 6.3 years in the HBV-group. The cumulative rate of newly diagnosed IPF in the HCV-group was higher than that in the HBV-group ($P = 0.021$).

Determinants of incidence of IPF

We then investigated the factors, except for virus marker, associated with the incidence of IPF in all the 8200 patients with HBV or HCV (Table 3). Univariate analysis identified the following three factors that influenced incidence of IPF: age ($P < 0.001$), state of liver disease ($P = 0.007$), and smoking index ($P = 0.015$). These three parameters were entered into multivariate Cox proportional hazard analysis. The IPF development rate of HCV positive patients was high with statistical significance in the following cases: (1) patients ≥ 55 years ($P < 0.001$); (2) patients who had smoking index (package per day × year) of ≥ 20 ($P = 0.002$), (3) patients who had liver cirrhosis ($P = 0.042$).

Next, we examined the factors associated with the incidence of IPF in all the 6150 patients with HCV multivariate Cox proportional hazard analysis. The IPF development rate of HCV positive patients was high with statistical significance in the following cases: (1) patients ≥ 55 years (OR: 14.24; 95% CI = 3.39-59.74, $P < 0.001$); (2) patients who had smoking index (package per day × year) of ≥ 20 (OR: 5.43; 95% CI = 1.74-16.88, $P = 0.003$); (3) patients who had liver cirrhosis (OR: 3.82; 95% CI = 1.23-11.86, $P = 0.02$).

Mortality and causes of death

Table 4 summarizes the characteristics of 15 patients who developed IPF in the HCV-group. Thirteen patients (12 men and 3 women; median age at the time of onset of IPF = 70 years, range = 57 to 79 years) with IPF were diagnosed by well-trained respiratory specialists.

During the observation period, 12 of the 15 patients with IPF in the HCV-group died. Seven patients died of liver-related disease (HCC, decompensated liver cirrhosis, rupture of esophageal varices) and five patients died of IPF. Liver-related death accounted for 58% (7/12) of all deaths and HCC was the major cause of liver-related deaths. IPF-related death accounted for 42% (5/12) of all deaths. On the other hand, of 6002 HCV positive patients without IPF, 1905 died of various diseases during the observation period. Of 1905 patients who died, 44 patients died of lung-related disease, such as acute pneumonitis or pulmonary tuberculosis. The incidence of death based on lung-related disease in patients with IPF was significantly higher than that in patients without IPF ($P < 0.001$).

DISCUSSION

We have described the development incidence of IPF in patients with HCV in the present study. The present study was limited by a retrospective cohort trial and age in patients. We selected patients with ages of 40-70 years at the first consultation. The reason is as follows: (1) onset of IPF is rare in young people with < 40 years; (2) the number of patients with > 70 years at the first consultation is few. Another limitation of the study was that HBsAg positive patients were selected as controlled group. Though there were no significant differences between the two groups with regard to sex ratio and age, there were significant differences in stage of the liver, AST, ALT, total bilirubin, total intake of alcohol, and smoking index. In Japan, HBV infection is usually

Table 4 Characteristics of patients with IPF in the HCV-group

Case	Age (yr)[1]	Sex	Liver disease[1]	Smoking index[2]	Age at the time of IPF onset	Period after IPF development[2]	Alive or death[2]	Cause of death
1	49	M	LC	25	57	5.7	Death	HCC
2	50	M	CH	38	62	12.3	Death	IPF
3	52	M	CH	0	73	3.8	Death	IPF
4	57	M	LC	28	68	8.7	Death	d-LC
5	58	M	LC	22	68	1.1	Death	IPF
6	61	M	LC	0	71	6	Death	IPF
7	61	F	LC	30	66	3.2	Death	IPF
8	62	M	LC	0	72	10.2	Death	HCC
9	62	F	LC	5	66	3.5	Death	d-LC
10	63	M	CH	26	75	10.6	Alive	
11	63	M	CH	34	64	12.1	Death	HCC
12	64	M	CH	40	69	10.1	Alive	
13	66	F	CH	24	79	1.8	Alive	
14	69	M	LC	0	70	2.3	Death	HCC
15	70	M	LC	42	76	4.1	Death	HCC

CH: Chronic hepatitis; d-LC: Decompensated liver cirrhosis; HCC: Hepatocellular carcinoma; IPF: Idiopathic pulmonary fibrosis; LC: Liver cirrhosis. [1]Characteristics of patients at the first consultation; [2]Smoking index = (package/d) × year, Smoking index indicates the sum of before and after first consultation; Period after IPF development, period (yr) between onset of IPF and final consultation; Alive or death, alive or death at the final consultation

acquired perinatally or in early childhood. Moreover, patients infected with HBV often have family history of HBV infection. Thus, patients infected with HBV might tend to avoid drinking alcohol or smoking on the merits of family advice. Next, AST, ALT, and total bilirubin levels were significantly high in HBV-group. These results may show that patients with HBV tend to have acute exacerbation of the liver. Another limitation is that we further explored the possibility that patient having IPF when a patient had any symptoms in relation to IPF (dry cough, dyspnea). However, Toranomon hospital was opened for attending patients who were officers or officials. Therefore, most of enrolled patients are officers, officials, or office workers. They have generally undergone chest checkup by the use of X-ray every year during incumbency and after retirement. This means that onset of IPF was checked constantly by respiratory specialists. Therefore, even if the diagnosis of IPF was late in cases in which hepatologists had no experience in the Department of Respiratory Disease, chest checkup by the use of X ray every year could assist on diagnosing IPF.

Among features of the present study are prolonged observation study and large number of the study population. The attending patients were followed closely base on the following reasons: (1) our hospital was opened for attending patients who were officers or officials; therefore, most of enrolled patients are officers, officials, or office workers; (2) our hospital is located in the center of the Tokyo metropolitan area in Japan, so it is convenient for patients to go to the our hospital. The present study shows several findings with regard to IPF in HCV positive patients. First, the IPF development rate in HC-group was higher than that in HB-group. Our retrospective study is the first to determine the annual incidence of IPF among patients with HCV at 0.03%-0.04%. The morbidity for IPF was estimated to be 0.003% to 0.004% in the general population in Japan[26]. Little is known about the relationship between the incidence of IPF and HCV. Conflicting studies on the incidence of HCV infection in patients with IPF have been published. Ueda *et al*[19] and Meliconi *et al*[20] reported a higher prevalence of HCV-antibody in patients with IPF compared with the general population. However, Irving *et al*[21] could not confirm the hypothesis that HCV may be a cause of IPF. The controversial results of the different research groups may be explained by the geographical differences of race and other factors. An accurate assessment of the exact risk could only come from a large cohort study. Thus, the present cohort study shows that prevalence of IPF tends to be slightly higher in the HC group compared to those in HB group in Japan. We believe this epidemiological study first elucidates the annual incidence of IPF among patients with HCV; the annual appearance rate was 0.03%-0.04%.

Second, the IPF development rate of HCV positive patients was high with statistical significance in the following cases: (1) patients $\geqslant$ 55 years; (2) patients who had smoking index of $\geqslant$ 20; (3) patients who had liver cirrhosis. Our results indicate that aging, liver cirrhosis and smoking enhance the development of IPF in patients with chronic hepatitis C infection. Idilman *et al*[27] have reported that HCV infection might be associated with an occult pulmonary inflammatory reaction manifested by an increased number of polymorphonuclear neutrophils in bronchoalveolar lavage fluid. Aging, liver cirrhosis, and smoking might enhance an occult pulmonary inflammatory reaction.

Third, the incidence rate of lung-related death of HCV positive patients with IPF was higher than that without IPF. IPF-related death corresponded to one-third of all deaths in patients with IPF. It can progress rapidly after such exacerbation and often proves fatal, despite treatment with oral corticosteroids and intravenous high-dose corticosteroid therapy. The fact that patients with IPF have high possibility dying from acute exacerbation due to IPF during the follow-

up shows the need to provide a high level of care to patients with IPF. In general, hepatologist regard the daily management of patients with HCV. When HCV patients complain dry cough and dyspnea, hepatologists should check the complication of IPF.

In the present study, hepatopulmonary syndrome was excluded by conventional chest radiographs or high-resolution computed tomography scans, electrocardiogram, and/or ultrasonic cardiography. However, hepatopulmonary syndrome tends to complicate in advanced liver disease. Therefore, it is still possible that some of these patients with IPF had complication of hepatopulmonary syndrome.

Despite extensive research, IPF remains a disease of unknown etiology with a poor prognosis after acute exacerbation. Idilman *et al*[27] have reported that an increased bronchoalveolar lavage neutrophil count in individuals with HCV induced chronic active hepatitis was identified. This finding suggests that HCV may have the potential to induce an alveolitis leading to fibrotic changes in the lung. In the formation of IPF in HCV positive patients, there are other mechanisms such as accumulation to lung tissue of immunoglobulin and/or immune complex or direct involvement of HCV-RNA. These mechanisms may be mutually related.

First mechanism could be explained by the following. Gut-derived antigens and antibodies from the bowel *via* portal circulation or other antigens and antibodies were not segregated in sufficient amounts in patients with severe liver dysfunction. Immune complexes formed by these antigens and antibodies were passed into the systemic circulation and finally, these immune complexes are accumulated in the glomeruli or lung. Owing to this mechanism, there could be a high prevalence of immunoglobulin deposition in glomeruli of patients with mesangial proliferative glomerulonephritis and membranoproliferative glomerulonephritis[28]. We also examined the lung in a few patients using immunofluorescence microscopy. However, we did not detect immunoglobulin in formalin-fixed lung tissue. These results might indicate that serum immunoglobulin play a minor role in IPF. However, there might be possibility of showing low sensitivities due to use the formalin-fixed tissue. On the other hand, Koike *et al*[29] reported that transgenic mice carrying the HCV envelope gene revealed an exocrinopathy resembling Sjogren syndrome. Similar to this, HCV might directly cause IPF. More studies are needed to confirm the mechanism producing IPF in HCV positive patients.

In conclusion, our retrospective study is the first to determine the annual incidence of IPF among patients with HCV at 0.03%-0.04%. Our results indicate that age, liver cirrhosis and smoking enhance the development of IPF in patients with chronic hepatitis C infection.

ACKNOWLEDGMENT

The authors are grateful to Drs. M Hara, Y Endo, K Ohashi (pathologist), S Tanimura, and K Nakada (respiratory specialist) for diagnosis of IPF. Moreover, the authors greatly acknowledged the editorial assistance of Thomas Hughes.

COMMENTS

Background

Idiopathic pulmonary fibrosis (IPF) is present in patients with chronic hepatitis C virus (HCV) infection. In any case, there is little or no information on the yearly cumulative incidence and risk factors on the development rate of IPF in patients with HCV.

Research frontiers

A few previous studies have presented conflicting results with some suggesting that an incidence of anti-HCV antibody positivity in patients with IPF is significantly higher than that in patients without IPF both in Italy and Japan, whereas others found that an incidence of anti-HCV antibody positivity is not high compared to controlled patients.

Innovations and breakthroughs

The morbidity for IPF was estimated to be 0.003% to 0.004% in the general population in Japan. This retrospective study is the first to determine the annual incidence of IPF among patients with HCV at 0.03%-0.04%. The results indicate that age, liver cirrhosis and smoking enhance the development of IPF in patients with chronic hepatitis C infection.

Applications

The fact that patients with IPF have a high possibility of dying from acute exacerbation due to IPF during the follow-up shows the need to provide a high level of care to patients with IPF. In general, hepatologists regard the daily management of patients with HCV. When HCV patients complain dry cough and dyspnea, hepatologists should check the complication of IPF.

Terminology

IPF was diagnosed by respiratory specialist based on the diagnostic criteria recommended by American Thoracic Society/European Respiratory Society. Smoking index was defined as (package/d) × year, indicating the sum of before and after first consultation; period after IPF development, period (year) between onset of IPF and final consultation.

Peer review

The manuscript is well written and the study is well designed. Authors investigated the cumulative development incidence and predictive factors for idiopathic pulmonary fibrosis in HCV positive patients.

REFERENCES

1 **Kobayashi T**, Kawa S, Tokoo M, Oguchi H, Kiyosawa K, Furuta S, Kanai M, Homma T. Comparative study of CA-50 (time-resolved fluoroimmunoassay), Span-1, and CA19-9 in the diagnosis of pancreatic cancer. *Scand J Gastroenterol* 1991; **26**: 787-797

2 **Alter MJ**, Margolis HS, Krawczynski K, Judson FN, Mares A, Alexander WJ, Hu PY, Miller JK, Gerber MA, Sampliner RE. The natural history of community-acquired hepatitis C in the United States. The Sentinel Counties Chronic non-A, non-B Hepatitis Study Team. *N Engl J Med* 1992; **327**: 1899-1905

3 **van Rossum TG**, Vulto AG, de Man RA, Brouwer JT, Schalm SW. Review article: glycyrrhizin as a potential treatment for chronic hepatitis C. *Aliment Pharmacol Ther* 1998; **12**: 199-205

4 **Colombo M**, Kuo G, Choo QL, Donato MF, Del Ninno E, Tommasini MA, Dioguardi N, Houghton M. Prevalence of antibodies to hepatitis C virus in Italian patients with hepatocellular carcinoma. *Lancet* 1989; **2**: 1006-1008

5 **Hasan F**, Jeffers LJ, De Medina M, Reddy KR, Parker T, Schiff ER, Houghton M, Choo QL, Kuo G. Hepatitis C-associated hepatocellular carcinoma. *Hepatology* 1990; **12**: 589-591

6 **Kew MC**, Houghton M, Choo QL, Kuo G. Hepatitis C virus antibodies in southern African blacks with hepatocellular carcinoma. *Lancet* 1990; **335**: 873-874

7 **Tsukuma H**, Hiyama T, Tanaka S, Nakao M, Yabuuchi T,

Kitamura T, Nakanishi K, Fujimoto I, Inoue A, Yamazaki H. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. *N Engl J Med* 1993; **328**: 1797-1801

8 **Ikeda K**, Saitoh S, Koida I, Arase Y, Tsubota A, Chayama K, Kumada H, Kawanishi M. A multivariate analysis of risk factors for hepatocellular carcinogenesis: a prospective observation of 795 patients with viral and alcoholic cirrhosis. *Hepatology* 1993; **18**: 47-53

9 **Gumber SC**, Chopra S. Hepatitis C: a multifaceted disease. Review of extrahepatic manifestations. *Ann Intern Med* 1995; **123**: 615-620

10 **Zignego AL**, Ferri C, Giannini C, Monti M, La Civita L, Careccia G, Longombardo G, Lombardini F, Bombardieri S, Gentilini P. Hepatitis C virus genotype analysis in patients with type II mixed cryoglobulinemia. *Ann Intern Med* 1996; **124**: 31-34

11 **Ferri C**, Monti M, La Civita L, Longombardo G, Greco F, Pasero G, Gentilini P, Bombardieri S, Zignego AL. Infection of peripheral blood mononuclear cells by hepatitis C virus in mixed cryoglobulinemia. *Blood* 1993; **82**: 3701-3704

12 **Johnson RJ**, Gretch DR, Yamabe H, Hart J, Bacchi CE, Hartwell P, Couser WG, Corey L, Wener MH, Alpers CE. Membranoproliferative glomerulonephritis associated with hepatitis C virus infection. *N Engl J Med* 1993; **328**: 465-470

13 **Yamabe H**, Johnson RJ, Gretch DR, Osawa H, Inuma H, Sasaki T, Kaizuka M, Tamura N, Tsunoda S, Fujita Y. Membranoproliferative glomerulonephritis associated with hepatitis C virus infection responsive to interferon-alpha. *Am J Kidney Dis* 1995; **25**: 67-69

14 **Pawlotsky JM**, Roudot-Thoraval F, Simmonds P, Mellor J, Ben Yahia MB, Andre C, Voisin MC, Intrator L, Zafrani ES, Duval J, Dhumeaux D. Extrahepatic immunologic manifestations in chronic hepatitis C and hepatitis C virus serotypes. *Ann Intern Med* 1995; **122**: 169-173

15 **Tran A**, Quaranta JF, Benzaken S, Thiers V, Chau HT, Hastier P, Regnier D, Dreyfus G, Pradier C, Sadoul JL. High prevalence of thyroid autoantibodies in a prospective series of patients with chronic hepatitis C before interferon therapy. *Hepatology* 1993; **18**: 253-257

16 **Boadas J**, Rodriguez-Espinosa J, Enriquez J, Miralles F, Martinez-Cerezo FJ, Gonzalez P, Madoz P, Vilardell F. Prevalence of thyroid autoantibodies is not increased in blood donors with hepatitis C virus infection. *J Hepatol* 1995; **22**: 611-615

17 **Haddad J**, Deny P, Munz-Gotheil C, Ambrosini JC, Trinchet JC, Pateron D, Mal F, Callard P, Beaugrand M. Lymphocytic sialadenitis of Sjogren's syndrome associated with chronic hepatitis C virus liver disease. *Lancet* 1992; **339**: 321-323

18 **Agnello V**, Chung RT, Kaplan LM. A role for hepatitis C virus infection in type II cryoglobulinemia. *N Engl J Med* 1992; **327**: 1490-1495

19 **Ueda T**, Ohta K, Suzuki N, Yamaguchi M, Hirai K, Horiuchi T, Watanabe J, Miyamoto T, Ito K. Idiopathic pulmonary fibrosis and high prevalence of serum antibodies to hepatitis C virus. *Am Rev Respir Dis* 1992; **146**: 266-268

20 **Meliconi R**, Andreone P, Fasano L, Galli S, Pacilli A, Miniero R, Fabbri M, Solforosi L, Bernardi M. Incidence of hepatitis C virus infection in Italian patients with idiopathic pulmonary fibrosis. *Thorax* 1996; **51**: 315-317

21 **Irving WL**, Day S, Johnston ID. Idiopathic pulmonary fibrosis and hepatitis C virus infection. *Am Rev Respir Dis* 1993; **148**: 1683-1684

22 **American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias.** This joint statement of the American Thoracic Society (ATS), and the European Respiratory Society (ERS) was adopted by the ATS board of directors, June 2001 and by the ERS Executive Committee, June 2001. *Am J Respir Crit Care Med* 2002; **165**: 277-304

23 **Desmet VJ**, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994; **19**: 1513-1520

24 **Lin DY**, Sheen IS, Chiu CT, Lin SM, Kuo YC, Liaw YF. Ultrasonographic changes of early liver cirrhosis in chronic hepatitis B: a longitudinal study. *J Clin Ultrasound* 1993; **21**: 303-308

25 **Fleming TR**, Harrington DP, O'Brien PC. Designs for group sequential tests. *Control Clin Trials* 1984; **5**: 348-361

26 **Iwai K**, Mori T, Yamada N, Yamaguchi M, Hosoda Y. Idiopathic pulmonary fibrosis. Epidemiologic approaches to occupational exposure. *Am J Respir Crit Care Med* 1994; **150**: 670-675

27 **Idilman R**, Cetinkaya H, Savas I, Aslan N, Sak SD, Bastemir M, Sarioqlu M, Soykan I, Bozdayi M, Colantoni A, Aydintuq O, Bahar K, Uzunalimoqlu O, Van Thiel DH, Numanoqlu N, Dokmeci A. Bronchoalveolar lavage fluid analysis in individuals with chronic hepatitis C. *J Med Virol* 2002; **66**: 34-39

28 **Arase Y**, Ikeda K, Murashima N, Chayama K, Tsubota A, Koida I, Suzuki Y, Saitoh S, Kobayashi M, Kobayashi M, Kobayashi M, Kumada H. Glomerulonephritis in autopsy cases with hepatitis C virus infection. *Intern Med* 1998; **37**: 836-840

29 **Koike K**, Moriya K, Ishibashi K, Yotsuyanagi H, Shintani Y, Fujie H, Kurokawa K, Matsuura Y, Miyamura T. Sialadenitis histologically resembling Sjogren syndrome in mice transgenic for hepatitis C virus envelope genes. *Proc Natl Acad Sci USA* 1997; **94**: 233-236

S- Editor Li DL **L- Editor** Li M **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5887

World J Gastroenterol 2008 October 14; 14(38): 5887-5892
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Gene profiles between non-invasive and invasive colon cancer using laser microdissection and polypeptide analysis

Jin-Shui Zhu, Hua Guo, Ming-Quan Song, Guo-Qiang Chen, Qun Sun, Qiang Zhang

Jin-Shui Zhu, Hua Guo, Ming-Quan Song, Guo-Qiang Chen, Qun Sun, Qiang Zhang, Department of Gastroenterology, Sixth People's Hospital Affiliated to Shanghai Jiao Tong University, Shanghai 200233, China
Guo-Qiang Chen, Shanghai Experimental Animal Center, Chinese Academy of Science, Shanghai 200233, China
Author contributions: Zhu JS, Guo H and Song MQ contributed equally to this work; Zhu JS designed research and wrote the paper; Guo H, Song MQ performed research; Chen GQ contributed new reagent; Sun Q and Zhang Q contributed analytic tools /analyzed data.
Supported by The Natural Science Foundation of Shanghai, No. 04ZB14072
Correspondence to: Jin-Shui Zhu, Professor, Department of Gastroenterology, Sixth People's Hospital Affiliated to Shanghai Jiao Tong University, Shanghai 200233, China. zhujs1803@hotmail.com
Telephone: +86-21-64369181 Fax: +86-21-64837019
Received: August 7, 2008 Revised: September 16, 2008
Accepted: September 23, 2008
Published online: October 14, 2008

Abstract

AIM: To explore the expression of differential gene expression profiles of target cell between non-invasive submucosal and invasive advanced tumor in colon carcinoma using laser microdissection (LMD) in combination with polypeptide analysis.

METHODS: Normal colon tissue samples from 20 healthy individuals and 30 cancer tissue samples from early non-invasive colon cancer cells were obtained. The cells from these samples were used LMD independently after P27-based amplification. aRNA from advanced colon cancer cells and metastatic cancer cells of 40 cases were applied to LMD and polypeptide analysis, semiquantitative reverse transcribed polymerase chain reaction (RT-PCR) and immunohistochemical assays were used to verify the results of microarray and further identify differentially expressed genes in non-invasive early stages of colon cancer.

RESULTS: Five gene expressions were changed in colon carcinoma cells compared with that of controls. Of the five genes, three genes were downregulated and two were upregulated in invasive submucosal colon carcinoma compared with non-invasive cases. The results were confirmed at the level of aRNA and gene expression. Five genes were further identified as differentially expressed genes in the majority of cases (> 50%, 25/40) in progression of colon cancer, and their expression patterns of which were similar to tumor suppressor genes or oncogenes.

CONCLUSION: This study suggested that combined use of polypeptide analysis might identify early expression profiles of five differential genes associated with the invasion of colon cancer. These results reveal that this gene may be a marker of submucosal invasion in early colon cancer.

Key words: Colon cancer; Laser microdissection; Polypeptide analysis

Peer reviewer: Peter L Lakatos, MD, PhD, Assistant Professor, 1st Department of Medicine, Semmelweis University, Koranyi S 2A, Budapest H1083, Hungary

Zhu JS, Guo H, Song MQ, Chen GQ, Sun Q, Zhang Q. Gene profiles between non-invasive and invasive colon cancer using laser microdissection and polypeptide analysis. *World J Gastroenterol* 2008; 14(38): 5887-5892 Available from: URL: http://www.wjgnet.com/1007-9327/14/5887.asp DOI: http://dx.doi.org/10.3748/wjg.14.5887

INTRODUCTION

Colon cancer ranks second of all gastrointestinal malignant tumors, it is one of the leading causes of cancer-related deaths worldwide. In the past five years, the incidence of colon cancer is reported to be especially high in Asia. Its clinical behavior depends on the potential metastasis of the tumor; its prognosis of advanced colon cancers remains very poor. Until now, several molecules have been reported to play an important role in gastroenterological tumorigenesis and tumor metastasis, but the molecular mechanisms involved tumor development and progression still remain unclear in colon cancer[1-3].

In this research, by using the combined methods of laser microdissection (LMD), P27-based RNA amplification, and polypeptide, we evaluated differentially expressed genes between early carcinoma and lymph node metastatic patients. Moreover, we further identified four differentially expressed genes in the progression of colon cancer in another group of 15 patients by means of semiquantitative reverse transcribed polymerase chain

reaction (RT-PCR), and the expression patterns of these four genes were similar to tumor suppressor genes or oncogenes.

It is now widely accepted that many malignant tumors contain heterogeneous subpopulations of cells. This heterogeneity is exhibited in a wide range of genetic, biochemical and immunologic characteristics. It is likely that specific tumor cells or colonies within the larger heterogeneous tumor specimen are the forerunners of distant metastases[4]. Therefore, many biologic differences exist between tumor cells in early carcinoma and those in metastatic lesions. Furthermore, the interaction of tumor cells within their living environment may add more differences between these two groups of cells[5]. As a result, tumor metastasis related genes can be identified by comparing the gene expression profiles between them.

LMD and polypeptide analysis are two new emerging technologies in the post-genomic era. LMD allows a specific area of tissue or group of cells to be obtained, and in this particular system, intracellular macromolecules remain intact. Since there is no heating of the sample or direct mechanical contact with the section, there is a reduced risk of contamination, sample loss or alteration of the protein sample[6,7].

The large-scale analysis of gene expression with a polypeptide allows us to evaluate the gene-expression profiles of hundreds to tens of thousands of genes in a single experiment[8]. This technology is a powerful tool for analyzing gene expression of which can be correlated with pathological phenotypes of various diseases. However, the expression profile of a specific cell type may be primarily masked or even lost because of the bulk of surrounding cells. Therefore, combined use of LMD and polypeptide analysis can provide a unique opportunity to study gene expression of subpopulations of cells in their native (*in vivo*) tissue environment.

MATERIAL AND METHODS

Patients with colon cancer and their pathological samples

Forty cases of advanced colon adenocarcinoma (TNM stage Ⅲ to Ⅳ) patients with lymph node metastasis were investigated in this study. There was 21 male and 19 female patients, with an average age of 58.7 ± 3.46 years old. Histologically, 22 cases had differentiated adenocarcinoma and 18 had poorly undifferentiated adenocarcinoma. Thirty cases of non-invasive submucosal colon adenocarcinoma patients (TNM stage I A) were obtained that included 19 male and 11 female patients. Histologically, 16 cases had differentiated adenocarcinoma and 14 had poorly undifferentiated adenocarcinoma, whose ages ranged from 45 to 80 years with an average age of 57.9 ± 3.24 years old (Table 1). All patients underwent gastrectomy with regional lymph nodes dissection and informed consent was obtained from each patient. Tissue blocks of normal colon epithelium from 20 healthy individuals, early tumors, advanced colon adenocarcinoma tissue and corresponding metastatic lymph nodes were obtained within 30 min after removal from the patient. Each block was cut into 2 pieces, one for routine pathologic diagnosis, and the other for molecular analysis. The latter samples were frozen in liquid nitrogen immediately and stored at -260℃ until use.

Table 1 Baseline clinical and pathological characteristics of the patients

Characteristic	Non-invasive tumor (*n*)	Invasive tumor (*n*)
Gender		
Male	19	19
Female	11	21
Age		
Median yr	57.9 ± 3.24	58.7 ± 3.46
Ranger yr	45-80	43-80
Cancer TNM stage		
IA	30	0
Ⅲ-Ⅳ	0	40
Histological type		
Differentiated	16	22
Undifferentiated	14	18

Laser microdissection and RNA extraction

Before sectioning, tissue blocks were embedded in Tissue Tek OCT compound medium (VWR Scientific Products, San Diego, CA, USA) in a cryostat. Then serial 8-micron-thick slices were prepared and mounted onto a foiled slide and stored at -70℃ until use.

Frozen section slides were stained just before LMD and the staining process was performed on ice. Briefly, the slides were fixed in 70% ethanol for 30 s and stained with 0.1% toluidine blue (TBO) for 15 s, followed by a 5-s dehydration step in each of 75%, 95% and 100% ethanol and a final 5-min dehydration step in xylene. Once air-dried, the sections were laser microdissected with a LMD system (Leica Microsystems, Wetzlar, Germany) and the target cells were selectively collected. Next, total RNA from the interest cells was extracted independently with the RNA-lyase Micro Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The integrity of the total RNA was measured by Lab-on-chip (Agilent, Palo Alto, USA).

Polypeptide

We carried out polypeptide analysis in 30 cases with early carcinoma, 40 cases with metastatic lymph node and 20 healthy individuals. All technical services were provided by Shanghai Biochip Corporation (Shanghai, China). Among the extracted total RNA, 200 ng was amplified for array analysis, while the other 100 ng was kept unamplified for later certification. After P27-based RNA amplification, aliquots (2.5 mg) of aRNA from early carcinoma tissues and the corresponding metastatic lymph node were labeled with Cy3-dCTP and Cy5-dCTP, respectively. The labeled probes were hybridized with Human cDNA Chip version 2.0 (SBC-R-HC-100-20, Shanghai, China) which contained 13 824 genes (including 10 positive controls and 6 negative ones), and the signals were detected by Agilent Scanner

(Agilent, Palo Alto, USA). We set the cutoff values for signal intensities, i.e., the signal to noise ratios of Cy3 or Cy5 must be greater than 2. Genes with Cy3:Cy5 ratios > 4 or < 0.25 in both cases were defined as commonly up- or down-regulated genes respectively.

Semiquantitative RT-PCR

Nonamplified total RNA (100 ng) was reverse transcribed into cDNA using Superscript Ⅱ reverse transcriptase (Invitrogen, USA) for 60 min at 42℃ and 15 min at 70℃. Each single-stranded cDNA was diluted for subsequent PCR amplification and the content of cDNA were semiquantitatively normalized by housekeeping gene *β-actin*. PCR conditions of different genes included an initial denaturation at 94℃ for 3 min, and 30-35 cycles of denaturation at 94℃ for 30 s, annealing for 30 s, and elongation at 72℃ for 1 min. The primer sequences, annealing temperatures and cycle numbers of each gene are available on request. Amplified PCR products were visualized by electrophoresis in 1% agarose gel containing ethidium bromide.

LMD

The target cells were successfully laser microdissected in every sample. Consequently, about 6-8 × 10^6 cells were collected for the total RNA extraction and near 200-400 ng total RNA was obtained. The integrity of every sample was proved by Lab-on-chip.

RESULTS

After P27-based amplification, we evaluated the expression profiles of the carcinoma cells from early colon cancer (ⅠA) in 30 cases in comparison with advanced colon adenocarcinoma and corresponding metastatic lymph node (TNM stage Ⅲ to Ⅳ) in 40 cases, scatter plots of polypeptide analysis showed in Figure 1. Amplified aRNA from early colon carcinoma cells (Cy-3) and metastatic carcinoma cells (Cy-5) were labeled and hybridized to the polypeptide.

The analysis of the polypeptide data showed that 49 genes (including 31 of unknown function) were commonly overexpressed (Cy3:Cy5 > 4) in 22 of 30 patients (TNM ⅠA) in early colon carcinoma, another 37 genes (including 9 of unknown function) were significantly suppressed (Cy3:Cy5 < 0.25) in the early carcinoma cells. The up-regulated genes were related to cell division, cell adhesion, cytoskeleton, and cell defense and cell metabolism. Meanwhile, the down-regulated genes included those associated with cell development, cell cycle, signal transduction, adhesion, cell defense, gene expression and cell metabolism (Table 2).

Expression of differential genes in the progression of colon cancer

By means of semiquantitative RT-PCR, we further evaluated the expression levels of 25 selected genes, which included 10 up-regulated and 15 down-regulated genes, in microdissected colon epithelium, early colon cancer cells and metastatic cells in lymph node from other 22 cases. First, we investigated the expression levels of these genes in early colon cancer and metastatic lymph node. The results showed that 12 genes were expressed in the same pattern in > 50% (8/15) cases as revealed by polypeptide analysis.

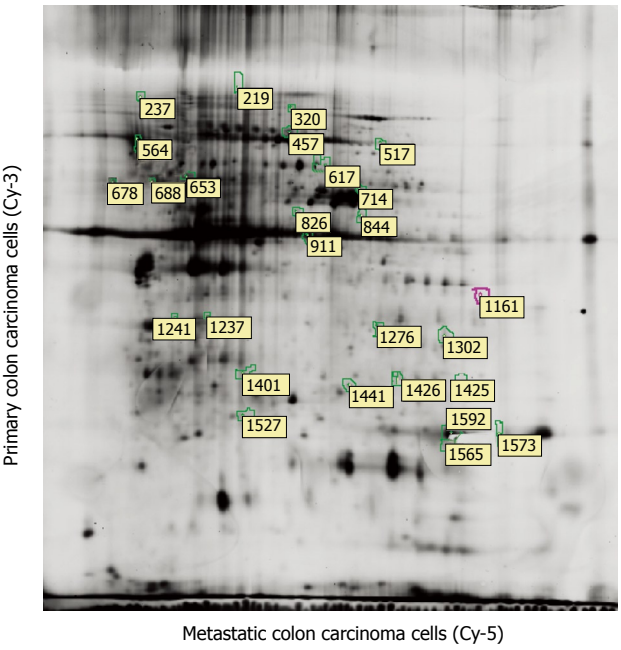


Figure 1 Scatter plots of polypeptide analysis. Amplified aRNA from early colon cancer cells (Cy-3) and metastatic cancer cells (Cy-5) are labeled and hybridized to the polypeptide.

We further measured the expression levels of these 12 genes in paired normal colon epithelium from healthy individuals and early carcinoma from the same 22 patients. We found that the expression pattern of 3 genes (*OPCML*, *RNASE1* and *YES1*) was similar to tumor suppressor genes in > 50% (8/15) cases, *e.g.* the expression level of these genes were highest in normal colon epithelium, decreased in early carcinoma, and further decreased in metastatic lymph node. Meanwhile, the expression level of ACK1 demonstrated the opposite tendency, the pattern of which was similar to oncogene in > 50% (8/15) cases (Figure 2). The expression pattern of OPCML was also confirmed at the gene level by immunohistochemical staining.

Confirmation of microarry finding

To examine the reliability of microarray data, we confirmed our data at the level of aRNA and gene expression. First, we selected three up-regulated genes (*SLC9A1*, *OPCML* and *UBAP*) and three down-regulated genes (*COX6C*, *SERPINB1* and *CCL22*) to measure their expression levels by semiquantitative RT-PCR. To obtain truly comparable results, we used the nonamplified total RNA (from the same batch that was used for the array hybridizations) as the template. The results were very similar to the microarray data for these genes.

Table 2 Representative list of differentially expressed genes detected by MALDI-TOF

GeneBank	Description	Cy3:Cy5[2]
NM_006500	MCAM (melanoma adhesion molecule)	10.79[1]
NM_002545	OPCML (opioid binding protein/cell adhesion molecule-like)	10.56[1]
NM_002933	RNASE1 (ribonuclease, RNase A family, 1)	6.8[1]
NM_001993	F3 (coagulation factor Ⅲ)	6.29[1]
NM_005433	YES1 (v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1)	5.916[1]
NM_016525	UBAP (ubiquitin associated protein)	5.876
NM_001428	ENO1 (enolase 1)	5.692[1]
S68616	SLC9A1 (Na+/H+ exchanger NHE-1 isoform)	5.484
NM_003254	TIMP1(tissue inhibitor of metalloproteinase 1)	5.29[1]
NM_005903	MADH5 (mothers against decapentaplegic, homolog 5)	5.083
U82828	ATM (ataxia telangiectasia)	0.205[1]
NM_006343	MERTK (c-mer proto-oncogene tyrosine kinase)	0.19
NM_002985	SCYA5 (small inducible cytokineA5)	0.19[1]
NM_005348	HSPCA (heat shock 90 kDa protein 1, alpha)	0.164
NM_003968	UBE1C (ubiquitin-activating enzyme E1C)	0.161
NM_004374	COX6C (cytochrome c oxidase subunit VIc)	0.131
NM_002990	SCYA22 (small inducible cytokine subfamily A, member22)	0.129
NM_005781	ACK1 (activated p21cdc42Hs kinase)	0.128[1]
NM_005139	ANXA3 (annexin A3)	0.124
AF053630	SERPINB1 (serine proteinase inhibitor, clade B, member 1)	0.123
NM_012090	MACF1 (microtubule-actin crosslinking factor 1)	0.098[1]
XM_042551	CAMK2A (calcium/calmodulin-dependent protein kinase)	0.0645
NM_000909	NPY1R (neuropeptide Y receptor Y1)	0.0252[1]
NM_015230	CENTD1 (centaurin, delta 1)	0.024
NM_004958	FRAP1 (FK506 binding protein 12-rapamycin associated protein 1)	0.0205

[1]Confirmed by semiquantitative RT-PCR in another group of 15 cases; [2]aRNA from primary colon carcinoma tissues and the corresponding metastatic lymph node were labeled with Cy3-dCTP and Cy5-dCTP, respectively.

As an additional means to confirm our data at the gene level, the results paralleled the differential expression pattern observed with polypeptide and RT-PCR.

Overall, the above results demonstrated that the samples obtained by means of P27-based amplification reflected the status of the original RNA in a proportional manner, and supported the reliability of our previous data[9].

DISCUSSION

Polypeptides allow an effective investigation of functional genomics. However, the existence of of the amount of surrounding cells can produce a lot of useless, noisy information because of the high sensitivity[9]. Therefore, the selection of cancer cells using LMD is of indispensable value in combination with polypeptide analysis. The LMD system used in this study integrated a UV laser of 337 nm wavelength with an upright microscope. The ultraviolet laser microbeam causes dissection by local photolysis of the supporter membrane and tissue section due to the high photon density of the microbeam rather than by local heating or coagulation. The cut sample falls down into PCR tubes placed underneath by gravity without any mechanical contact or further destruction of energy, and the integrity of the extracted aRNA is maximally kept.

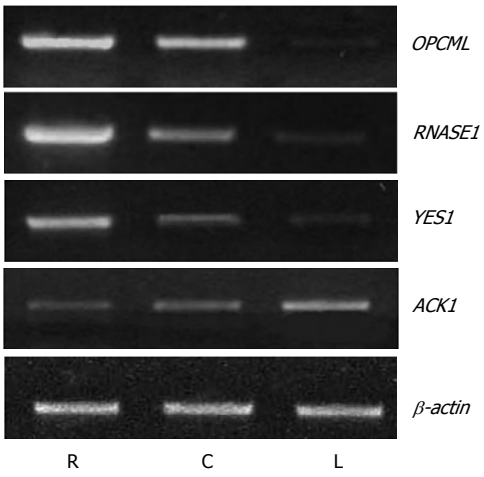


Figure 2 Identification of differentially expressed genes in progression of colon cancer by means of semiquantitative RT-PCR. R signifies a sample from normal colon epithelium, C signifies a sample from the colon carcinoma cells, and L signifies a sample from the metastatic carcinoma cells.

Metastasis of cancer is a highly selective sequential step, which favors the survival of a subpopulation of metastatic cells preexisting within the early tumor mass to produce clinically relevant metastases, the successfully metastatic cells must exhibit a complex phenotype that is regulated by transient or permanent changes in different genes at the DNA and/or aRNA level(s). This was also proved in colon cancer by work of other researchers[10].

Previously, our former study had discovered differential gene expression profiles between primary tumor and metastatic lesions in gastric cancer patients using LMD and cDNA microarray[11]. The results clearly demonstrated that an analysis of the gene-expression profile could be performed by using LMD, P27-based RNA amplification and polypeptide. Moreover, it was confirmed at the level of aRNA and gene. Although the majority of these genes have been implicated in various aspects of tumor biology, few have been demonstrated to be associated with colon cancer.

Among the above genes, some may be differentially expressed because of different living environments[12-15]. Therefore, to further confirm and screen the results of polypeptide analysis, we measured the expression levels of 25 selected genes in another group using semiquantitative RT-PCR. These target cells were collected by LMD, and the normal colon epithelium were included. As a result, we identified four genes, the expression level of were not only different between early carcinoma and metastatic lymph node (same as the results of polypeptide), but also different between normal mucosa and early tumor. These results suggested that these four genes could play a role in

the tumorigenesis and metastasis of colon cancer .The expression pattern of three genes (*OPCML*, *RNASE1* and *YES1*) was similar to tumor suppressor genes[16], *e.g.* the expression level of these genes were highest in normal colon epithelium, decreased in early carcinoma, and further decreased in metastatic lymph node. *OPCML* (opioid binding gene/cell adhesion molecule-like) encodes a member of the IgLON subfamily in the immunoglobulin gene superfamily and acted as a GPI-anchored gene[17,18]. Sellar *et al* had found that *OPCML* has tumor-suppressor function in epithelial ovarian cancer, which was in accordance with our findings in gastric cancer[19-22]. Interestingly, *YES1* is the cellular homolog of a virus oncogene and was considered to be associated with esophageal tumorigenesis[23-25], which was on the contrary to our results. In respect to the gene *RNASE1*, it encoded a member of the pancreatic-type of secretory ribonucleases, a subset of the ribonuclease A superfamily, and it had not been shown to have any relationship with human cancers previously[26].

On the contrary, the expression pattern of gene *ACK1* demonstrated the opposite tendency to the genes mentioned above, which was similar to oncogene[27]. *ACK1* encodes a tyrosine kinase that binds Cdc42Hs in its GTP-bound form and inhibited both the intrinsic and GTPase-activating gene (*GAP*)-stimulated GTPase activity of Cdc42Hs[28]. It was directly linked to a tyrosine phosphorylation signal transduction pathway. Up to now, its effect on tumor progression had not been reported.

Tumor suppressor genes and oncogenes were important target molecules in the clinical diagnosis and therapy of tumors. We reported the different expression of these four genes during the progression of colon cancer. In conclusion, using carefully controlled conditions, analysis of gene expression profile by means of LMD, P^{27}-based amplification, and polypeptide has been provided.

Useful information for clarifying the mechanism underlying development and metastasis of colon cancers[29,30]. These results not only reveal the differentially expressed genes in progression of colon cancer, but also provide information that may prove useful for identifying novel diagnostic and therapeutic targets.

COMMENTS

Background

Colon cancer is the second leading cause of cancer death in the worldwide. Its clinical behavior depends on the potential metastasis of the tumor. However, up to now, the difference of genes had rarely been reported between early colon adenocarcinoma (TNM stage Ⅰ) patients and advanced adenocarcinoma (TNM stage Ⅲ-Ⅳ) cases with lymph node metastasis.

Research frontiers

In this study, the authors revealed the gene profiles in non-invasive and invasive colon cancer using laser microdissection (LMD) and polypeptide analysis. Using these techniques they have identified early expression profiles of five differential genes associated with invasive colon cancer.

Innovations and breakthroughs

These studies have shown that certain genes are involved in the development of colon cancer and that the gene expression of non-invasive early colon adenocarcinoma was obviously different from that of invasive cases. This offers a new potential molecular mechanism of invasive colon cancer using LMD and polypeptide analysis. Our major finding in the study is that five genes are consequently differently expressed between early and advanced colorectal cancer (CRC) cases. Three of these resembled the expression pattern of tumor suppression genes (*OPCML*, *RNASE1* and *YES1*).

Applications

These results revealed that expression profiles of differential genes were associated with the progression of colon cancer, and these insights might play a role in identifying novel diagnostic and therapeutic markers in colon cancer.

Peer review

This is an interesting manuscript investigating the expression profile differences of early and advanced CRC at aRNA level using laser microdissection and polypeptide analysis. The major finding of the study was that five genes were consequently differently expressed between early and advanced CRC cases. Three of these resembled the expression pattern of tumor suppression genes (*OPCML*, *RNASE1* and *YES1*) while one further gene showed an expression pattern similar to oncogenes.

REFERENCES

1 **Zhu JS**, Shen B, Chen JL, Chen GQ, Yu XH, Yu HF, Zhu ZM. Molecular action mechanisms of NM-3 on human gastric cancer SGC-7901 cells in vivo or in vitro. *World J Gastroenterol* 2003; **9**: 2366-2369

2 **Zhu JS**, Jiang XH, Cheng WX. Studies on peritoneal chemotherapy treatment of gastric cancer ascites and correlative effects. *Hepato-Gastroenterol* 2000; **15** (Suppl): B108

3 **Wang ZN**, Xu HM, Jiang L, Zhou X, Lu C, Zhang X. Expression of survivin mRNA in peritoneal lavage fluid from patients with gastric carcinoma. *Chin Med J* (Engl) 2004; **117**: 1210-1217

4 **Portera CA Jr**, Berman RS, Ellis LM. Molecular determinants of colon cancer metastasis. *Surg Oncol* 1998; **7**: 183-195

5 **Fidler IJ**. Critical determinants of metastasis. *Semin Cancer Biol* 2002; **12**: 89-96

6 **Emmert-Buck MR**, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR, Weiss RA, Liotta LA. Laser capture microdissection. *Science* 1996; **274**: 998-1001

7 **Böhm M**, Wieland I, Schütze K, Rübben H. Microbeam MOMeNT: non-contact laser microdissection of membrane-mounted native tissue. *Am J Pathol* 1997; **151**: 63-67

8 **Liotta L**, Petricoin E. Molecular profiling of human cancer. *Nat Rev Genet* 2000; **1**: 48-56

9 **Wang L**, Zhu JS, Song MQ, Chen GQ, Chen JL. Comparison of gene expression profiles between primary tumor and metastatic lesions in gastric cancer patients using laser microdissection and cDNA microarray. *World J Gastroenterol* 2006; **12**: 6949-6954

10 **Versteeg HH**, Spek CA, Peppelenbosch MP, Richel DJ. Tissue factor and cancer metastasis: the role of intracellular and extracellular signaling pathways. *Mol Med* 2004; **10**: 6-11

11 **Kakinuma T**, Hwang ST. Chemokines, chemokine receptors, and cancer metastasis. *J Leukoc Biol* 2006; **79**: 639-651

12 **Cui Y**, Ying Y, van Hasselt A, Ng KM, Yu J, Zhang Q, Jin J, Liu D, Rhim JS, Rha SY, Loyo M, Chan AT, Srivastava G, Tsao GS, Sellar GC, Sung JJ, Sidransky D, Tao Q. OPCML is a broad tumor suppressor for multiple carcinomas and lymphomas with frequently epigenetic inactivation. *PLoS ONE* 2008; **3**: e2990

13 **Ye F**, Zhang SF, Xie X, Lu WG. OPCML gene promoter methylation and gene expression in tumor and stroma cells of invasive cervical carcinoma. *Cancer Invest* 2008; **26**: 569-574

14 **Hayashida T**, Ueda M, Aiura K, Tada H, Onizuka M, Seno M, Yamada H, Kitajima M. Anti-angiogenic effect of an insertional fusion protein of human basic fibroblast growth factor and ribonuclease-1. *Protein Eng Des Sel* 2005; **18**:

321-327
15 **Mahajan NP**, Whang YE, Mohler JL, Earp HS. Activated tyrosine kinase Ack1 promotes prostate tumorigenesis: role of Ack1 in polyubiquitination of tumor suppressor Wwox. *Cancer Res* 2005; **65**: 10514-10523
16 **Lynch G**, Kohler S, Leser J, Beil M, Garcia-Marin LJ, Lutz MP. The tyrosine kinase Yes regulates actin structure and secretion during pancreatic acinar cell damage in rats. *Pflugers Arch* 2004; **447**: 445-451
17 **Ntougkos E**, Rush R, Scott D, Frankenberg T, Gabra H, Smyth JF, Sellar GC. The IgLON family in epithelial ovarian cancer: expression profiles and clinicopathologic correlates. *Clin Cancer Res* 2005; **11**: 5764-5768
18 **Reed JE**, Dunn JR, du Plessis DG, Shaw EJ, Reeves P, Gee AL, Warnke PC, Sellar GC, Moss DJ, Walker C. Expression of cellular adhesion molecule 'OPCML' is down-regulated in gliomas and other brain tumours. *Neuropathol Appl Neurobiol* 2007; **33**: 77-85
19 **Sellar GC**, Watt KP, Rabiasz GJ, Stronach EA, Li L, Miller EP, Massie CE, Miller J, Contreras-Moreira B, Scott D, Brown I, Williams AR, Bates PA, Smyth JF, Gabra H. OPCML at 11q25 is epigenetically inactivated and has tumor-suppressor function in epithelial ovarian cancer. *Nat Genet* 2003; **34**: 337-343
20 **Guo W**, Shang F, Liu Q, Urim L, West-Mays J, Taylor A. Differential regulation of components of the ubiquitin-proteasome pathway during lens cell differentiation. *Invest Ophthalmol Vis Sci* 2004; **45**: 1194-1201
21 **Wei W**, Ayad NG, Wan Y, Zhang GJ, Kirschner MW, Kaelin WG Jr. Degradation of the SCF component Skp2 in cell-cycle phase G1 by the anaphase-promoting complex. *Nature* 2004; **428**: 194-198
22 **Kudo Y**, Kitajima S, Sato S, Ogawa I, Miyauchi M, Takata T. Transfection of p27 (Kip1) threonine residue 187 mutant type gene, which is not influenced by ubiquitin-mediated degradation, induces cell cycle arrest in oral squamous cell carcinoma cells. *Oncology* 2002; **63**: 398-404
23 **Defatta RJ**, De Benedetti A. Translational upregulation of yes accompanies eIF4E-mediated oncogenic transformation. *Int J Oncol* 2003; **23**: 1709-1713
24 **Lawson C**, Goupil S, Leclerc P. Increased activity of the human sperm tyrosine kinase SRC by the cAMP-dependent pathway in the presence of calcium. *Biol Reprod* 2008; **79**: 657-666
25 **Barraclough J**, Hodgkinson C, Hogg A, Dive C, Welman A. Increases in c-Yes expression level and activity promote motility but not proliferation of human colorectal carcinoma cells. *Neoplasia* 2007; **9**: 745-754
26 **Psarras K**, Ueda M, Tanabe M, Kitajima M, Aiso S, Komatsu S, Seno M. Targeting activated lymphocytes with an entirely human immunotoxin analogue: human pancreatic RNase1-human IL-2 fusion. *Cytokine* 2000; **12**: 786-790
27 **Mahajan NP**, Liu Y, Majumder S, Warren MR, Parker CE, Mohler JL, Earp HS, Whang YE. Activated Cdc42-associated kinase Ack1 promotes prostate cancer progression via androgen receptor tyrosine phosphorylation. *Proc Natl Acad Sci USA* 2007; **104**: 8438-8443
28 **Modzelewska K**, Newman LP, Desai R, Keely PJ. Ack1 mediates Cdc42-dependent cell migration and signaling to p130Cas. *J Biol Chem* 2006; **281**: 37527-37535
29 **Castiglione F**, Taddei A, Buccoliero AM, Garbini F, Gheri CF, Freschi G, Bechi P, Degl'Innocenti DR, Taddei GL. TNM staging and T-cell receptor gamma expression in colon adenocarcinoma. Correlation with disease progression? *Tumori* 2008; **94**: 384-388
30 **Iddings D**, Bilchik A. The biologic significance of micrometastatic disease and sentinel lymph node technology on colorectal cancer. *J Surg Oncol* 2007; **96**: 671-677

S- Editor Li DL **L- Editor** Alpini GD **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5893

World J Gastroenterol 2008 October 14; 14(38): 5893-5899
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Effect of b value on monitoring therapeutic response by diffusion-weighted imaging

Zhao-Xia Jiang, Wei-Jun Peng, Wen-Tao Li, Feng Tang, Shi-Yuan Liu, Xu-Dong Qu, Jian-Hua Wang, Hong-Feng Lu

Zhao-Xia Jiang, Wei-Jun Peng, Wen-Tao Li, Feng Tang, Department of Radiology, Affiliated Cancer Hospital of Fudan University, Shanghai 200032, China
Zhao-Xia Jiang, Wei-Jun Peng, Wen-Tao Li, Feng Tang, Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China
Shi-Yuan Liu, Department of Radiology, Affiliated Changzheng Hospital of the Second Military Medical University, Shanghai 200032, China
Xu-Dong Qu, Jian-Hua Wang, Department of Radiology, Affiliated Zhongshan Hospital of Fudan University, Shanghai 200032, China
Hong-Feng Lu, Department of Pathology, Affiliated Cancer Hospital of Fudan University, Shanghai 200032, China
Author contributions: Jiang ZX, Peng WJ designed the research; Jiang ZX, Li WT, Liu SY, Qu XD and Wang JH performed the research; Jiang ZX, Tang F and Lu HF analyzed data; Jiang ZX wrote the manuscript; Peng WJ revised the paper.
Supported by The National Natural Science Foundation of China, NO. 30470503; The Key Program of Shanghai Science and Technology Commission, NO. 04JC14074; The Foundation of Shanghai Educational commission, NO. 03J405037
Correspondence to: Wei-Jun Peng, Department of Radiology, Affiliated Cancer Hospital of Fudan University, 270 Dong'an Road, Shanghai 200032, China. weijunpeng@yahoo.com
Telephone: +86-21-64433384 Fax: +86-21-64433384
Received: July 7, 2008 Revised: August 26, 2008
Accepted: September 2, 2008
Published online: October 14, 2008

Abstract

AIM: To explore the diffusion gradient b-factor that optimizes both apparent diffusion coefficient (ADC) measurement and contrast-to-noise (CNR) for assessing tumor response to transarterial chemoembolization (TACE) in a rabbit model.

METHODS: Twelve New Zealand white rabbits bearing VX2 tumors in the liver were treated with TACE. Diffusion-weighted imaging (DWI) with various b values was performed using the same protocol before and 3 d after treatment with TACE. ADC values and CNR of each tumor pre- and post-treatment with different b factors were analyzed. Correlation between ADC values and extent of necrosis in histological specimens was analyzed by a Pearson's correlation test.

RESULTS: The quality of diffusion-weighted images diminished as the b value increased. A substantial decrease in the mean lesion-to-liver CNR was observed on both pre- and post-treatment DW images, the largest difference in CNR pre- and post-treatment was manifested at a b value of 1000 s/mm^2 (P = 0.036). The effect of therapy on diffusion early after treatment was shown by a significant increase in ADCs (P = 0.007), especially with large b factors ($\geqslant$ 600 s/mm^2). The mean percentage of necrotic cells present within the tumor was 76.3%-97.5%. A significant positive correlation was found between ADC values and the extent of necrosis with all b values except for b200, a higher relative coefficient between ADC values and percentage of necrosis was found on DWI with b1000 and b2000 (P = 0.002 and 0.006, respectively).

CONCLUSION: An increasing b value of up to 600 s/mm^2 would increase ADC contrast pre- and post-treatment, but decrease image quality. Taking into account both CNR and ADC measurement, diffusion-weighted imaging obtained with a b value of 1000 s/mm^2 is recommended for monitoring early hepatic tumor response to TACE.

Key words: Hepatic carcinoma; Diffusion-weighted MR; Treatment response; Apparent diffusion coefficient

Peer reviewer: Dr. Li-Qin Zhao, Department of Radiology, Beijing Friendship Hospital Affiliate of Capital University of Medical Science, 95 Yongan Road, Xuanwu Distric, Beijing 100050, China

Jiang ZX, Peng WJ, Li WT, Tang F, Liu SY, Qu XD, Wang JH, Lu HF. Effect of b value on monitoring therapeutic response by diffusion-weighted imaging. *World J Gastroenterol* 2008; 14(38): 5893-5899 Available from: URL: http://www.wjgnet.com/1007-9327/14/5893.asp DOI: http://dx.doi.org/10.3748/wjg.14.5893

INTRODUCTION

Transcatheter hepatic arterial chemoembolization (TACE) remains the initial treatment for unresectable hepatocellular carcinoma (HCC)[1]. Evaluating the therapeutic response of HCC to TACE is critical

in assessing the success of treatment and deciding therapeutic plan. Diffusion-weighted imaging (DWI) enables noninvasive characterization of biologic tissues based on their water diffusion properties. It is theoretically possible to quantify the combined effects of capillary perfusion and water diffusion *in vivo* by an apparent diffusion coefficient (ADC)[2], and its value is equal to the true diffusion coefficient *D* when diffusion is the only type of motion. Diffusion-weighted images are obtained by acquiring T2-weighted images with the addition of diffusion weighting gradient known as the "b value". Generally, the larger the b values used, the lower the ADC values owing to the contribution of perfusion. Large b factors should be chosen for more precise evaluation of ADC values of the tumor. However, image quality will be greatly diminished if large b factors are used. Thus, it is very important to select a suitable b value to evaluate the tumor response accurately. Diffusion-weighted imaging has recently been used to monitor tumor response after therapy. However, there are considerable discrepancies in the selection of b values in previous reports[3-7]. In our study, we compared different b-value DWI in evaluation of hepatic tumor necrosis after TACE in rabbits to explore the optimal b value.

MATERIALS AND METHODS

Animal model and study design

Fifteen male adult New Zealand white rabbits including three carrier rabbits (Animal Laboratory, Fudan University) with an average weight of 2.5 kg, were used in this experiment. The Animal Care and Use Subcommittee at Fudan University approved this experimental procedure. VX2 carcinoma strain was maintained by successive transplantation into the hind limb of a carrier rabbit. Tumor cell suspension was implanted with one subcutaneous injection into the hind leg of each carrier rabbit and grown for 2 wk. All animals were intramuscularly anesthetized with a mixture of ketamine hydrochloride (0.1 g/kg) and diazepam (5 mg/kg). The tumor was surgically excised from the carrier rabbit and placed in normal saline. Tumor tissue was dissociated into approximately 1-2 mm^3 fragments and suspended in 5 mL normal saline, then aspirated into a 1 mL injector. A midline laparotomy was performed to expose the liver of the recipient rabbit, and tumor suspension (0.1-0.2 mL) was implanted in the left lobe of liver *via* an 18-gauge needle. The abdomen was closed in two layers. The tumors were allowed to grow to 1-2 cm in diameter, which typically required 2 wk.

Conventional and diffusion-weighted imaging was performed before and 3 d after therapy using the same protocol.

TACE

TACE was performed under the guide of digital subtraction angiography (Infinix Vc-i, Toshiba, Japan). Animals were intramuscularly anesthetized with a mixture of ketamine hydrochloride (0.1 g/kg) and diazepam (5 mg/kg). Vascular access was achieved in the femoral artery through surgical cut down. Celiac angiography was performed to identify the hepatic arterial anatomy and the feeder artery of the tumor using a 3-F catheter (Cook, Bloomington, India). The left hepatic artery, which exclusively supplies blood flow to the tumor, was catheterized selectively. When the catheter was adequately positioned in the left hepatic artery after celiac arteriography was performed, a chemoembolization mixture consisting of 5 mg doxorubicin (adriamycin; Farmitalia Carlo Erba, Italy) and 1 mL ethiodized oil (lipiodol; Andre Guerbet, France) was injected carefully into the artery. Digital spot images were obtained after chemoembolization. The catheter was then removed, and the femoral artery was ligated.

MR imaging protocol

MR scanning was performed on a 1.5 T superconducting magnet (Signa Twinspeed excite, GE Medical Systems, USA) equipped with a maximum gradient strength of 40 mT/m. All images were acquired using a phased array knee coil. In all rabbits, unenhanced and contrast-enhanced T1-, T2-, and diffusion-weighted images were obtained in the axial, and/or coronal, sagittal plane, respectively. Diffusion-weighted images were obtained before contrast medium injection. The rabbits were anesthetized with a combination of ketamine hydrochloride and diazepam as described above. Each animal was placed in the knee coil at supine position with its abdomen fastened using a belt to control the motion artifacts caused by breathing.

T2-weighted fast spin-echo images (TR/TE, 2800/72.4; matrix, 512 × 512), and T1-weighted spin-echo images (TR/TE, 350/9.0; matrix, 256 × 256) were obtained. DWI was performed in the axial plane using a single-shot echo planar imaging (EPI) sequence with the following parameters: TR/TE = 3000-4000 /50.9-70.2 ms, FOV = 12 cm × 12 cm, pixel matrix = 256 × 256, section thickness = 3 mm, intersection gap= 0.5 mm. Different values of b factor (0, 200, 400, 600 or 1000, 2000 s/mm^2) were used. Then, 0.05 mmol/kg gadopentetate dimeglumine (Magnevist; Schering, Germany) was administered intravenously, and fat-saturated spoiled gradient-echo T1W sequences were obtained. Therefore, the overall scan time was approximately 15 min.

Image analysis

ADC maps were automatically generated on the post processing workstation (ADW4.0, GE Medical Systems, USA). One experienced radiologist established ROIs in the tumors, lesion-free liver parenchyma, and background on all series of diffusion-weighted images. For heterogeneous tumors, regions of interest (ROIs) covered the entire tumor at the maximum section consisting of at least 100 pixels, and then were copied to the corresponding ADC maps, from which the ADC value with different b factors for each ROI was calculated. Each of the signal intensities (SI) and ADCs was measured three times, and the measurements

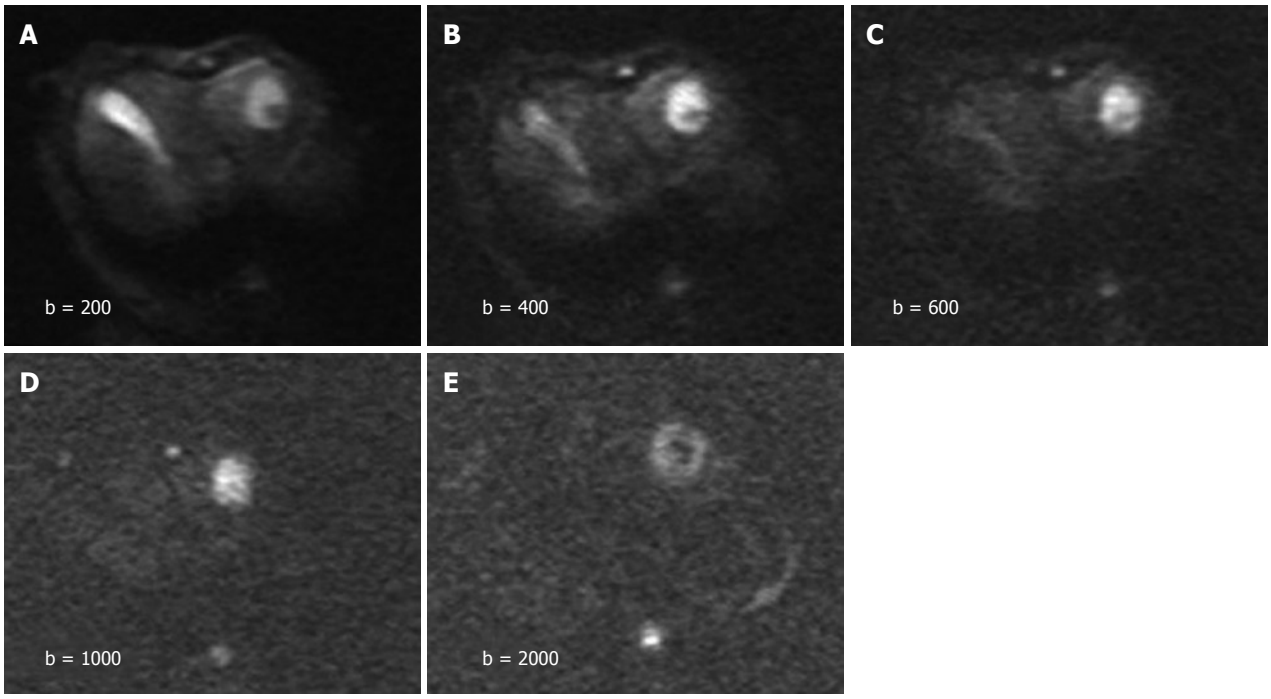


Figure 1 Diffusion-weighted images obtained with different b-values. **A** and **B**: High signal intensity in tumors and hyperintense gallbladder on b200 and b400 DWI; **C**-**E**: Hyperintensive tumors and invisible gallbladder on b600, b1000 and b2000 DWI. The image qualities degrade with increasing b values.

were averaged. CNR performed on all DWI series was calculated using the following formula: CNR = $(SI_{lesion}-SI_{liver})/SD_{noise}$, where SI_{lesion} and SI_{liver} are the signal intensity of the tumor and liver, respectively, and SD is the standard deviation from the background noise.

Histological analysis and comparison with MR images

All animals were sacrificed by giving an intravenous pentobarbital overdose immediately after the completion of MR imaging. The tumors were surgically removed and fixed in a 10% formaldehyde solution. From each tumor three 5 μm thick sections corresponding to the image planes were cut and stained with hematoxylin and eosin (H&E) and analyzed under a light microscope. Viable tumor and tumor necrosis were identified on these sections and correlated to the corresponding spin-echo images and the ADC maps. The percentage of necrotic area in each tumor was then calculated by an experienced pathologist.

Statistical analysis

Statistical analysis was performed using SPSS 13.0. ADC values were presented as mean ± standard deviation (SD). Pearson's correlation test was used to test for the relationship between ADC values and extent of necrosis. Differences in the ADCs pre- and post-treatment were assessed with the paired Student's *t*-test. The correlation between ADC, CNR and b factors was analyzed. $P < 0.05$ was considered statistically significant.

RESULTS

Chemoembolization

TACE was performed successfully in all animals, and no animals died within 3 d after the procedure. The left hepatic artery exclusively supplied blood flow to the tumor. A region of hypervascular blush was noted on the left side of the upper abdomen. Selective accumulation of deposits of iodized oil was observed in the hepatic tumors at digital spot images performed immediately after TACE.

MR imaging

The tumors were slightly inhomogeneous hyperintense on T2-weighted images, hypointense on T1-weighted images. A marked peripheral enhancement pattern was noted on contrast-enhanced T1WI before and after TACE, with centrally non-enhancing regions corresponding to the necrotic area. However, the margin between the viable tumor and its surrounding liver parenchyma was inconspicuous. On DWI obtained before treatment with a b value of 200 or 400 s/mm^2, both the tumor and gallbladder presented with hyperintense signals (Figure 1). On DWI with a b value equal to or larger than 600 s/mm^2, the gallbladder was depicted as a region of hypointense signal attenuation, whereas the tumor remained hyperintense (Figure 1). On DWI obtained after treatment, the viable tumor presented with hyperintense signals indicating a restricted diffusion capacity, whereas a necrotic area was depicted as hypointense indicating free diffusion. The image quality diminished greatly with increasing b value especially on b2000 DWI (Figure 1). A substantial decrease in the mean lesion-to-liver CNR was observed on both pre- and post-treatment DW images. On these series of DW images, the largest difference in CNR pre- and post-treatment was manifested at a b value of 1000 s/mm^2 ($P = 0.036$, Figure 2).

Table 1 ADCs obtained with different b-values on DWI before and after therapy

B value(s/mm^2)	ADC(10^{-3} mm^2/s) pre-treatment	ADC(10^{-3} mm^2/s) post-treatment
200	2.32 ± 0.53	2.41 ± 0.62
400	1.88 ± 0.45	1.99 ± 0.60
600	1.49 ± 0.26	1.79 ± 0.43
1000	1.19 ± 0.32	1.52 ± 0.42
2000	0.81 ± 0.29	1.22 ± 0.36

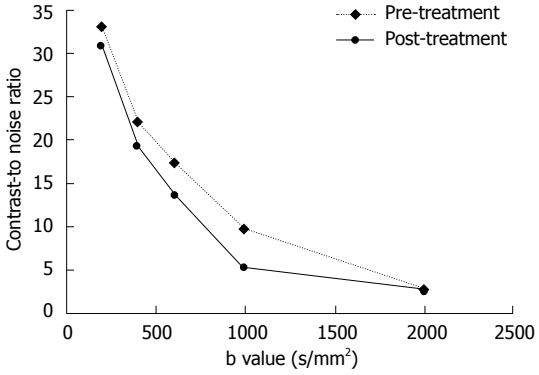


Figure 2 Contrast-to-noise ratio (CNR) of the diffusion-weighted images *vs* different sets of b-values of hepatic tumors pre- and post-treatment. CNR decreases with increasing b values after TACE, especially at b1000 on DWI.

The early effect of TACE on diffusion was shown by a substantial increase in ADC (P = 0.007), especially with large b factors (≥ 600 s/mm^2, Figure 3). These data are summarized in Table 1.

Histopathologic analysis

At microscopic examination, tumors treated with TACE revealed massive necrosis involving both the peripheral and central regions of the tumor. The mean percentage of necrotic cells present within the tumor was 76.3%-97.5% (mean 89.1%). A significant positive correlation was found between ADC values and extent of necrosis with all b values except for b200. A higher relative coefficient between ADC values and percentage of necrosis was found on DWI with b1000 and b2000 (P = 0.002 and 0.006, Figure 4).

DISCUSSION

Total necrosis after TACE has been reported to be as low as 10%-20%[8,9], and the presence of residual or recurrent tumor is inevitable. Precise evaluation with imaging modalities at an early stage is important to determine whether the tumor needs further treatment. The tumor volume has generally been used as an indicator of therapeutic response. However, necrotic tumors do not shrink until 1-2 mo after chemoembolization[10]. Besides, the apparent post-treatment increase may be due to the visualization of surrounding edematous changes. Thus, changes in tumor volume fail to predict the histological tumor response.

Diffusion-weighted imaging provides insights into tumor behavior for monitoring treatment response,

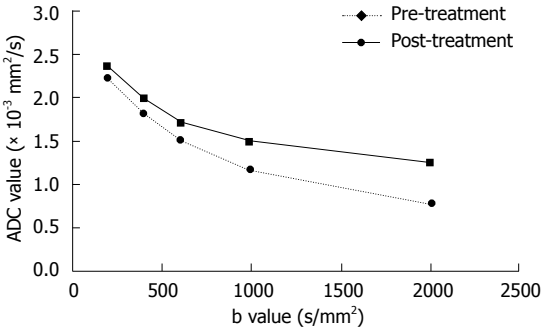


Figure 3 Plots of averaged ADC values for all 12 tumors vs different sets of b-values of hepatic tumors pre- and post-treatment. ADC values present as a function of b factors. The early effect of therapy on diffusion is shown by a substantial increase in ADCs, especially at a large b factor (≥ 600 s/mm^2).

thus enabling noninvasive depiction of molecular diffusion which is the Brownian motion of water protons in biologic tissues. Calculation of the ADC allows quantification of that motion[11-16]. The sensitivity of diffusion-weighted imaging to water motion can be varied by changing b value, which is a function of diffusion gradient strength, duration of the gradient, and interval between diffusion gradients. Diffusion-weighted imaging has been used to predict and monitor the effect of several treatment options and to differentiate between viable and necrotic tumor tissues[3-7]. In these studies, a variety of b values ranging from 0 s/mm^2 to 4000 s/mm^2 were used[3-7]. To our knowledge, however, comparison between DW images obtained with different b values on evaluation of the efficacy of TACE has not been reported.

In this study, we treated rabbits bearing VX2 tumors with TACE, whose vascularization is similar to that of human liver tumors[17-19]. Subsequently, the early tumor response to TACE was assessed *via* different b values on DWI to determine which b value is most suitable for evaluation.

In the present study, the tumor and gallbladder presented with hyperintense signals on DWI obtained with a low b value of 200 or 400 s/mm^2. When the b value increased to 600 s/mm^2, the gallbladder was depicted as a region of hypointense signal attenuation whereas the tumor remained hyperintense (Figure 1). Visual assessment of signal attenuation on DWI has been applied in tumor detection and characterization, however, signal intensity observed on DWI depends on both water diffusion and T2 relaxation time[20]. The relative contribution of T2 signal intensity to DWI, namely "T2 shine-through" effect, is a source of error in image interpretation. Tissues of organs with a long T2 relaxation time, such as gallbladder or cystic lesions, may appear hyperintense on DWI because of the T2 shine-through effect. This effect can be reduced by increasing the b value, but cannot be easily avoided. The ADC value is independent of magnetic field strength and can overcome the effects of T2 shine-through, thus allowing a more meaningful evaluation of tumor response to therapy.

In our study, the ADC values corresponded to the

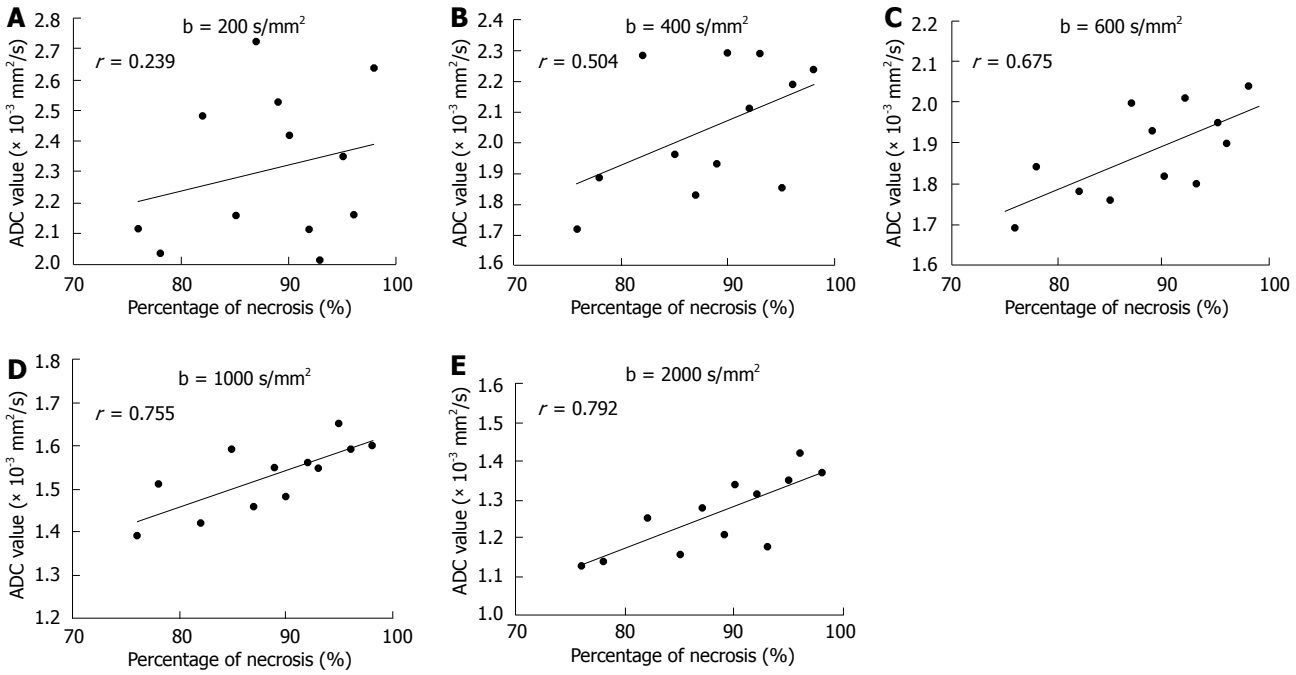


Figure 4 Plots of post-treatment ADC values as a percentage of necrosis measured on the sections of tumors. The r values of 0.239, 0.504, and 0.675 found at b200, b400 and b600 (**A**-**C**), and a higher *r* value at b1000 and b2000 are found on DWI (**D** and **E**).

histopathologic rate of necrosis within the tumors, suggesting that diffusion-weighted imaging has a potential for early detection of tumor necrosis after TACE, which is in agreement with the findings in theoretical diffusion models and *in vitro* and *in vivo* studies[3,5-7,11]. DWI can differentiate viable and necrotic tissues by calculating ADC values because in the former, cell and intracellular membranes are intact, restricting molecular diffusion into viable tumors. Conversely, necrotic tumors are characterized by a breakdown of these membranes, thereby allowing free diffusion and an increase of diffusing molecules, resulting in an increased ADC value[11,14].

Comparison between different b-values on DWI revealed that the ADC values decreased with the increasing b value, and the difference in ADC values pre- and post-treatment was significant. A higher relative coefficient between ADC values and percentage of necrosis was found on DWI with b1000 and b2000, indicating that a high b-value on DWI is more sensitive in early detection of tumor necrosis. The signal intensity on diffusion-weighted images is a mixture of diffusion and perfusion. On DWI obtained with a low b-value, perfusion effects usually cause larger signal attenuation than diffusion effects[21,22]. In the presence of perfusion, the ADC value calculated from images with a low b value would be overestimated due to this additional cause of signal attenuation. At a high b value, ADC measurement will be relatively perfusion insensitive and theoretically more reflective of tissue cellularity and the integrity of cellular membranes[23,24]. A few studies on evaluation of tumor response to therapy with high b values on DWI have recently been reported[25,26]. Mardor *et al*[25] reported that a high-b-value on DWI is highly correlated to radiotherapy-treated human brain tumors. Roth *et al*[26] demonstrated that high-b-value diffusion-weighted MR imaging can be potentially used in early detection of response to chemotherapy.

However, in the present study, the image quality diminished with the increasing b value especially at b2000 on DWI, CNR measurements showed that b1000 on DWI increased the contrast pre- and post-treatment, suggesting that higher b values may increase the diffusion sensitivity by diminishing the T2 shine-through effect. High b values may also decrease the absolute difference in signal intensity between tumor and liver parenchyma. The results of our study suggest that an intermediate b value (i.e., 1000 s/mm^2) may provide optimal visualization. In terms of the accuracy of ADC measurement, because the single-shot echo planar pulse sequence is very sensitive to magnetic susceptibility, resulting in geometric distortion artifacts that tend to be more severe when the b value is 2000 s/mm^2, image distortion may cause significant errors in the measurement of ADC values.

In abdominal diffusion-weighted imaging, the most challenging technical difficulty is to overcome the effects of breathing motion, while retaining the sensitivity to the microscopic motion. To reduce the artifact caused by breathing, several attempts were made in our study. First, fast imaging acquisition was applied. The most common form of data acquisition is a single-shot read out, in particular single-shot echo planar imaging[27-30], since the acquired phase error due to bulk motion is equal in each phase encoding step and therefore does not affect the image reconstruction. With EPI, the fastest MR imaging technique, an image can be acquired within 50 ms and physiologic motion can be literally frozen-out, so it has been widely applied in DW imaging.

Second, respiratory rate should be controlled

efficiently. To minimize the potential confounding artifacts caused by motion, Geschwind *et al*[13] acquired images shortly after the death of animals. We modified our protocol by fastening the abdomen of rabbits with a belt and using combined deep anesthesia. By this means, we obtained high quality images without virtually visible motion artifacts. The whole acquisition time was 15 min, and no animals died during the MR scanning procedure.

Our study had two major limitations. First, we placed the ROI covering the entire tumor. The ADC values of viable and necrotic area were not calculated. Second, due to the small population of animals, the results were not analyzed according to the tumor response or disease progress.

In summary, DWI can be used to assess the degree of tumor necrosis based on significant differences in ADC values. Based on the maximal contrast and changes in ADC values pre- and post-treatment, b values above 600 s/mm^2 are recommended. We hold that the optimal b value should be 1000 s/mm^2.

COMMENTS

Background

Necrotic tumor remnants or inflammatory fibrosis may not be accurately differentiated from residual tumor by conventional MR imaging early after therapy. DWI enables noninvasive characterization of biologic tissues based on their water diffusion properties and could provide information that is not readily available from conventional MR imaging. The sensitivity of diffusion-weighted imaging to water motion can be varied by changing the b value, which is a function of diffusion gradient strength. Thus, it is very important to select a suitable b value to evaluate the tumor response accurately.

Research frontiers

Diffusion-weighted imaging has been used to predict and monitor the effect of several treatment options and to differentiate between viable and necrotic tumor tissues. A few studies on evaluation of tumor response to therapy using at high b values on diffusion-weighted imaging (DWI) have recently been reported. Mardor *et al* reported that a high-b-value on DWI has a higher correlation to radiotherapy-treated human brain tumors. Roth et al demonstrated that a high-b-value on diffusion-weighted MR imaging can be potentially used in detecting early response of tumors to chemotherapy in an animal model.

Innovations and breakthroughs

A major limitation of previous studies is the lack of comparison between DW images obtained with different b values. In this study, we treated rabbits bearing VX2 tumors with transarterial chemoembolization (TACE). Subsequently, the early response of tumor to TACE was assessed *via* different b values on DWI to determine which b value is most suitable for evaluation.

Applications

DWI can be used to assess the degree of tumor necrosis based on significant differences in the apparent diffusion coefficient (ADC) values. With regard to maximal contrast, changes in ADC values pre- and post-treatment, b values above 600 s/mm^2 are recommended. Physicians should be aware of the trade-off between increased image distortion and ADC difference at higher b values, the optimal b value should be 1000 s/mm^2.

Terminology

DWI exploits the random, translational motion of water protons in biologic tissues, which causes phase dispersion of the spins resulting in signal loss. This signal loss can be quantified by calculating the ADC value, which refers to the specific diffusion capacity of a biologic tissue. The sensitivity of diffusion-weighted imaging to water motion can be varied by changing the b value, which is a function of diffusion gradient strength.

Peer review

In the study, the authors tried to find the diffusion gradient b-factor that optimizes ADC measurement and contrast-to-noise (CNR) for assessing tumor response to TACE in a rabbit model. The image modalities and technique they used in this study were advanced both at home and abroad. The study design is perfect. The conclusion about the optimal b value for monitoring early hepatic tumor response to TACE is of clinical importance.

REFERENCES

1 **Ramsey DE**, Kernagis LY, Soulen MC, Geschwind JF. Chemoembolization of hepatocellular carcinoma. *J Vasc Interv Radiol* 2002; **13**: S211-S221

2 **Le Bihan DJ**. Differentiation of benign versus pathologic compression fractures with diffusion-weighted MR imaging: a closer step toward the "holy grail" of tissue characterization? *Radiology* 1998; **207**: 305-307

3 **Hayashida Y**, Yakushiji T, Awai K, Katahira K, Nakayama Y, Shimomura O, Kitajima M, Hirai T, Yamashita Y, Mizuta H. Monitoring therapeutic responses of primary bone tumors by diffusion-weighted image: Initial results. *Eur Radiol* 2006; **16**: 2637-2643

4 **Dzik-Jurasz A**, Domenig C, George M, Wolber J, Padhani A, Brown G, Doran S. Diffusion MRI for prediction of response of rectal cancer to chemoradiation. *Lancet* 2002; **360**: 307-308

5 **DeVries AF**, Kremser C, Hein PA, Griebel J, Krezcy A, Ofner D, Pfeiffer KP, Lukas P, Judmaier W. Tumor microcirculation and diffusion predict therapy outcome for primary rectal carcinoma. *Int J Radiat Oncol Biol Phys* 2003; **56**: 958-965

6 **Seierstad T**, Folkvord S, Roe K, Flatmark K, Skretting A, Olsen DR. Early changes in apparent diffusion coefficient predict the quantitative antitumoral activity of capecitabine, oxaliplatin, and irradiation in HT29 xenografts in athymic nude mice. *Neoplasia* 2007; **9**: 392-400

7 **Tomura N**, Narita K, Izumi J, Suzuki A, Anbai A, Otani T, Sakuma I, Takahashi S, Mizoi K, Watarai J. Diffusion changes in a tumor and peritumoral tissue after stereotactic irradiation for brain tumors: possible prediction of treatment response. *J Comput Assist Tomogr* 2006; **30**: 496-500

8 **Okusaka T**, Okada S, Ueno H, Ikeda M, Yoshimori M, Shimada K, Yamamoto J, Kosuge T, Yamasaki S, Iwata R, Furukawa H, Moriyama N, Sakamoto M, Hirohashi S. Evaluation of the therapeutic effect of transcatheter arterial embolization for hepatocellular carcinoma. *Oncology* 2000; **58**: 293-299

9 **Choi BI**, Kim HC, Han JK, Park JH, Kim YI, Kim ST, Lee HS, Kim CY, Han MC. Therapeutic effect of transcatheter oily chemoembolization therapy for encapsulated nodular hepatocellular carcinoma: CT and pathologic findings. *Radiology* 1992; **182**: 709-713

10 **Pacella CM**, Bizzarri G, Cecconi P, Caspani B, Magnolfi F, Bianchini A, Anelli V, Pacella S, Rossi Z. Hepatocellular carcinoma: long-term results of combined treatment with laser thermal ablation and transcatheter arterial chemoembolization. *Radiology* 2001; **219**: 669-678

11 **Lyng H**, Haraldseth O, Rofstad EK. Measurement of cell density and necrotic fraction in human melanoma xenografts by diffusion weighted magnetic resonance imaging. *Magn Reson Med* 2000; **43**: 828-836

12 **Lang P**, Wendland MF, Saeed M, Gindele A, Rosenau W, Mathur A, Gooding CA, Genant HK. Osteogenic sarcoma: noninvasive in vivo assessment of tumor necrosis with diffusion-weighted MR imaging. *Radiology* 1998; **206**: 227-235

13 **Geschwind JF**, Artemov D, Abraham S, Omdal D, Huncharek MS, McGee C, Arepally A, Lambert D, Venbrux AC, Lund GB. Chemoembolization of liver tumor in a rabbit model: assessment of tumor cell death with diffusion-weighted MR imaging and histologic analysis. *J Vasc Interv Radiol* 2000; **11**: 1245-1255

14 **Herneth AM**, Guccione S, Bednarski M. Apparent diffusion coefficient: a quantitative parameter for in vivo tumor characterization. *Eur J Radiol* 2003; **45**: 208-213

15 **Thoeny HC**, De Keyzer F, Vandecaveye V, Chen F, Sun X, Bosmans H, Hermans R, Verbeken EK, Boesch C, Marchal G, Landuyt W, Ni Y. Effect of vascular targeting agent in rat

tumor model: dynamic contrast-enhanced versus diffusion-weighted MR imaging. *Radiology* 2005; **237**: 492-499
16 **Baur A**, Huber A, Arbogast S, Durr HR, Zysk S, Wendtner C, Deimling M, Reiser M. Diffusion-weighted imaging of tumor recurrencies and posttherapeutical soft-tissue changes in humans. *Eur Radiol* 2001; **11**: 828-833
17 **Zhao JG**, Feng GS, Kong XQ, Li X, Li MH, Cheng YS. Changes of tumor microcirculation after transcatheter arterial chemoembolization: first pass perfusion MR imaging and Chinese ink casting in a rabbit model. *World J Gastroenterol* 2004; **10**: 1415-1420
18 **Kuszyk BS**, Boitnott JK, Choti MA, Bluemke DA, Sheth S, Magee CA, Horton KM, Eng J, Fishman EK. Local tumor recurrence following hepatic cryoablation: radiologic-histopathologic correlation in a rabbit model. *Radiology* 2000; **217**: 477-486
19 **Quan XY**, Xie W, Zhang XL, Sun XJ, Zhu XL, Yan Z, Liang W. [Modification of the method for preparing rabbit liver VX2 tumor model and its MRI findings] *Nanfang Yike Daxue Xuebao* 2006; **26**: 747-749
20 **Provenzale JM**, Engelter ST, Petrella JR, Smith JS, MacFall JR. Use of MR exponential diffusion-weighted images to eradicate T2 "shine-through" effect. *AJR Am J Roentgenol* 1999; **172**: 537-539
21 **Le Bihan D**, Breton E, Lallemand D, Aubin ML, Vignaud J, Laval-Jeantet M. Separation of diffusion and perfusion in intravoxel incoherent motion MR imaging. *Radiology* 1988; **168**: 497-505
22 **Morvan D**. In vivo measurement of diffusion and pseudo-diffusion in skeletal muscle at rest and after exercise. *Magn Reson Imaging* 1995; **13**: 193-199
23 **Thoeny HC**, De Keyzer F, Boesch C, Hermans R. Diffusion-weighted imaging of the parotid gland: Influence of the choice of b-values on the apparent diffusion coefficient value. *J Magn Reson Imaging* 2004; **20**: 786-790
24 **Wheeler-Kingshott CA**, Thomas DL, Lythgoe MF, Guilfoyle D, Williams SR, Doran SJ. Burst excitation for quantitative diffusion imaging with multiple b-values. *Magn Reson Med* 2000; **44**: 737-745
25 **Mardor Y**, Pfeffer R, Spiegelmann R, Roth Y, Maier SE, Nissim O, Berger R, Glicksman A, Baram J, Orenstein A, Cohen JS, Tichler T. Early detection of response to radiation therapy in patients with brain malignancies using conventional and high b-value diffusion-weighted magnetic resonance imaging. *J Clin Oncol* 2003; **21**: 1094-1100
26 **Roth Y**, Tichler T, Kostenich G, Ruiz-Cabello J, Maier SE, Cohen JS, Orenstein A, Mardor Y. High-b-value diffusion-weighted MR imaging for pretreatment prediction and early monitoring of tumor response to therapy in mice. *Radiology* 2004; **232**: 685-692
27 **Manenti G**, Squillaci E, Di Roma M, Carlani M, Mancino S, Simonetti G. In vivo measurement of the apparent diffusion coefficient in normal and malignant prostatic tissue using thin-slice echo-planar imaging. *Radiol Med* (Torino) 2006; **111**: 1124-1133
28 **Abdel Razek AA**, Kandeel AY, Soliman N, El-shenshawy HM, Kamel Y, Nada N, Denewar A. Role of diffusion-weighted echo-planar MR imaging in differentiation of residual or recurrent head and neck tumors and posttreatment changes. *AJNR Am J Neuroradiol* 2007; **28**: 1146-1152
29 **Koh DM**, Scurr E, Collins DJ, Pirgon A, Kanber B, Karanjia N, Brown G, Leach MO, Husband JE. Colorectal hepatic metastases: quantitative measurements using single-shot echo-planar diffusion-weighted MR imaging. *Eur Radiol* 2006; **16**: 1898-1905
30 **Taouli B**, Vilgrain V, Dumont E, Daire JL, Fan B, Menu Y. Evaluation of liver diffusion isotropy and characterization of focal hepatic lesions with two single-shot echo-planar MR imaging sequences: prospective study in 66 patients. *Radiology* 2003; **226**: 71-78

S- Editor Zhong XY **L- Editor** Wang XL **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5900

World J Gastroenterol 2008 October 14; 14(38): 5900-5906
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

A systematic review and meta-analysis of the Chinese literature for the treatment of achalasia

Lan Wang, You-Ming Li, Lan Li, Chao-Hui Yu

Lan Wang, You-Ming Li, Lan Li, Chao-Hui Yu, Department of Gastroenterology, the First Affiliated Hospital, Medical College, Zhejiang University, Hangzhou 310003, Zhejiang Province, China

Author contributions: Wang L and Li L were responsible for data collection and preparing the manuscript; Li YM was responsible for study design; all of the authors read and approved the final manuscript.

Correspondence to: You-Ming Li, the 1st Affiliated Hospital, Medical College, Zhejiang University, No. 79 Qingchun Road, Hangzhou 310003, Zhejiang Province, China. zlym@zju.edu.cn
Telephone: +86-571-87236603 Fax: +86-571-87236611

Received: May 5, 2008 Revised: August 26, 2008
Accepted: September 2, 2008
Published online: October 14, 2008

Abstract

AIM: To evaluate the effect of different approaches in the treatment of achalasia in China.

METHODS: We performed a systematic review and meta-analysis of Chinese literature by searching the Chinese Biomedical Database and Chinese scientific Journals database (up to March 2008). All cohort studies (controlled or uncontrolled) in which the patients were observed for more than a year were reviewed in detail. Dichotomous outcomes were reported as relative risks (RR) with 95% confidence interval (CI) for controlled trials. The efficacy in uncontrolled trials was assessed by a pooled estimate of response rate with individual studies weighted in proportion to the sample size.

RESULTS: Seven controlled trials compared the effect of botulinum toxin injection (BoTx) with pneumatic dilation (PD). PD was superior to BoTx [65.2% *vs* 45.3%; RR 1.47 (95% CI 1.23-1.77), $P < 0.0001$], and had a lower clinical relapse rate (BoTx 30.2% *vs* PD 10%, RR 0.32 (0.16-0.65), $P = 0.001$). Heller myotomy (HM) had superior remission rate compared to PD [HM 94.0% *vs* PD 64.1%, RR 1.48 (1.15-1.99), $P = 0.002$]. In uncontrolled trials, the effectiveness of PD was 86.6% (23.9%) *vs* 94.8% (10.6%) for HM. The main complications of PD were perforation and gastroesophageal reflux disease.

CONCLUSION: HM is the most effective long-term treatment for patients with achalasia in China. In the future, controlled clinical trials on the treatment of achalasia should focus on comparing laparoscopic myotomy with or without antireflux procedure, including different partial and total fundoplication techniques.

Key words: Meta-analysis; Achalasia; Esophageal; Treatment; Pneumatic dilation; Botulinum toxin injection; Laparoscopic myotomy

Peer reviewer: Luigi Bonavina, Professor, Department of Surgery, Policlinico San Donato, University of Milano, via Morandi 30, Milano 20097, Italy

Wang L, Li YM, Li L, Yu CH. A systematic review and meta-analysis of the Chinese literature for the treatment of achalasia. *World J Gastroenterol* 2008; 14(38): 5900-5906 Available from: URL: http://www.wjgnet.com/1007-9327/14/5900.asp DOI: http://dx.doi.org/10.3748/wjg.14.5900

INTRODUCTION

Achalasia is an idiopathic primary motility disorder, characterized manometrically by poor relaxation of the lower esophageal sphincter (LES) and complete loss of primary peristalsis, which leads to a compromise in the primary function of the esophagus[1]. The cause of achalasia is not known. Untreated, it leads to an extremely poor quality of life because of progressive dysphagia, esophageal dilation, and stasis[2]. Botulinum toxin (BoTx) injection of the LES, pneumatic dilation (PD) and Heller myotomy (HM) are the most commonly used techniques for the treatment of achalasia[3].

BoTx prompted a great deal of enthusiasm because it is safe, simple to use and can be easily repeated[4]. There are no reports of any serious complications after BoTx injection. However, symptoms often recur after the first injection and serial injections are required, with the efficacy waning over time due to the production of antibodies[5]. Currently, newly diagnosed achalasia patients are offered serial PDs, followed by surgical intervention if the dilatations fail[6]. With the introduction of HM, there has been a change in the initial management approach to the treatment of achalasia. However, there is much controversy as to which treatment provides the best efficacy, sustained symptom relief and low complication rate[7], especially in China[8]. Therefore, the aim of the present study was to

evaluate the effects of different treatment modalities by reviewing the published trials in China.

MATERIALS AND METHODS

Study design

All controlled and uncontrolled studies were included in the analysis if the patients underwent clinical, manometric, radiographic and endoscopic evaluation to confirm the diagnosis of primary achalasia. The presence of symptoms and esophageal function were reassessed at 6 mo and 1 year after therapy. Treatment efficacy was determined by the improvement rate (%) in the symptom score, 1 year after therapy. Treatment failure was defined as the lack of any reduction in the symptom grade of > 1, or a recurrence of symptoms 1 year after therapy.

Search strategy and data extraction

Two investigators independently searched the Chinese literature published in the Chinese Biomedical Database and Chinese scientific Journals database (up to March 2008). All controlled or uncontrolled trials involving patients with achalasia who were given any form of treatment were included in the analysis. The search terms used were "esophageal" or "oesophagus", "achalasia", and "therapy" or "treatment". Data was extracted by the same investigators using standardized forms. The date obtained included the number of patients, the exact methods of therapy, the outcome variables listed above, and any reported adverse effects of therapy. The quality of all selected articles was ranked in accordance with the Jadad composite scale. According to this scale, low quality studies had a score of $\leqslant 2$ and high quality studies had a score of $\geqslant 3$.

Statistical analysis

We performed the meta-analysis using the RevMan 4.2.10 software (provided by the Cochrane Collaboration, Oxford, UK) for the controlled studies. The relative risk (RR) was calculated with 95% confidence intervals (CI). We used χ^2 to assess statistical heterogeneity and the Higgins I^2 statistic to determine the percentage of total variations across studies due to heterogeneity. If the I^2 statistic was $\leqslant$ 50%, the fixed effect model was used to pool studies, otherwise, the random effects model was used. The efficacy in uncontrolled trials was assessed by a pooled estimate of the response rate with individual studies weighted proportionally to the sample size[9]. In calculating the weighted mean response for each treatment modality ($\hat{p}$), the studies were characterized by the number of subjects included (n) and the response rate in those subjects (p). Ellipses represents scant data.

$$\hat{p} = (n_1 p_1 + n_2 p_2 + n_x p_x)/(n_1 + n_2 + n_3)$$

$$SE(\hat{p}) = \text{Sqrt}\{[p_1(1-p_1)/n_1] + [p_2(1-p_2)/n_2] + [p_x(1-p_x)/n_x]\}$$

RESULTS

Study characteristics

The search strategy generated 612 studies. From these, we identified 43 studies (12 controlled studies and 31 uncontrolled studies) that met the inclusion criteria. Only studies that included more than 10 patients, and had a follow-up of at least 12 mo were tabulated (Table 1). Sample sizes in the trials ranged from 10 to 125 participants (total 1791); 46.9% of the participants were male. Ten controlled studies were considered to be of low quality, and two controlled trials were graded as high quality (Table 2).

Effects of the treatment of achalasia

Control studies: There were five controlled studies[10-14], while two studies[15,16] compared the clinical effect of BoTx with that of PD. Our meta-analysis showed that BoTx was significantly more effective than PD in the incidence of symptom remission [PD 65.2% *vs* BoTx 45.3%, *RR* 1.47 (95% CI 1.23-1.77), $P < 0.0001$, Figure 1A], whereas PD had a lower relapse rate than BoTx [PD 10% *vs* BoTx 30.2%, *RR* 0.32 (0.16-0.65), $P = 0.001$, Figure 1B]. The main adverse effect of BoTx injection was chest pain which was controlled by medical therapy. Complications with PD consisted of perforation and gastroesophageal reflux disease (GERD). Some patients were referred for Heller myotomy. We believe that PD is the preferred medical treatment for achalasia, if performed by a skillful expert.

One trial[17] compared PD with BoTX-PD. The one year remission rate in the BoTX-PD group was 61% compared with 28% in the PD group ($P < 0.05$). It was suggested that BoTx injection before PD improved the efficacy of PD. However, some studies have shown that primary BoTx treatment increased the risk for PD[53]. Therefore, more randomized controlled studies are required to evaluate whether combined therapy is superior to single treatment in achalasia.

Two controlled studies[18,19] compared the effect of PD with HM. Our meta-analysis showed that HM had superior remission rate compared to PD [HM 94.0% *vs* PD 64.1%, *RR* 1.48 (1.15-1.99), $P = 0.002$), (Figure 1C]. There was no difference in the complication rate between HM and PD. These findings suggest that HM offers better long-term clinical results than PD. Two trials[20,21] assessed the efficacy of laparoscopic myotomy (LM) with thoracoscopic myotomy (TM). The results showed that good or excellent relief of symptoms was obtained in 93.8% after TM and 98% after LM, with no difference in the remission rate [RR 1.48 (0.91-1.05, $P = 0.58$), Figure 1D]. These findings need further confirmation and validation.

Uncontrolled trials: There were 31 uncontrolled trials on different treatment methods for achalasia. Table 3 summarizes the results obtained with endoscopic and surgical treatment. Some studies were conducted prospectively[22-26], while others[27-31] were retrospective reviews. These studies evaluated the clinical effect

Table 1 Demographic data of studies included in systematic review

	No. of patients	Age (yr) (median) (range)	Sex (M/F)	Remission rate (%) (mo)	Relapse rate (%) (mo)	Follow up time (mo)	Complications (*n*/N, type)
Cai *et al* 2003[10]	62 BoTx	38.7 ± 32.8	72/46	67.35 (12)	25.8 (12)	24	
	56 PD			92	5.40		1/56 EF
Liu *et al* 2003[11]	16 BoTx	36.2 ± 13.4	7/9	18.75 (12)	NA	12	0/16
	16 PD	35.2 ± 10.2	6/10	37.50			
	16 BoTx + PD	38.1 ± 12.6	8/8	87.50			
Gui *et al* 2006[12]	16 BoTx	32.3 ± 10.2	5/11	87.5 (12)	37.5 (12)	12	0/16
	16 PD		7/9	93.80	18.80		
Yang *et al* 2002[13]	24 BoTx	42.2 ± 13.1	10/14	16.67 (12)	NA	12	0/24
	24 PD		12/12	33.33			
Jin *et al* 2004[14]	14 BoTx	35.3 ± 15.3	6/8	21.4 (12)	NA	24	
	16 PD		7/9	62.50	NA		1/16 PF
Qian *et al* 2006[15]	20 BoTx	53.4 ± 10.4	12/8	50 (12)	NA	24	0/20
	20 PD		10/10	85			2/20
Yin *et al* 2003[16]	18 BoTx	36.8 ± 12.1	10/8	50 (12)	38.9 (12)		
	13 PD		7/6	76.90	23.10		
Yang *et al* 2006[17]	25 PD	37.1 ± 13.6	13/12	28 (12)	36 (12)	24	13 MH
	18 BoTx + PD		8/10	61	16.70		3 MH
Jia *et al* 2001[18]	20 PD	43.4 ± 15.1	12/8	52.63 (12)	47.06 (12)	unclear	
	19 HM	41.8 ± 13.9	10/9	94.4 (12)	0		8 GER
Ge *et al* 1997[19]	20 PD	39.5 ± 11.2	16/24	75 (12)	NA	24	1 GER
	20 HM			93 (12)		168	3 GER
Jiang *et al* 2007[20]	27 TM	43.4 ± 15.1	12/15	85.2 (24)	11 (12)	24	3 PF
	8 LM + Dor	50.3 ± 13.5	3/5	87.50	12.50		1 PF
Guo *et al* 2005[21]	29 LM	38.2 ± 10.6	15/14	100 (24)	0	2-60	
	54 TM	39.1 ± 12.1	21/33	98.1 (24)	1.9 (12)	1-80	1 PA
Li *et al* 1994[22]	15 HM	38.6 ± 15.6	28/44	86.4 (12)	NA	36	3/15 GER
	52 HM + Dor			96			5/52 GER
Jiang *et al* 2002[23]	30 PD	36 ± 10.6	19/21	87 (12)	13 (12)		
	10 HM			100	0 (120)		3/10 GER
Jiang *et al* 2007[24]	30 TM	45.2 ± 13.1	12/18	90 (48)	3.3 (2y)	96	3 PF
Gu *et al* 2007[25]	125 PD	48.3 ± 19.3	57/68	92.14 (12)	7.86 (60)	60	1 PF; 1 GER
Zhang *et al* 2005[26]	90 Open - TM	37.1 ± 20.1	40/50	100 (12)	0 (12)	3	1 PF
Xu *et al* 2007[27]	29 BoTx - PD	40.6 ± 13.3	12/17	93.1 (12)	6.9 (12)	12	NA
Xu *et al* 2003[28]	28 PD	32.6 ± 14.2	4/24	85.7 (12)	14.3 (12)	24	None
Li *et al* 2004[29]	75 PD	37 ± 11.1	39/36	91 (12)	10.7 (12)	12	2 PF
Jia *et al* 2003[30]	38 PD	31.3 ± 12.0	12/26	89.5 (12)	10.5 (12)	36	1 MH
Chen *et al* 2005[31]	32 PD	40.2 ± 10.6	8/24	81.25 (24)	18.6 (24)	24	3 MH
Zhu *et al* 2005[32]	23 PD	33.4 ± 12.3	9/14	75 (24)	25 (24)	24	17 MH
Ma *et al* 2002[33]	26 PD	34.2 ± 11.3	14/12	92.3 (24)	3.8 (24)	4-36	7 CP
Wu *et al* 2007[34]	29 PD	38.2 ± 14.1	21/8	82.7 (12)	10.3 (12)	12-102	3 GER
Lin *et al* 2004[35]	37 PD	33.1 ± 12.3	21/16	91.9 (24)	8 (12)	12	8 CP
Jia *et al* 2003[36]	19 PD	26.2 ± 8.90	8/11	82 (24)	18.8 (24)	24	8 GER
Wei *et al* 2005[37]	18 HM	32.1 ± 10.1	12/6	94.4 (12)	5.6 (12)	24	2 GER
Zhong *et al* 2003[38]	58 PD	42.5 ± 2.5	28/27	93.1 (12)	6.9 (12)	16-24	4 GER
Du *et al* 2003[39]	48 HM	35.0 ± 11.2	22/26	100 (24)	0 (24)	75	3 GER
Li *et al* 2005[40]	19 HM	32.8 ± 11.6	20/12	94.4 (12)	5.3 (12)	12-120	4 GER
	13 HM + Dor			100 (12)	0		1 GER
Song *et al* 2001[41]	21 HM	34.5 ± 9.30	12/9	90.5 (12)	0	12-144	2 GER
Li *et al* 2005[42]	15 HM	29 ± 7.80	5/10	100 (12)	0	36-156	0
Xiao *et al* 2004[43]	27 LM	48.1 ± 12.6	15/12	96.3 (24)	3.7 (24)	14	0
Sun *et al* 2003[44]	39 HM	31.3 ± 7.60	15/24	83.8 (24)	1.7 (24)	12-120	9 LM
Wang *et al* 2005[45]	21 HM+Dor	45.5 ± 13.5	6/15	100 (24)	0	24-60	0
Lu *et al* 2002[46]	56 HM	37 ± 11.2	24/32	96.4 (36)	3.6 (36)	3-178	16 GER
Zhu *et al* 2007[47]	56 PD	37.5 ± 10.7	17/39	84.4 (12)	15 (12)	1-48	0
Chen *et al* 2003[48]	32 PD	35 ± 13.2	14/18	81.3 (12)	3.8 (12)	1-6	6 CP
Wang *et al* 2007[49]	25 PD	35.8 ± 9.30	10/15	89 (12)	8 (12)	12-36	5 GER
Han *et al* 2004[50]	34 PD	32.3 ± 11.8	20/14	92 (24)	5.9 (12)	24-35	1 PF
Yang *et al* 2007[51]	16 TM	42.5 ± 10.2	6/10	93.3 (12)	6.7 (12)	14	1 ML
Huang *et al* 2005[52]	18 TM	39 ± 8.90	7/11	94 (12)	6 (12)	1-80	1 PA; 3 ML

BoTx: Botulinum toxin; PD: Pneumatic dilation; BoTx-PD: Botulinum toxin plus Pneumatic dilation; HM: Heller myotomy; HM + Dor: Heller myotomy and Dor fundoplication; EF: Esophageal fistula; PF: Perforation; MH: Mucosae hemorrhage; PA: Pulmonary atelectasis; CP: Chest pain; MIS: Metal internal stent; ML: Mucosae laceration; CP: Chest pain; PA: Pulmonary atelectasis.

of PD[32-36] and the long term efficacy of HM by the abdominal approach[37,39-42]. One study[43] assessed the efficacy of LM. Some studies[44-46] evaluated the remission rate of HM with fundoplication, while others[38,47-50]

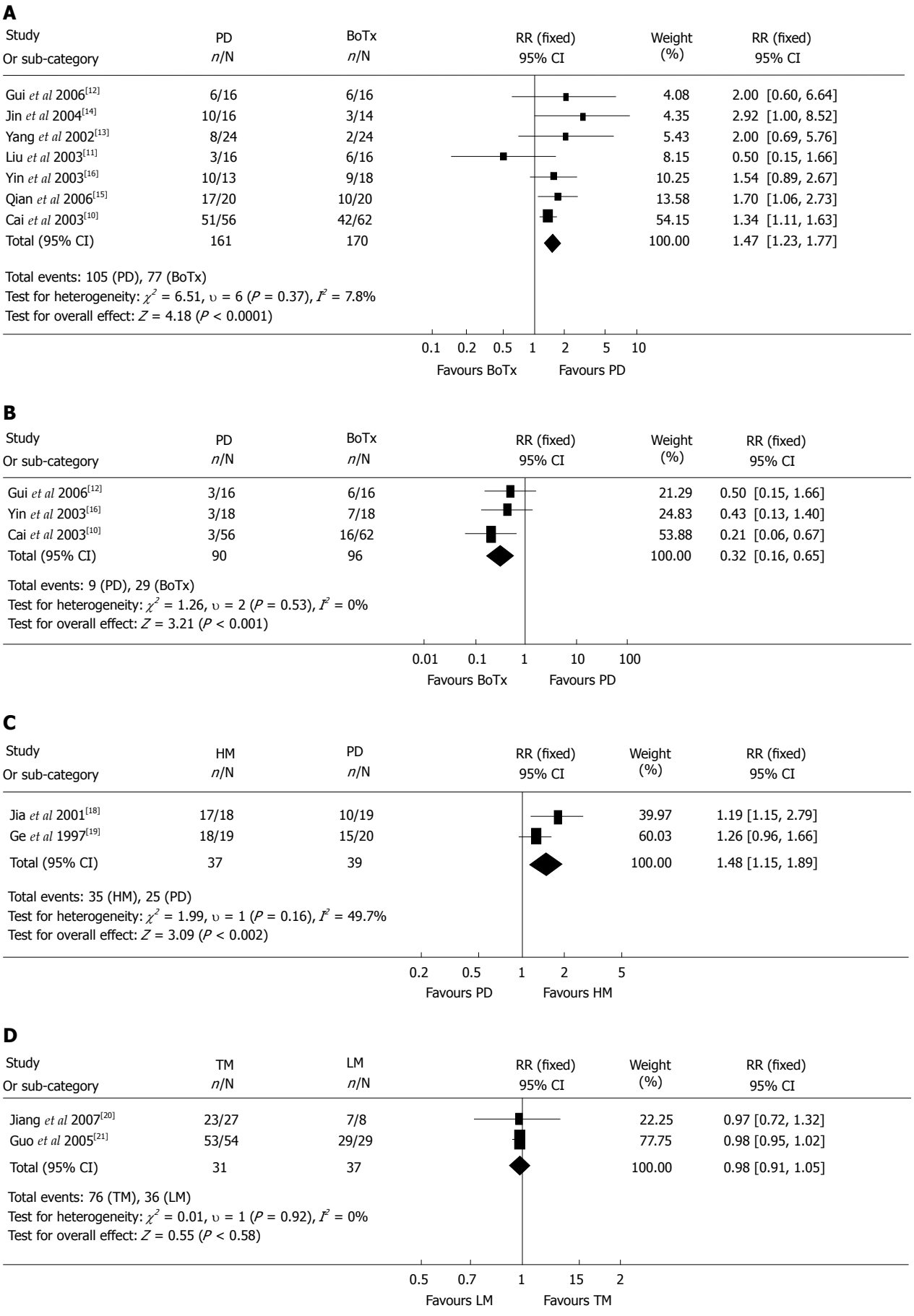


Figure 1 Meta-analysis. **A**: Treatment effect of pneumatic dilation *vs* botulinum toxin injection; **B**: Relapse rate of pneumatic dilation *vs* botulinum toxin injection; **C**: Treatment effect of pneumatic dilation *vs* Heller myotomy; **D**: Treatment effect of laparoscopic myotomy *vs* thoracoscopic myotomy.

Table 2 Methodological quality of randomized controlled trials included in systematic review

	Yr	Randomisation method	Blind	Explanation for withdrawals	Allocation concealment	Jadad score
Cai *et al*[10]	2003	Random	Unclear	Yes	Unclear	3
Liu *et al*[11]	2003	Random	Unclear	Yes	Unclear	3
Gui *et al*[12]	2006	Unclear	Unclear	Yes	Unclear	2
Yang *et al*[13]	2002	Unclear	Unclear	Yes	Unclear	2
Jin *et al*[14]	2004	Unclear	Unclear	Yes	Unclear	2
Qian *et al*[15]	2006	Unclear	Unclear	Yes	Unclear	2
Yin *et al*[16]	2003	Unclear	Unclear	Yes	Unclear	2
Yang *et al*[17]	2006	Unclear	Unclear	Yes	Unclear	2
Jia *et al*[18]	2001	Random	Unclear	Yes	Unclear	3
Ge *et al*[19]	1997	Unclear	Unclear	Yes	Unclear	2
Jiang *et al*[20]	2007	Unclear	Unclear	Yes	Unclear	2
Guo *et al*[21]	2005	Unclear	Unclear	Yes	Unclear	2

Table 3 Pooled estimate of response rate of non-controlled trials for achalasia treatments across referenced trials

Therapy and references	Total No. of patients	Weighted response of treatment methods for achalasia (1 yr after treatment)		
		Remission rate (þ) mean ± SE, %	Relapse rate (þ) mean ± SE, %	Weighted follow-up mean (range), mo
PD	667	86.6 ± 23.9	10.7 ± 21.0	26 (4-102)
HM	354	94.8 ± 10.6	1.5 ± 6.3	46 (1-178)
TM	64	92.0 ± 10.0	4.91 ± 9.0	30 (1-80)

PD: Pneumatic dilation; BoTx-PD: Botulinum toxin plus Pneumatic dilation; HM: Heller myotomy. In calculating the weighted mean response for each treatment modality (þ), included studies were characterized by the number of subjects included (*n*) and the response rate for those subjects (p). Ellipses represent scant data.

reported the efficacy of large diameter balloon dilation. Three trials[24,51-52] evaluated the efficacy of TM. The results were combined (shown in Table 3) because the findings were identical. In calculating the response rate of each study, we extracted the number of individuals with a good-to-excellent response that was sustained until the end of the observation period, without further therapy. Thus, if a patient required a second dilation or a second injection, or if a laparoscopic operation was converted to an open procedure, these were considered as failures of initial treatment. The results show that PD and HM are the most popular treatment methods for achalasia in China; the effectiveness of PD [weighted mean (SD)] was 86.6% (23.9%) *vs* 94.8% (10.6%) for HM. Only 3 of the 31 uncontrolled trials evaluated the clinical effect of TM [weighted mean (SD)] [92.0% (10.0%)], while one study evaluated the effect of LM (96.3%). However, in most western countries, the majority of patients undergo LM, with excellent results[54]. Some workers regard LM as the gold standard treatment for achalasia[55]. More randomized controlled trials should be carried out in China to evaluate the effect of LM. There was a significant difference in the effect of PD between controlled trials and uncontrolled trials (controlled 65.2% *vs* uncontrolled 86.6%). Therefore, a uniform standard for evaluating the effect of achalasia treatment should be developed.

DISCUSSION

The present review indicates that HM is the best choice for the treatment of achalasia in China. HM has gained widespread popularity for its excellent results and the advantage of a 1-shot therapy. There was no significant difference in the complication rate between LM and PD. PD was also a popular therapy in Chinese patients. Although side effects, such as perforation and bleeding occur with PD, the remission rate was higher with PD compared to BoTx. Moreover, the recurrence rate was higher with BoTx compared to PD.

However, Bassotti *et al*[56] using a different BoTx regimen, consisting of two injections within a 4-wk period, reported a success rate 80% at 12 mo. These findings may encourage new interest in this therapy. Moreover, BoTx treatment is less expensive, is virtually risk-free, is easy to administer by any endoscopist, and the results are reproducible. Therefore, there is an urgent need for high quality studies to evaluate the efficacy of repeated injections of BoTx, and BoTx combined with other treatment methods.

HM is an invasive and risky treatment modality for achalasia, and requires surgical skills that are not always available (the results obtained with HM depend largely on the surgeon's expertise). To reduce the complications and risks of HM, several modifications have been developed[57], including HM with partial fundoplication, and minimally invasive myotomy, using different endoscopic techniques. In the past, both laparoscopy and thoracoscopy were used to perform HM. However, it soon became clear that laparoscopy offers several inherent advantages, including superior visualization of the gastroesophageal junction, a single

lumen endotracheal intubation, the ability to add an anti-reflux procedure, and a shorter hospital stay. In addition, laparoscopy provided better symptomatic outcome and lower incidence of postoperative gastroesophageal reflux (GER)[58]. Our meta-analysis showed that there was no significant difference in the remission rate between LM and TM within 12 mo of the initial intervention. However, LM has a relatively higher response rate in uncontrolled studies (LM 96.3% *vs* TM 91.95%). Patients experienced greater relief of dysphagia after LM compared to TM. Moreover, GER is the most common complication after HM, and fundoplication cannot be performed at the same time with TM. Therefore, more studies are required to determine whether LM is superior to TM in the treatment of achalasia.

The number of trials included in the present meta-analysis, and the number of patients randomized to receive the different treatment modalities was relatively small. This was compounded by stratification of the subjects into smaller subgroups. Moreover, poor randomization techniques and inadequate follow-up further limited the interpretation of the findings. Few studies provided proper definitions of the postoperative events. Future trials should include standard measures to allow objective and comparable assessment of the outcomes. Standardization was lacking in the reporting of treatment outcomes, length of trial, and the proportion of recruited sample that was kept under follow-up. Several trials failed to accurately present such information. Disease recurrence may also be underestimated, since some studies used telephone contact or questionnaire-based follow-up. Therefore it is possible that a small number of patients with macroscopic but asymptomatic recurrence may not be detected using such assessment techniques.

Our results indicate that in China, HM is the most effective treatment for achalasia and can safely and durably relieve the symptoms of dysphagia. BoTx injection is recommended if multiple injections are given or is combined with other treatment modalities. PD provides better dysphagia control, but is associated with the risk of perforation. It is important that more randomized controlled clinical trials are carried out in China to compare LM, with and without an antireflux procedure, and with partial or total fundoplication.

ACKNOWLEDGMENTS

We are grateful to Dr. Yinglong Tao for his excellent assistance in the statistical analysis.

COMMENTS

Background

Botulinum toxin injection (BoTx), pneumatic dilatation and Heller myotomy (HM) are the most commonly used techniques for the treatment of achalasia in China. However, it unclear as to which is the best treatment modality for achalasia.

Research frontiers

The aim of the present study was to evaluate the outcome of different approaches for the treatment of achalasia in Chinese patients.

Innovations and breakthroughs

Previous studies in China suggest that pneumatic dilatation is as effective as HM. It remains unclear whether pneumatic dilatation can achieve sustained symptom remission. In the present study, HM was found to provide better results compared with other treatment modalities in China.

Applications

Our results helped to identify the best treatment method for Chinese patients. They also offer directions for randomized controlled trials in the future.

Terminology

Achalasia is a primary motor disorder, characterized by incomplete relaxation of the lower esophageal sphincter and aperistalsis of the esophageal body, secondary to the loss of the inhibitory ganglion cells in the myenteric plexus. The etiology of achalasia is unknown; genetic, autoimmune, infectious, and environmental factors have been implicated.

Peer review

This is a systematic review and meta-analysis of the current treatments options for achalasia, namely BoTx, pneumatic dilation, and Heller myotomy. The authors concluded that Heller myotomy is superior to endoscopic approaches. This is a very interesting study.

REFERENCES

1 **Massey BT**. Management of idiopathic achalasia: short-term and long-term outcomes. *Curr Gastroenterol Rep* 2000; **2**: 196-200
2 **Pohl D**, Tutuian R. Achalasia: an overview of diagnosis and treatment. *J Gastrointestin Liver Dis* 2007; **16**: 297-303
3 **Da Silveira EB**, Rogers AI. Achalasia: a review of therapeutic options and outcomes. *Compr Ther* 2002; **28**: 15-22
4 **Zaninotto G**, Vergadoro V, Annese V, Costantini M, Costantino M, Molena D, Rizzetto C, Epifani M, Ruol A, Nicoletti L, Ancona E. Botulinum toxin injection versus laparoscopic myotomy for the treatment of esophageal achalasia: economic analysis of a randomized trial. *Surg Endosc* 2004; **18**: 691-695
5 **Vaezi MF**, Richter JE, Wilcox CM, Schroeder PL, Birgisson S, Slaughter RL, Koehler RE, Baker ME. Botulinum toxin versus pneumatic dilatation in the treatment of achalasia: a randomised trial. *Gut* 1999; **44**: 231-239
6 **Kostic S**, Kjellin A, Ruth M, Lonroth H, Johnsson E, Andersson M, Lundell L. Pneumatic dilatation or laparoscopic cardiomyotomy in the management of newly diagnosed idiopathic achalasia. Results of a randomized controlled trial. *World J Surg* 2007; **31**: 470-478
7 **Lake JM**, Wong RK. Review article: the management of achalasia - a comparison of different treatment modalities. *Aliment Pharmacol Ther* 2006; **24**: 909-918
8 **Zhang L**, Wang MW, Wu BY. Botulinum toxin versus pneumatic dilatation in the treatment of achalasia and a review analysis. *Zhongguo Shiyong NeikKe Zazhi* 2005; **25**: 938-939
9 **Spiess AE**, Kahrilas PJ. Treating achalasia: from whalebone to laparoscope. *JAMA* 1998; **280**: 638-642
10 **Cai FC**, Liu QS, Guo RB, Men JY, Cheng LF. The kinetic changes in esophageal achalasia treated by pneumatic dilation or intrasphincteric injection of botulinum toxin. *Shiyong Yixue Zazhi* 2003; **19**: 341-343
11 **Liu JY**, Yang CM, Zhang AZ, Cui Y, Wang HB. Clinical study on intrasphincteric injection of botulinum toxin and/or small balloon dilation in patients with esophageal achalasia. *Zhonghua Xiaohua Neijing Zazhi* 2003; **20**:158-160
12 **Gui RH**, Zheng XP, Leng JH, Wang XP, Duan XM. Clinical study on intrasphinctive injection of botul inum toxin or small balloon dilation in patients with esophageal achalasia. *Zhongguo Xiandai Yu Linchuang* 2006; **3**: 31-32
13 **Yang CM**, Liu JY, Liu QY, Zhang AZ, Xiu HW, Wang HB. Clinical study on intrasphincteric injection of botulinum toxin in the treatment of achalasia. *Shandong Yixue Zazhi* 2002; **42**: 1-2
14 **Jin Y**. The efficacy of patients with achalasia by intrasphincteric injection of botul inum toxin versus balloon

dilation. *Xiandai Yiyao Weisheng* 2004; **20**: 1728-1729
15 **Qian F**. Clinical study on intrasphincteric injection of botulinum toxin versus balloon dilation in the treatment of achalasia. *Shiyong Zhenduan Yu Zhiliao* 2006; **20**: 908-909
16 **Yin YM**, Lin L, Shi RH, Zhao ZQ. A comparative study on treating achalasia by balloon dilatation and botulinum toxin lical injection. *Nanjing Yike Daxue Xuebao* 2003; **23**: 51-53
17 **Yang ZW**, Shao XY, Bu LX, Ma LX, Zhang LP. Clinical study on intrasphinctive injection of botulinum toxin A and balloon dilation in patients with esophageal achalasia. *Zhonghua Neijing Zazhi* 2006; **12**: 706-709
18 **Jia DQ**, Ouyang Q, Wang ZQ, Luo CF. The value of pneumatic dilation in treament of esophageal achalasia. *Zhonghua Xiaohua Neijing Zazhi* 2001; **18**: 358-359
19 **Ge F**, Zhang LJ, Li ZJ, Ke MY, Wang ZF. To assess the effect of pneumatic dilation and surgery treament of esophageal achalasia. *Zhonghua Xiongxin Xueguan Waike Zazhi* 2001; **18**: 301-302
20 **Jiang W**, Tan LJ, Wang Q, Xu ZL, Zheng RH, Zhang Y. Comparison of thoracoscopic and laparoscopic Heller myotomy for achalasia. *Zhongguo Linchuang Yixue* 2007; **14**: 55-57
21 **Guo L**, Gu JW, Zhu JY, Wang CS, Tao WD, Gu ML, Huang YG. Laparoscopic management of esophageal achalasia. *Fuqiangjing Waike Zazhi* 2005; **10**: 326-327
22 **Li ZM**, Dong WH, Jiang XM. The surgery treatment for achalasia. *Zhongguo Xinxueguan Waike Linchuang Zazhi* 1994; **1**: 97-98
23 **Jiang M**, Zhang L, Chu YL, Bai XP, Zhu JX. A comparative study on treating achalasia by Pharmacological therapy, balloon dilatation and botulinum toxin lical injection. *Xiandai Xiaohua Ji Jieru Zhenliao* 2002; **7**: 47-49
24 **Jiang W**, Wang Q, Tan LJ, Xu ZL, Zheng RH. The clinical experience of thoracoscopic Heller myotomy for achalasia. *Fudan Xuebao* (Yixue Ban) 2007; **34**: 296-299
25 **Gu XH**, Zhang ZB, Wang B, Sheng JW. Evaluation of Pneumatic dilation for treatment of primary achalasia under gastroendoscopy. *Linchuang Xiaohuabing Zazhi* 2007; **19**: 233-235
26 **Zhang WX**, Wang BX, Chen SX, Luo WJ, Jiang HH, Long L, Zhang CF. Treatment of achalasia of cardia by transthoracic Heller myotomy with tricavity biocapsule tube. *Zhongguo Xiandai Yixue Zazhi* 2005; **15**: 2517-2518
27 **Xu EB**, Bai HX, Cai YG, Huang ZW, Xiong YH, Zhang ZB. Study on Intrasphincteric Injection of Botulinum Toxin Combined with Small Balloon Dilation. *Shiyong Linchuang Yixue* 2007; **8**: 14-16
28 **Xu MF**, Yu WH, Liu ZW. Evaluation of Pneumtic dilation for treatment of achalasia with 28 patients. *Yixue Lilun Yu Shijian* 2003; 16: 1042-1043
29 **Li YF**, Cui Y, Li CJ, Nie XY. The effect of Pneumtic dilation for treatment of achalasia in the long term. *Xin Yixue* 2004; **35**: 677-679
30 **Jia JZ**, Ma YP, Geng JM, Kou HW. Endoscopic direct optional balloon dilatation Therapy for achalasia. *Zhongguo Neijing Zazhi* 2003; **9**: 17-19
31 **Chen Z**, Zou YB. Endoscopic Balloon Dilatation for Achalasia. *Keji Tongbao* 2005; **21**: 577-579
32 **Zhu X**, Chi BR, Meng XW, Chen LG. The evaluation of endoscopic direct balloon dilation therapy for achalasia. *Jiling Yixue* 2005; **26**: 169-170
33 **Ma YC**, Xiong YZ, Zhou CP, Du J, Yang YG. Endoscopic pneumatic dilation for Achalasia. *Zhonghua Xiaohua Neijing Zazhi* 2002; **19**: 174-175
34 **Wu J**, Jia YG, Li HY, Chen S, Gan HY. Inside endoscopy next air sac expansion treatment achalasia long-term curative effect in observe. *Zhongguo Neijing Zazhi* 2007; **13**: 117-119
35 **Lin ZG**, Liang YG, Huang LZ, Huang Y. Pneumatic dilation treatment for Achalasia with 37 patients. *Xiandai Xiaohua Ji Jieru Zhenliao* 2004; **9**: 101
36 **Jia JZ**, Ma YP, Geng JM, Kou HW, Lei XH. Endoscopic direct optical balloon dilatation therapy for achalasia. *Ningxia Yixue Zazhi* 2003; **25**: 264-265
37 **Wei K.** Heller myotomy for the treatment of achalasia with 18 patients. *Youjiang Mingzu Yixueyuan Xuebao* 2005; **27**: 836
38 **Zhong M**. Pneumatic dilation for the treatment of achalsia by the Endoscopic direct. *Zhongguo Neijing Zazhi* 2003; **9**: 41-42
39 **Du C**, Wang RW, Jiang YG, Zhao YP, Tian QY. Treatment of achalasia of cardia by transabdominal cardiomyotomy plus posteroesophageal partial gastric fundoplication. *Disan Junyi Daxue Xuebao* 2003; **25**: 528-530
40 **Li JY**, Jiang J, Wei B, Yu T. Long-term results of modified Heller myotomy for achalasia by abdominal approach. *Zhonghua Xiongxin Xueguan Waike Zazhi* 2005; **21**: 149-121
41 **Song QQ**, Zhang YR, Zhou Z, Wang YJ, Wei DQ. Modified Heller myotomy for the treatment of achalasia. *Zhongguo Putong Waike Zazhi* 2002; **11**: 187-188
42 **Li B**, Zhu LY, Dong ZY, Wang WZ. Heller myotomy for the treatment of achalasia with 15 patients and review. *Zhongguo Yiyao Daobao* 2007; **4**: 118-119
43 **Xiao HB**, Melvin WS. Robot-assisted laparoscopic Heller cardiomyotomy. *Zhonghua Weichang Waike Zazhi* 2004; **7**: 33-34
44 **Sun JT**, Dai WC, Du MX. Heller and fundoplication for achalasia with 39 patients. *Heilongjiang Yixue* 2003; **27**: 823
45 **Wang XZ**, Gao WJ, Fu JS, Wang CJ, Hou XS, Meng QY. Heller and fundoplication for the treatment of achalasia. *Zhongguo Xiongxin Xueguan Waike Zazhi* 2005; **12**: 295-296
46 **Lu SR**. The treatment of achalasia with 56 patients. *Jiangsu Daxue Xuebao* (Yi Xue Ban) 2002; **12**: 172
47 **Zhu X**, Zhong MR, Li BM, Liu ZJ, Xu P, Yu GH. The clinical research of endoscopic balloon dilation for achalasia. *Zhongguo Neijing Zazhi* 2007; **13**: 193-196
48 **Chen X**, Qiao XL. The evaluation of endoscopic balloon dilation for achalasia. *Shanxi Yiyao Zazhi* 2003; **32**: 46-48
49 **Wang QZ**, Yan SJ, Wang JC, Li DP, Wang QW. The treatment of endoscopic balloon dilation for achalasia. *Shiyong Quanke Yixue* 2007; **15**: 668-669
50 **Han XW**, Wu G, Xing GS, Ma B, Li TX, Liu BS, Shi J. The big balloon dilatation for the treatment of cardiac achalasia. *Yixue Yingxiangxue Zazhi* 2004; **14**: 226-228
51 **Yang HD**, Song SM, Song XY. TV-assisted thoracoscopy Heller myotomy for the treatment of achalasia. *Zhongguo Weichuang Waike Zazhi* 2007; **7**: 543-544
52 **Huang YQ**, Li XG, Liu J, Zhao H, Li Y, Wang J. TV-assisted thoracoscopy myotomy for the treatment of achalasia with 18 patients. *Zhonghua Waike Zazhi* 2005; **43**: 636-637
53 **Mikaeli J**, Bishehsari F, Montazeri G, Mahdavinia M, Yaghoobi M, Darvish-Moghadam S, Farrokhi F, Shirani S, Estakhri A, Malekzadeh R. Injection of botulinum toxin before pneumatic dilatation in achalasia treatment: a randomized-controlled trial. *Aliment Pharmacol Ther* 2006; **24**: 983-989
54 **Omura N**, Kashiwagi H, Ishibashi Y, Yano F, Tsuboi K, Kawasaki N, Suzuki Y, Yanaga K. Laparoscopic Heller myotomy and Dor fundoplication for the treatment of achalasia. Assessment in relation to morphologic type. *Surg Endosc* 2006; **20**: 210-213
55 **Sharp KW**, Khaitan L, Scholz S, Holzman MD, Richards WO. 100 consecutive minimally invasive Heller myotomies: lessons learned. *Ann Surg* 2002; **235**: 631-638; discussion 638-639
56 **Bassotti G**, D'Onofrio V, Battaglia E, Fiorella S, Dughera L, Iaquinto G, Mazzocchi A, Morelli A, Annese V. Treatment with botulinum toxin of octo-nonagerians with oesophageal achalasia: a two-year follow-up study. *Aliment Pharmacol Ther* 2006; **23**: 1615-1619
57 **Bonavina L**. Minimally invasive surgery for esophageal achalasia. *World J Gastroenterol* 2006; **12**: 5921-5925
58 **Hunter JG**, Trus TL, Branum GD, Waring JP. Laparoscopic Heller myotomy and fundoplication for achalasia. *Ann Surg* 1997; **225**: 655-664; discussion 664-665

S- Editor Li DL **L- Editor** Anand BS **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5907

World J Gastroenterol 2008 October 14; 14(38): 5907-5912
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Choledochoscope manometry about different drugs on the Sphincter of Oddi

Jing Kong, Shuo-Dong Wu, Xiao-Bo Zhang, Zhen-Sheng Li, Gang Shi, Wei Wang, Jun-Zhi Chen

Jing Kong, Shuo-Dong Wu, Xiao-Bo Zhang, Zhen-Sheng Li, Gang Shi, Jun-Zhi Chen, Department of General Surgery, Shengjing Hospital of China Medical University, Shenyang 110004, Liaoning Province, China
Wei Wang, Department of Pediatric Surgery, Shengjing Hospital of China Medical University, Shenyang 110004, Liaoning Province, China
Author contributions: Kong J and Wu SD contributed equally to this work; Kong J and Wu SD designed research; Kong J, Zhang XB, Li ZS, Shi G, Chen JZ, and Wang W performed research; Wang W contributed analytic tools; Zhang XB, Li ZS and Shi G analyzed data; and Kong J, Wu SD and Chen JZ wrote the paper.
Correspondence to: Shuo-Dong Wu, Department of General Surgery, Shengjing Hospital of China Medical University, Shenyang 110004, Liaoning Province, China. wushuodong@yahoo.cn
Telephone: +86-24-83956291 Fax: +86-24-86368876
Received: June 17, 2008 Revised: September 17, 2008
Accepted: September 24, 2008
Published online: October 14, 2008

Abstract

AIM: To assess the effects of H_2-receptor blocking pharmacon, protease inhibitor, and gastro kinetic agents on the human Sphincter of Oddi (SO) motility by choledochoscope manometry.

METHODS: One hundred and seventy-five patients with T tube installed after cholecystectomy and choledochotomy were assessed by choledochoscope manometry. They were randomly assigned into groups of H_2-receptor blocking pharmacon, protease inhibitor, and gastro kinetic agents. The Sphincter of Oddi basal pressure (SOBP), amplitude (SOCA), frequency of contractions (SOF), duodenal pressure (DP), and common bile duct pressure (CBDP) were scored and analyzed.

RESULTS: SOBP and SOCA were significantly decreased after Cimetidine administration, and no statistical difference was seen in the Famotidine group. In the Gabexate mesilate group, SOBP had decreased significantly. In the Ulinastatin group, SOCA decreased when Ulinastatin was given at the rate of 2500 U/min; when Ulinastatin administration was raised to 5000 U/min, SOBP, SOF and SOCA all experienced a fall. SOBP and SOCA for Domperidone and SOCA for Mosapride groups all decreased distinctly after administration.

CONCLUSION: The regular dosage of Cimetidine showed an inhibitory effect on the motility of SO, while Famotidine had no obvious effects otherwise. Gabnexata mesilate, Ulinastatin and gastro kinetic agents also showed inhibitory effects on the SO motility.

Key words: Sphincter of Oddi; Medicamentum; Choledochoscope manometry

Peer reviewers: Dr. Yuji Sakai, Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, Inohana 1-8-1, Chuou-ku, Chiba City 260-8670, Japan; Dr. Yang Tian, Department of Biliary Surgery, Easten Hepatobiliary Surgery Hospital, 225 Changhai Road, Yangpu District, Shanghai 200438, China

Kong J, Wu SD, Zhang XB, Li ZS, Shi G, Wang W, Chen JZ. Choledochoscope manometry about different drugs on the Sphincter of Oddi. *World J Gastroenterol* 2008; 14(38): 5907-5912 Available from: URL: http://www.wjgnet.com/1007-9327/14/5907.asp DOI: http://dx.doi.org/10.3748/wjg.14.5907

INTRODUCTION

The Sphincter of Oddi (SO) plays a vital role in maintaining the normal bile duct pressure, promoting gallbladder excretion and preventing from reflux. When Sphincter of Oddi dysfunction (SOD) occurs, the incidence rate for bile duct infection and cholelithiasis will greatly increase. The Sphincter of Oddi Manometry (SOM) is the gold standard for examining the SOD- in which the SO pressure is well accommodated by the abundance of nerves and hormone receptors on the SO. This study assessed the effects of H_2-receptor blocking pharmacon, protease inhibitor and gastro kinetic agents on the human SO pressure measured by choledochoscope manometry, and reveals the effects of different drugs on the SO pressure, which in turn provides the theoretical basis for clinical medication.

MATERIALS AND METHODS

Patients

Between the years 2002 and 2006, 175 individuals (59 men and 116 women, with a mean age of 56

years old, ranging from 21 to 80 years old) were subject to choledochofiberscope examination from the Department of the First Minimally-Invasive and Biliary Surgery of Shengjing hospital affiliated to the China Medical University were chosen. According to the random number table, adult subjects were further randomized into groups of H_2-receptor blocking pharmacon, protease inhibitor, and gastro kinetic agents. Every patient in each group has had cholecystectomy, biliary exploration, and T tube drainage troubled by biliary calculi. Choledochoscope examination was conducted two months after the procedures prior to manometry measurement. All the patients can close their T tube at 15 days post-operation continuously. The common bile duct diameter varied from 0.8 cm to 1.2 cm. Before the manometry was performed, all the patients were informed in detail of the procedure aims and probable dangers, with informed consent form signed.

Manometry system

The apparatus used were PC polygram HR (CTD-Synectics Medical Company, Sweden), triple lumen polyethylene manometry catheter (Wilson-Cook Medical Company, America), low compliance water perfusion system, nitrogen pump and PENTEX LX-750 Fiber Choledochoscope (PENTEX Company, Japan). A triple lumen polyethylene manometry catheter was 200 cm in length with an outer diameter of 1.7 mm. The three sided holes in the distal end were 2 mm apart facing different directions.

Method

Every patient was fasted overnight and kept drug-free to avoid biliary passage pressure changes before the examination. The computer manometry parameter was set at nitrogen pressure 40 kPa and the water velocity at 0.5 mL/min. After the manometry tubes were connected and the T tube removed, choledochoscope was inserted through the T tube sinus tract to observe the papilla vermicular movements at the end of the common bile duct to see whether if there was stenosis, fibrosis or calculus present. All the tested patients had never had EST, balloon dilation of the papilla or papilla plastic repair. During the operation we often used mollis urinary canal of F8 as an alternative to the metallic probe to pass the papilla avoiding the damage of papilla. The papilla moved properly, no parenchyma diseases were found and the regular lockage movements were found. For those patients who had stones in the common bile duct, the stones were removed first. Thirty minutes after the removal, the manometry catheter was introduced *via* the side-pore of choledochoscope, through the papilla into the duodenum under the euthyphoria, and 30 s later the manometry was performed. The catheter is then dragged to the SO; its position in the SO could be confirmed by direct observation through choledochoscope and by the phase waves displayed on the monitor. The SO contractions were recorded in one continuous act by the manometry. Following the SO, the catheter then moved into the common bile duct where the pressure was measured. Lastly, the drugs were then administered. The entire procedure described runs in repetition for every 10 min. The manometry was repeated and the curves were recorded. The administration program is shown in Table 1. The Medication dosage abides by clinic. Ulinastatin was given in different dosage due to clinical application.

Parameter observation

The Sphincter of Oddi basal pressure (SOBP), amplitude (SOCA), frequency of contractions (SOF), duodenal pressure (DP), and common bile duct pressure (CBDP) were scored and analyzed. The pressure of duodenum was used as null point.

Statistical analysis

SPSS 11.5 statistical package was adopted for analysis. The statistical data of our research were all measurement data in a normal distribution. DP was used as null point, and the relative magnitude of the remaining indexes were computed. The results were expressed by mean ± SD. The means of the sample before and after the administration were compared, and we also conducted the matched-pairs interclass *t*-test.

RESULTS

Effects of H_2-receptor blocking pharmacon on SO pressure (Table 2)

SOBP and SOCA were decreased after Famotidine administration, but there was no statistical difference. SOBP and SOCA decreased noticeably after Cimetidine administration, which indicated that Cimetidine had negative inotropic effects on the SO.

Effects of protease inhibitor on SO pressure (Table 3)

In the Gabexate mesilate group, SOBP decreased apparently with the rate of 2500 U/min, which had a statistical significance. SOCA and SOF experienced a tendency to drop but it was insignificant statistically. In the Ulinastatin group, SOCA decreased evidently with Ulinastatin when given at 2500 U/min. SOBP, SOF and SOCA all decreased obviously with Ulinastatin at 5000 U/min, and 20 min later such decrease became more apparent.

Effects of gastro kinetic agents on SO pressure (Table 4)

SOBP and SOCA decreased markedly 20 min after oral administration of Domperidone, which had a statistical significance. SOCA decreased markedly 10 min after administration of Mosapride. There was no obvious change in the indexes after oral administration of Tegaserod.

DISCUSSION

The focus of biliary tract kinetic research is the SO, and

Table 1 Single administration program

Group	Cases	Medication name	Medication dosage	Medication channel
H_2-receptor blocking pharmacon	20	Famotidine	40 mg	intravenous injection
	20	Cimetidine	200 mg	intravenous injection
Protease inhibitor	25	Gabexate mesilate	2.5 mg/min	intravenous injection with micro pump continuing
	25	Ulinastatin	2500 U/min	intravenous injection with micro pump continuing
	25	Ulinastatin	5000 U/min	intravenous injection with micro pump continuing
Gastro kinetic agents	20	Domperidone	10 mg	Take orally
	20	Mosapride	5 mg	Take orally
	20	Tegaserod	12 mg	Take orally

Table 2 Effects of H_2-receptor blocking pharmacon on SO pressure (mean ± SD)

	Famotidine (n = 20)			Cimetidine (n = 20)		
	Before administration	10 min after administration	20 min after administration	Before administration	10 min after administration	20 min after administration
SOBP (mmHg)	9.63 ± 8.48	5.92 ± 4.41	4.97 ± 6.53	9.63 ± 8.16[a]	4.96 ± 4.43[a]	3.10 ± 3.16[b]
SOCA (mmHg)	96.21 ± 53.12	85.31 ± 59.48	78.18 ± 64.01	106.55 ± 73.58	79.24 ± 59.93	52.09 ± 37.11[c]
SOF (times/min)	9.82 ± 3.18	10.57 ± 3.88	8.35 ± 2.01	8.30 ± 2.84	10.57 ± 2.82	11.44 ± 4.39
CDBP (mmHg)	5.09 ± 7.74	5.63 ± 5.33	2.42 ± 5.28	6.54 ± 4.16	9.03 ± 9.53	5.87 ± 5.07

9.63 ± 8.16 *vs* 4.96 ± 4.43, [a]$P < 0.05$; 9.63 ± 8.16 *vs* 3.10 ± 3.16, [b]$P < 0.01$; 106.55 ± 73.58 *vs* 52.09 ± 37.11, [c]$P < 0.01$.

Table 3 Effects of protease inhibitor on SO pressure (mean ± SD)

		Ulinastatin 2500 U (n = 25)	Ulinastatin 5000 U (n = 25)	Gabexate mesilate (n = 25)
SOBP (mmHg)	Before administration	11.53 ± 4.22	11.81 ± 5.21	14.33 ± 3.74
	10 min	11.96 ± 7.91	9.16 ± 5.97	12.31 ± 4.15
	20 min	9.25 ± 4.43	8.70 ± 4.50[a]	11.61 ± 4.50[b]
SOCA (mmHg)	Before administration	78.63 ± 35.96	65.21 ± 23.46	94.84 ± 39.64
	10 min	64.79 ± 22.25	63.73 ± 33.52	90.60 ± 38.69
	20 min	53.35 ± 25.52[c]	47.13 ± 21.53[d]	80.55 ± 27.58
SOF (t/min)	Before administration	6.67 ± 2.15	7.38 ± 2.58	7.01 ± 1.57
	10 min	6.03 ± 2.10	5.29 ± 2.39[e]	6.45 ± 1.24
	20 min	5.57 ± 1.92	4.65 ± 2.47[f]	6.14 ± 1.73
CDBP (mmHg)	Before administration	7.34 ± 3.06	9.06 ± 4.50	9.68 ± 3.25
	10 min	8.03 ± 3.97	8.51 ± 5.41	10.87 ± 4.55
	20 min	7.47 ± 4.21	8.80 ± 3.50	11.07 ± 4.05

11.81 ± 5.21 *vs* 8.70 ± 4.50, [a]$P < 0.05$; 14.33 ± 3.74 *vs* 11.61 ± 4.50, [b]$P < 0.05$; 78.63 ± 35.96 *vs* 53.35 ± 25.52, [c]$P < 0.01$; 65.21 ± 23.46 *vs* 47.13 ± 21.53, [d]$P < 0.01$; 7.38 ± 2.58 *vs* 5.29 ± 2.39, [e]$P < 0.01$; 7.38 ± 2.58 *vs* 4.65 ± 2.47, [f]$P < 0.01$.

Table 4 Effects of gastro kinetic agents on SO pressure

		Domperidone (n = 20)	Mosapride (n = 20)	Tegaserod (n = 20)
SOBP (mmHg)	Before administration	10.30 ± 4.99	12.56 ± 13.02	7.11 ± 4.08
	10 min	12.79 ± 13.47	5.36 ± 6.70	9.44 ± 5.25
	20 min	6.40 ± 3.66[a]	6.00 ± 5.74	12.93 ± 31.97
SOCA (mmHg)	Before administration	110.52 ± 37.80	83.44 ± 46.16	74.62 ± 26.06
	10 min	97.20 ± 59.96	45.06 ± 31.32[c]	71.74 ± 37.63
	20 min	68.67 ± 41.02[b]	52.48 ± 44.19	58.54 ± 37.19
SOF (t/min)	Before administration	6.84 ± 2.43	7.13 ± 3.21	6.55 ± 2.49
	10 min	8.03 ± 6.52	6.33 ± 3.07	6.07 ± 3.31
	20 min	8.86 ± 7.56	5.74 ± 2.41	5.88 ± 1.47
CDBP (mmHg)	Before administration	5.78 ± 6.59	3.92 ± 3.58	2.9 ± 3.71
	10 min	5.38 ± 2.23	1.74 ± 1.77	3.36 ± 2.98
	20 min	4.34 ± 4.75	1.95 ± 3.43	4.07 ± 5.27

10.30 ± 4.99 *vs* 6.40 ± 3.66, [a]$P < 0.05$; 110.52 ± 37.80 *vs* 68.67 ± 41.02, [b]$P < 0.01$; 83.44 ± 46.16 *vs* 45.06 ± 31.32, [c]$P < 0.01$.

the SO pressure is recognized as the index reflecting the SO motor function. SO manometry plays an important role in illustrating the disease etiology, pathogenesis, and turnover.

Thus far, the direct manometry is the gold standard to evaluate the SO motor function. At present, the most

common and recognized method is the endoscopic retrograde cholangiopancreatography (ERCP) simultaneous manometry. Although the endoscope manometry is widely used, its shortcomings do exist, such as the profuse endoscopic technique experience needed, the short manometry time and the pancreatitic risk[1]. Choledochoscope manometry adopts the same manometry theorem, manometry system and software with ERCP but uses different ways of catheterization manometry. Before the T tube is removed after a bile duct operation, the regular choledochoscopy is needed to avoid residual stones. When manometry is performed through choledochoscope, the measuring tube can reach the biliary tract directly and transit the SO. It is easy to operate, and no medication is required for the operation. Long term manometry research can be conducted with rare complications. In addition, the position of the measuring tube can be defined under the orthophoria in choledochoscope manometry, which makes the result more precise. The effects of the drugs on SO can be observed in choledochoscope manometry. However the shortcomings of choledochoscope manometry do exist, the subjects are all the patients with bile duct stone and post-operation, it is difficult to obtain the relative normal values. In this experiment, all the patients had clear pressure graphs, and no complications occurred. The effects of H_2-receptor blocking pharmacon, protease inhibitor, and gastro kinetic agents had on the SO composed to a pleasant result.

The confirmed SO activity rhythm increasing drugs at present are opium, Anticholinesterase drug, α-adrena receptor blocking pharmacon and H_1-receptor blocking pharmacon. While the SO activity rhythm decreasing drugs are M cholinergic receptor blocker, nitroester drugs, Ca-ion channel blocker, gastro kinetic agents and ®- receptor blocking pharmacon. We studied many different drugs according to the clinical practical medication.

Nitroester drugs can relax vascular smooth muscle, gastrointestinal tract smooth muscle and the SO. M cholinergic receptor has blocking function by inhibiting the cholinergic receptor on the smooth muscle. Our previous research had shown that Nitroester drugs and M cholinergic receptor have inhibitory effect on the SO[2,3]. It is commonly believed that morphine functions through the μ acceptor. It was found in the former research that morphine had excitatory effects on the SO motility[4-6]. Our experiment had shown that morphine had excitatory effect and tramadol had inhibitory effect on the SO, while pethidine had no apparent effect[7]. The effects of somatostatin and its analogue octreotide on the SO are distinct in different genera, and the mechanism of somatostatin accommodation on the SO motility has not been known yet[8]. Our previous research showed that administration density of somatostatin and its analogues should be paid special attention in clinical application, because small dose can excite the SO, which may increase the basic pressure, restrain biliary and pancreatic fluid, exacerbate the obstruction factors and finally affect the curative effect[9].

Histamine, H_2-receptor blocking pharmacon, Famotidine and Cimetidine are often clinically used for the treatment of gastric acid related diseases such as peptic ulcer, gastrinoma and gastroesophageal reflux. Recently their effects on gastrointestinal motility have been given more and more attention, but the inhibitory mechanism hasn't been known. In the experiment regarding the effects of histamine on the SO of opossum, Toouli *et al*[10] found that histamine' inhibitory effect on the SO was aroused by stimulating the H_1 receptor mediated inhibitory nerve, and the inhibitory nerve was non-adrenergic and non-cholinergic nerve having nothing to do with H_2 receptor. Sand *et al*[11] also found that histamine' inhibitory effect on the SO was mediated by H_1 receptor. While the research of Maples *et al*[12] showed that H_2 receptor agonist betazole could increase CDBP and was correlated to duodenum myoelectricity activity. After the antagonistic effect of Cimetidine, the duodenum myoelectricity activity and CDBP decreased to the baseline level, which showed that H_2-receptor blocking pharmacon had inhibitory effect on the biliary tract. The effect of Cimetidine on gastrointestinal motility might vary in different species and sites of action, but the mechanism was controversial. Some people believed that Cimetidine had the anti-effect on acetylcholine, so the effect on gastrointestinal motility was related to M receptor. Others believed that the inhibitory effect of Cimetidine occurred *via* the endogenous prostaglandin receptor. In our research, we found that SOBP and SOCA had the decreasing tendency after the administration of Famotidine, but there was no statistical difference. SOD, SOF and CDBP showed no difference, which indicated that they had no obvious effect on the SO. The experiment also showed that common dose of Cimetidine had inhibitory effect on the SO. Famotidine and Cimetidine were both H_2-receptor blocking pharmacon and mainly inhibited gastric acid secretion, however Famotidine was more effective. However, there was no identical display in the effect on the SO motility, so it might be concluded that their effect on the SO had nothing to do with the effect of H_2 receptor.

Gabexate mesilate and Ulinastatin are the common clinical protease inhibitor; proteinase inhibitor, and the common drugs for pancreatitis treatment. They have been reported to be effective on the prevention of post-ERCP pancreatitis[13,14]. However, at present, there are few studies on the effect of Gabexate mesilate on the SO. Research by Kobayashi *et al*[15] on Gabexate mesilate's inhibitory effect on the SO in dogs was realized by non-adrenergic and non-cholinergic passageway. Di Francesco *et al*[16] adopted the endoscope manometry to study Gabexate mesilate's effect on the SO. SOCA and SOF decreased after the administration, while SOBP showed no apparent

change. The pertinent literatures and reports on Ulinastatin's effect on the SO have not been seen yet. We found that SOBP decreased with the intravenous injection of Ulinastatin 2.5 mg/min, and SOCA and SOF showed no apparent change. SOBP, SOCA, and SOF decreased obviously 20 min after the administration of Ulinastatin at 2500 U/min. SOBP and SOF decreased obviously 10 min after the administration of Ulinastatin at 5000 U/min, and 20 min later the decrease became more apparent and SOCA also decreased apparently. Therefore, it could be supposed that Ulinastatin's effect on the SO was related to the medication time and drug concentration. Our results of manometry indicate that Gabexate mesilate and Ulinastatin had some influence on the SO. The administration of these agents could reduce the incidence of post-ERCP pancreatitis.

Domperidone is the peripheral dopamine receptor blocker, and has strong affinity with D2 receptor especially the gastrointestinal dopamine receptor. It has no cholinergic activity and it is free of atropine inhibition[17]. The results of the studies on Domperidone's effect on the SO are different. Tankurt *et al*[18] found in the experiment that in the Domperidone group Gallbladder contraction increased apparently after administration. He believed that Domperidone's effect on the Gallbladder was nonspecific, and might not depend on dopamine receptor. However, its effect on the SO has not been reported. We found in the experiment that SOBP and SOCA decreased after the administration of Domperidone, and it might have non-adrenergic and non-cholinergic mechanisms. Mosapride stimulates the release of acetylcholine by exciting the 5-HT_4 of the myenteric nerve plexus, and strengthens the movement of the gaster and duodenum[19]. No reports on Mosapride's effect on the SO have been known yet. However, for the homoplastic cisapride there are studies and reports on its inhibitory effect on the SO, and it can be inferred that the effect is regulated by non-adrenergic and non-cholinergic nerves[20]. Our study results showed that Mosapride had an inhibitory effect on the SO, and its' mechanism of action might be similar to or the same as cisapride. Tegaserod is 5-HT_4 receptor partial agonist[21]. In the Tegaserod group, we didn't find the similar inhibitory effect on the SO, which might be related to Tegaserod's high selectivity towards the receptor. In addition, the result might also be influenced by the short administration time we spent on manometry.

Above all, we adopted simple but effective choledochoscope manometry to study the SO motility, and explored the effect of many kinds of drugs on the SO rhythm, which profited our clinical practice. However, their mechanisms of action are not the same, and they may function in different aspects such as nerves, hormone etc. Their influence on different species may be also different. Their mechanisms of action warrant further study.

REFERENCES

1 **Lans JL**, Parikh NP, Geenen JE. Application of sphincter of Oddi manometry in routine clinical investigations. *Endoscopy* 1991; **23**: 139-143

2 **Wu SD**, Zhang ZH, Li DY, Jin JZ, Kong J, Tian Z, Wang W, Wang MF. Nitroester drug's effects and their antagonistic effects against morphine on human sphincter of Oddi motility. *World J Gastroenterol* 2005; **11**: 2319-2323

3 **Wu SD**, Kong J, Wang W, Zhang Q, Jin JZ. Effect of morphine and M-cholinoceptor blocking drugs on human sphincter of Oddi during choledochofiberscopy manometry. *Hepatobiliary Pancreat Dis Int* 2003; **2**: 121-125

4 **Radnay PA**, Brodman E, Mankikar D, Duncalf D. The effect of equi-analgesic doses of fentanyl, morphine, meperidine and pentazocine on common bile duct pressure. *Anaesthesist* 1980; **29**: 26-29

5 **Helm JF**, Venu RP, Geenen JE, Hogan WJ, Dodds WJ, Toouli J, Arndorfer RC. Effects of morphine on the human sphincter of Oddi. *Gut* 1988; **29**: 1402-1407

6 **Blaut U**, Marecik J, Hartwich A, Herman RM, Laskiewicz J, Thor PJ. The effect of transcutaneous nerve stimulation on intraductal biliary pressure in post-cholecystectomy patients with T-drainage. *Eur J Gastroenterol Hepatol* 2003; **15**: 21-26

7 **Wu SD**, Zhang ZH, Jin JZ, Kong J, Wang W, Zhang Q, Li DY, Wang MF. Effects of narcotic analgesic drugs on human Oddi's sphincter motility. *World J Gastroenterol* 2004; **10**: 2901-2904

8 **Di Francesco V**, Angelini G, Zoico E, Zamboni M, Frulloni L, Cavallini G. Effect of native somatostatin on Sphincter of Oddi motility in patients with acute recurrent pancreatitis. A pilot study with Ultrasound-Secretin test. *Dig Liver Dis* 2006; **38**: 268-271

9 **Wu SD**, Zhang ZH, Kong J, Li YJ, Jin JZ, Wang W, Li DY, Wang MF. Effects of somatostatin analogues on human sphincter of Oddi pressure. *Hepatobiliary Pancreat Dis Int* 2005; **4**: 302-305

10 **Toouli J**, Dodds WJ, Honda R, Hogan WJ. Effect of histamine on motor function of opossum sphincter of Oddi. *Am J Physiol* 1981; **241**: G122-G128

11 **Sand J**, Arvola P, Porsti I, Jantti V, Oja OS, Baer G, Nordback I. Histamine in the control of porcine and human sphincter of Oddi activity. *Neurogastroenterol Motil* 2000; **12**: 573-579

12 **Maples MD**, Lea JW 4th, O'Leary JP. Effects of betazole hydrochloride and cimetidine on common bile pressure and duodenal myoelectric activity in the dog. *Am Surg* 1981; **47**: 519-521

13 **Cavallini G**, Tittobello A, Frulloni L, Masci E, Mariana A, Di Francesco V. Gabexate for the prevention of pancreatic damage related to endoscopic retrograde cholangiopancreatography. Gabexate in digestive endoscopy--Italian Group. *N Engl J Med* 1996; **335**: 919-923

14 **Tsujino T**, Komatsu Y, Isayama H, Hirano K, Sasahira N, Yamamoto N, Toda N, Ito Y, Nakai Y, Tada M, Matsumura M, Yoshida H, Kawabe T, Shiratori Y, Omata M. Ulinastatin for pancreatitis after endoscopic retrograde cholangiopancreatography: a randomized, controlled trial. *Clin Gastroenterol Hepatol* 2005; **3**: 376-383

15 **Kobayashi T**, Hosoba T, Mori M, Mimura H, Miyake J, Hamazaki K, Tsuge H, Orita K, Yamasato T, Neya T. Effects of gastrectomy on motility, perfusion pressure, and caerulein-induced relaxation of sphincter of Oddi in dogs. *J Smooth Muscle Res* 1994; **30**: 85-96

16 **Di Francesco V**, Mariani A, Angelini G, Masci E, Frulloni

L, Talamini G, Passaretti S, Testoni P, Cavallini G. Effects of gabexate mesilate, a protease inhibitor, on human sphincter of Oddi motility. *Dig Dis Sci* 2002; **47**: 741-745

17 **Marzio L**, Neri M, Pieramico O, Delle Donne M, Peeters TL, Cuccurullo F. Dopamine interrupts gastrointestinal fed motility pattern in humans. Effect on motilin and somatostatin blood levels. *Dig Dis Sci* 1990; **35**: 327-332

18 **Tankurt E**, Apaydin S, Ellidokuz E, Igci E, Guven H, Simsek I, Gonen O. The prokinetic effect of domperidone in gallbladder--not upon dopaminergic receptors. *Pharmacol Res* 1996; **34**: 153-156

19 **Sasaki N**, Okamura K, Yamada H. Effects of mosapride, a 5-hydroxytryptamine 4 receptor agonist, on electrical activity of the small intestine and cecum in horses. *Am J Vet Res* 2005; **66**: 1321-1323

20 **Baker RA**, Saccone GT, Toouli J. Cisapride inhibits motility of the sphincter of Oddi in the Australian possum. *Dig Dis Sci* 1990; **35**: 711-715

21 **Beattie DT**, Smith JA, Marquess D, Vickery RG, Armstrong SR, Pulido-Rios T, McCullough JL, Sandlund C, Richardson C, Mai N, Humphrey PP. The 5-HT4 receptor agonist, tegaserod, is a potent 5-HT2B receptor antagonist in vitro and in vivo. *Br J Pharmacol* 2004; **143**: 549-560

S- Editor Tian L **L- Editor** Alpini GD **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5913

World J Gastroenterol 2008 October 14; 14(38): 5913-5915
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Porphyria cutanea tarda as a complication of therapy for chronic hepatitis C

James Azim, Heather McCurdy, Richard H Moseley

James Azim, Heather McCurdy, Richard H Moseley, Division of Gastroenterology, Department of Internal Medicine, Department of Veterans Affairs Medical Center and University of Michigan School of Medicine, Ann Arbor, Michigan, United States

Author contributions: All authors contributed equally to this work.

Correspondence to: Richard H Moseley, MD, Chief, Medical Service (111), Ann Arbor VA Healthcare System, 2215 Fuller Road, Ann Arbor, Michigan 48105, United States. rmoseley@med.umich.edu

Telephone: +1-734-8455849 Fax: +1-734-9130883
Received: July 15, 2008 Revised: September 16, 2008
Accepted: September 23, 2008
Published online: October 14, 2008

Abstract

There is a strong association between porphyria cutanea tarda (PCT) and chronic viral hepatitis C. Therapy for chronic viral hepatitis C may improve PCT. However, there are only a few reports of the *de novo* development of PCT during therapy for chronic viral hepatitis C. We describe the development of PCT in a 56-year-old patient with chronic viral hepatitis C after 12 wk of peginterferon/ribavirin therapy. In addition, the patient was homozygous for the H63D hereditary hemochromatosis gene (*HFE*) mutation. The association of PCT with chronic viral hepatitis C and the possible role of hepatic iron overload and ribavirin-induced hemolytic anemia in the development of PCT during therapy for chronic viral hepatitis C are discussed.

Key words: Porphyria; Hepatitis C virus; Ribavirin; Pegylated interferon; Hemochromatosis

Peer reviewers: Stephan Menne, Assistant Professor of Virology, Department of Clinical Sciences/GI Unit, College of Veterinary Medicine, Cornell University, C2-005 Veterinary Medical Center, Ithaca, NY 14853, United States; Eva Herrmann, Professor, Saarland University, Kirrberger Street, Homburg/Saar 66421, Germany

Azim J, McCurdy H, Moseley RH. Porphyria cutanea tarda as a complication of therapy for chronic hepatitis C. *World J Gastroenterol* 2008; 14(38): 5913-5915 Available from: URL: http://www.wjgnet.com/1007-9327/14/5913.asp DOI: http://dx.doi.org/10.3748/wjg.14.5913

INTRODUCTION

Porphyria cutanea tarda (PCT), the most common of the porphyrias, results from reduced activity of uroporphyrinogen decarboxylase (UROD), an enzyme in the heme biosynthetic pathway[1]. Clinical signs of PCT include photodistributed erythema, skin fragility, bullae, erosions and hypertrichosis. A strong association between PCT and hepatitis C virus (HCV) infection is well established[2]. Improvement of PCT during HCV treatment with interferon has been frequently described[3-6]. On the other hand, *de novo* occurrence of PCT during interferon plus ribavirin therapy for chronic viral hepatitis C has previously been described in only four patients[7-9]. Herein we describe the *de novo* development of PCT in a patient with chronic HCV infection undergoing combined peginterferon/ribavirin therapy, who was also found to be homozygous for the H63D mutation of the *HFE* gene.

CASE REPORT

Chronic hepatitis C (genotype 1a) was diagnosed in a 56-year-old man with a history of blood transfusions while serving in Vietnam. Past medical history included alcohol and cocaine dependency, as well as a history of treatment for rheumatoid arthritis, which was later characterized as osteoarthritis. He denied alcohol use for more than four years prior to initiation of ribavirin/interferon therapy. Prior tests for hepatitis B and HIV were negative. He did not smoke. Family history was noncontributory. At presentation, liver biopsy showed mildly active chronic hepatitis C with portal fibrosis (Batts and Ludwig grade 2 inflammation and stage 1 fibrosis). Hepatic iron deposition was not commented on. HCV-RNA PCR showed 102 500 copies and serum transaminases were mildly elevated (AST 53 IU/L and ALT 100 IU/L). The patient was treated with peginterferon-α2b (150 μg/wk) and ribavirin (1200 mg/day) for 44 weeks of a planned 48-week course. Treatment ceased early secondary to anemia and neutropenia. Viral clearance was achieved after 16 wk of treatment and HCV-RNA PCR remained negative at the

end of and 6 mo following treatment.

After 12 wk of therapy, the patient presented with multiple hand blisters and erosions in various stages of healing, consistent with PCT. Diagnosis of PCT was confirmed by detection of elevated urinary uroporphyrin (599 μg/24 h, N < 44) and heptacarboxyl-porphyrin (799 μg/24 h, N < 12) levels. Because of the patient's anemia (hemoglobin dropped from 15.7 g/dL pretreatment to 10.4 g/dL at onset of PCT), treatment of PCT with phlebotomy was deferred until completion of his peginterferon/ribavirin treatment. Clinical resolution of PCT occurred thereafter with repeated phlebotomy. Prior to initiation of phlebotomy, ferritin was markedly elevated (2920 ng/mL, N < 464) and the transferrin saturation was 81%. The patient was found to be homozygous for the H63D *HFE* gene mutation.

DISCUSSION

PCT arises from decreased activity of hepatic uroporphyrinogen decarboxylase (UROD), an enzyme in the heme synthetic pathway[1,10]. Although about 20% of cases involve mutations in the UROD gene (type Ⅱ PCT), nearly all other occurrences (approximately 80%) are considered to arise sporadically (type Ⅰ PCT)[1]. The genetic defects in type Ⅱ PCT alone are not sufficient to cause phenotypic PCT, for they reduce UROD activity by no more than 50%[10]. Other contributing factors, including those that may be associated with type I PCT, must be present for phenotypic expression. Whether sporadic or familial, the clinical manifestations of PCT are the same, including skin fragility, erythema, bullae, erosions, hypertrichosis and milia in sun-exposed areas of the skin[11]. These lesions result from the photosensitizing effects of porphyrins that accumulate in the skin or dermal blood vessels[12]. The pathogenesis of the enzymatic defect in sporadic PCT is unclear, but is postulated to involve increased oxidative stress in the liver, modulated by multiple exogenous and endogenous factors[12,13].

Well-recognized risk factors for PCT include HCV infection, hereditary hemochromatosis and other iron overload syndromes, as determined by mutations in the *HFE* gene, excessive alcohol use, HIV infection, and exposure to estrogen (including pregnancy) and polyhalogenated aromatic compounds[10]. The association of PCT with chronic HCV infection is particularly strong. The prevalence of HCV infection in patients with PCT was found to be about 50% in a recent systematic review and meta-analysis, though there was marked regional variation[2]. The underlying mechanism is unclear, despite this strong association of PCT with chronic HCV infection[11].

Mainstays of PCT management include phlebotomy, chloroquine and avoidance of sun exposure[12]. HCV-associated PCT has, similarly, been reported to improve following initiation of interferon monotherapy[3-6], though improvement with interferon administration is not universally described[14]. Likewise, we found a single report describing the onset of HCV-associated PCT 2 years following initiation of interferon monotherapy in a patient who also had non-Hodgkins lymphoma and dermatomyositis[15]. The skin lesions resolved with chemotherapy and the authors hypothesized that the lymphoma had triggered PCT.

In addition to the case we report here, *de novo* occurrence of PCT during interferon and ribavirin therapy for chronic HCV infection has been described in only 4 other cases[7-9]. Jessner *et al*[7] reported what they considered to be *de novo* occurrence of PCT after 4 months of therapy in a woman who had noticed occasional self-resolving small blisters prior to HCV therapy. A liver biopsy showed moderate iron deposition. She was found to have both type Ⅰ ("familial") PCT and heterozygosity for the C282Y HFE gene defect. Thevenot *et al*[8] described two patients without HFE gene defects who had onset of PCT one and two months, respectively, after initiating ribavirin and interferon therapy. One patient was noted to have a ferritin of 2408 ng/mL and the other patients' liver biopsy showed mild iron deposition in Kupffer cells. Mutation analysis of the UROD gene was not reported in either case. Finally, Frider *et al*[9] reported a 47-year-old patient who developed PCT after 44 weeks of ribavirin and interferon therapy. HFE genotyping revealed C282Y/H63D compound heterozygosity.

In all 5 reported cases of *de novo* PCT during ribavirin/interferon therapy, including that we describe here, the patients had clinical evidence of systemic or hepatic iron overload. Although the mechanism is unclear, iron overload is believed to play a significant role in the pathogenesis of PCT[16,17], possibly modulated by an iron-dependent reversible inactivation of hepatic UROD[16]. Supporting this are the oft-noted observations that the majority of patients with PCT exhibit some iron overload, iron depletion improves PCT, and iron administration produces relapse[13,16,17].

Possibly contributing to hepatic iron loading in the reported *de novo* occurrences of PCT was the administration of ribavirin. A well-recognized major side effect of ribavirin is dose-dependent reversible hemolytic anemia[18]. Although Thevenot *et al*[8] did not comment on anemia, Jessner *et al*[7] noted onset of anemia concomitant with diagnosis of PCT. In addition to anemia, hepatic iron stores have been found to increase following prolonged ribavirin administration[19,20], with a relatively greater concentration of iron demonstrated in hepatocytes *versus* Kupffer cells[20]. The additional hepatic iron loading attributable to ribavirin administration may reduce the level of UROD activity sufficiently for PCT to become manifest in otherwise susceptible individuals. Consequently, screening for treatable PCT-associated conditions, such as hemochromatosis, should be considered prior to initiation of peginterferon/ribavirin therapy for chronic HCV.

REFERENCES

1 **Elder GH**. Porphyria cutanea tarda. *Semin Liver Dis* 1998; **18**: 67-75
2 **Gisbert JP**, Garcia-Buey L, Pajares JM, Moreno-Otero R. Prevalence of hepatitis C virus infection in porphyria cutanea tarda: systematic review and meta-analysis. *J Hepatol* 2003; **39**: 620-627
3 **Okano J**, Horie Y, Kawasaki H, Kondo M. Interferon treatment of porphyria cutanea tarda associated with chronic hepatitis type C. *Hepatogastroenterology* 1997; **44**: 525-528
4 **Sheikh MY**, Wright RA, Burruss JB. Dramatic resolution of skin lesions associated with porphyria cutanea tarda after interferon-alpha therapy in a case of chronic hepatitis C. *Dig Dis Sci* 1998; **43**: 529-533
5 **Siegel LB**, Eber BB. Porphyria cutanea tarda remission. *Ann Intern Med* 1994; **121**: 308-309
6 **Takikawa H**, Yamazaki R, Shoji S, Miyake K, Yamanaka M. Normalization of urinary porphyrin level and disappearance of skin lesions after successful interferon therapy in a case of chronic hepatitis C complicated with porphyria cutanea tarda. *J Hepatol* 1995; **22**: 249-250
7 **Jessner W**, Der-Petrossian M, Christiansen L, Maier H, Steindl-Munda P, Gangl A, Ferenci P. Porphyria cutanea tarda during interferon/ribavirin therapy for chronic hepatitis C. *Hepatology* 2002; **36**: 1301-1302
8 **Thevenot T**, Bachmeyer C, Hammi R, Dumouchel P, Ducamp-Posak I, Cadranel JF. Occurrence of porphyria cutanea tarda during peginterferon/ribavirin therapy for chronic viral hepatitis C. *J Hepatol* 2005; **42**: 607-608
9 **Frider B**, Buonsante ME, Tiscornia J, Alessio A, Pellerano G. [Development of porphyria cutanea tarda in a chronic hepatitis C patient with indetectable viremia under treatment with peg-interferon plus ribavirin] *Acta Gastroenterol Latinoam* 2006; **36**: 38-41
10 **Lambrecht RW**, Thapar M, Bonkovsky HL. Genetic aspects of porphyria cutanea tarda. *Semin Liver Dis* 2007; **27**: 99-108
11 **Berk DR**, Mallory SB, Keeffe EB, Ahmed A. Dermatologic disorders associated with chronic hepatitis C: effect of interferon therapy. *Clin Gastroenterol Hepatol* 2007; **5**: 142-151
12 **Chemmanur AT**, Bonkovsky HL. Hepatic porphyrias: diagnosis and management. *Clin Liver Dis* 2004; **8**: 807-838, viii
13 **Bonkovsky HL**, Poh-Fitzpatrick M, Pimstone N, Obando J, Di Bisceglie A, Tattrie C, Tortorelli K, LeClair P, Mercurio MG, Lambrecht RW. Porphyria cutanea tarda, hepatitis C, and HFE gene mutations in North America. *Hepatology* 1998; **27**: 1661-1669
14 **Furuta M**, Kaito M, Gabazza E, Fujita N, Ishida S, Tamaki S, Ikeda R, Wakisawa S, Hayashi H, Watanabe S, Adachi Y. Ineffective interferon treatment of chronic hepatitis C-associated porphyria cutanea tarda, but with a transient decrease in HCV RNA levels. *J Gastroenterol* 2000; **35**: 60-62
15 **Bauza A**, Espana A, Lloret P. Porphyria cutanea tarda, dermatomyositis and non-Hodgkin lymphoma in virus C infection. *Eur J Dermatol* 2003; **13**: 302-304
16 **Alla V**, Bonkovsky HL. Iron in nonhemochromatotic liver disorders. *Semin Liver Dis* 2005; **25**: 461-472
17 **Fargion S**, Fracanzani AL. Prevalence of hepatitis C virus infection in porphyria cutanea tarda. *J Hepatol* 2003; **39**: 635-638
18 **De Franceschi L**, Fattovich G, Turrini F, Ayi K, Brugnara C, Manzato F, Noventa F, Stanzial AM, Solero P, Corrocher R. Hemolytic anemia induced by ribavirin therapy in patients with chronic hepatitis C virus infection: role of membrane oxidative damage. *Hepatology* 2000; **31**: 997-1004
19 **Di Bisceglie AM**, Bacon BR, Kleiner DE, Hoofnagle JH. Increase in hepatic iron stores following prolonged therapy with ribavirin in patients with chronic hepatitis C. *J Hepatol* 1994; **21**: 1109-1112
20 **Fiel MI**, Schiano TD, Guido M, Thung SN, Lindsay KL, Davis GL, Lewis JH, Seeff LB, Bodenheimer HC Jr. Increased hepatic iron deposition resulting from treatment of chronic hepatitis C with ribavirin. *Am J Clin Pathol* 2000; **113**: 35-39

S- Editor Zhong XY **L- Editor** Wang XL **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5916

World J Gastroenterol 2008 October 14; 14(38): 5916-5919
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Jejunal diverticulosis is not always a silent spectator: A report of 4 cases and review of the literature

Vishal Arun Patel, Helen Jefferis, Ben Spiegelberg, Quamar Iqbal, Ashish Prabhudesai, Simon Harris

Vishal Arun Patel, Helen Jefferis, Ben Spiegelberg, Quamar Iqbal, Ashish Prabhudesai, Simon Harris, Department of Colorectal Surgery, Hillingdon Hospital, Uxbridge, Middlesex UB8 3NN, United Kingdom
Author contributions: Patel VA contributed most to this work; Patel VA, Jefferis H and Iqbal Q reviewed the cases; Patel VA, Spiegelberg B and Harris S made the literature review; Patel VA and Prabhudesai A wrote the paper.
Correspondence to: Vishal Arun Patel, Department of Colorectal Surgery, Hillingdon Hospital, 4 Potter Street, Northwood, Middlesex HA6 1QE, United Kingdom. vish079@hotmail.com
Telephone: +44-19-23836418 Fax: +44-19-23820547
Received: January 31, 2008 Revised: June 6, 2008
Accepted: July 13, 2008
Published online: October 14, 2008

Abstract

Jejunal diverticulosis (JD) is a rare clinical entity. The potential complications of this condition are discussed here through a series of cases presented to our centre. A retrospective analysis of four cases, which were diagnosed and treated, was performed. These included two cases of gastrointestinal haemorrhage, one case of perforation and one case of enterolith obstruction. All of these cases were secondary to jejunal diverticulosis and treated surgically. This was accompanied by a literature search to identify the different modalities for diagnosis and treatment of this condition. JD is rare and may lead to a diagnostic delay. Awareness of the wide spectrum of potential complications can prevent this delay.

Key words: Jejunum; Diverticulosis; Gastrointestinal; Haemorrhage; Perforation; Enterolith; Obstruction

Peer reviewers: Henrike Maria Hamer, Division of Gastroenterology, Department of Internal Medicine, Maastricht University, PO Box 616, Maastricht 6200 MD, Netherlands; Damian Casadesus, MD, PhD, Calixto Garcia University Hospital, J and University, Vedado, Havana City, Cuba

Patel VA, Jefferis H, Spiegelberg B, Iqbal Q, Prabhudesai A, Harris S. Jejunal diverticulosis is not always a silent spectator: A report of 4 cases and review of the literature. *World J Gastroenterol* 2008; 14(38): 5916-5919 Available from: URL: http://www.wjgnet.com/1007-9327/14/5916.asp DOI: http://dx.doi.org/10.3748/wjg.14.5916

INTRODUCTION

The majority of patients with jejunal diverticula are asymptomatic[1]. Jejunal diverticula are the rarest of all small bowel diverticula. An incidence of 0.5%-2.3% of small bowel contrast studies and 0.3%-4.5% of autopsies have been reported in the literature[2,3]. Chronic abdominal symptoms like abdominal pain/nausea and vomiting/flatulence/diarrhoea and malabsorption have been described in some reports. However, jejunal diverticulosis (JD) may present more acutely. Major complications include diverticulitis, gastrointestinal (GI) haemorrhage, intestinal obstruction and acute perforation[4,5]. We discuss four patients who presented with complications of JD.

CASE REPORT

Case 1: Gastrointestinal haemorrhage

A 71-year-old male was admitted as an emergency with a day history of lower abdominal pain associated with five episodes of passing altered blood per rectum (PR) and vomiting. He had no recent history of change in bowel habit. Past medical history included a laparotomy for a perforated appendix, duodenal ulcer and deep vein thrombosis. He was haemodynamically stable with a soft, non-tender abdomen. Rectal examination revealed dark blood with no masses. His Hb was 12.0 g/dL, white cell count was 12.0 cells/mm^3 and a raised urea of 12.9 mmol/L.

The following day he passed approximately 750 mL of blood PR with associated hypotension and a drop in his Hb to 5.5 g/dL. He was resuscitated and an upper gastrointestinal endoscopy was performed, which revealed a hiatus hernia. A flexible sigmoidoscopy showed dark blood but no identifiable bleeding source. An urgent mesenteric angiogram demonstrated extravasation of contrast into the proximal jejunum from the 2nd jejunal branch of the superior mesenteric artery.

He then underwent a laparotomy which revealed a 1.5 cm solitary jejunal diverticulum 25 cm from the duodeno-jejunal flexure. An enteroscopy *via* jejunal access showed a pulsating vessel at the neck of the diverticulum. A local jejunal resection was carried out with a functional end-to-end stapled anastomosis. The postoperative period was unremarkable and he was discharged home on day 9. His out patient review at 6 wk showed a good recovery with no further bleeding.

Case 2: Gastrointestinal haemorrhage

A 62-year-old Indian male presented with a day history of altered PR bleeding. He had a similar episode of this per rectal bleeding four years prior to this episode with no apparent cause established. He had an episode of loss of consciousness and his blood pressure dropped to 93/54 mmHg with 6.8 g/dL haemoglobin. After fluid resuscitation and 3 unit blood transfusion, he had a normal upper gastrointestinal endoscopy. He was discharged with an out-patient colonoscopy requested and had no further bleeding.

He re-presented on the 4th day with further PR bleeding with a blood pressure of 60/40 mmHg and 5.8 g/dL haemoglobin. Despite resuscitation with blood transfusion, his bleeding continued with a further drop of his haemoglobin to 3.9 g/dL. As he was haemodynamically unstable, he underwent an emergency laparotomy, which revealed blood in the proximal small bowel extending to the transverse colon with multiple jejunal diverticula. These first appeared 15 cm distal to the duodeno-jejunal flexure. Following cross clamping of this jejunal segment containing diverticula, no further bleeding was evident. This segment was resected and a side to side anastomosis was performed.

This gentleman spent a further 6 d in the Intensive Care Unit where he required ventilatory and inotropic support. The remainder of his in patient stay was unremarkable and he was discharged on day 12. His out patient follow-up has revealed a large, symptomatic incisional hernia for which he is currently awaiting repair.

Case 3: Perforation with abscess formation

A 66-year-old female presented with a 5-d history of right sided abdominal pain and watery mucoid diarrhoea. She was reviewed two days ago in the emergency department and diagnosed with exacerbation of irritable bowel syndrome. Her past medical history included angina/Sjorgen's syndrome and a previous appendicectomy. On examination she was haemodynamically stable and tender in both iliac fossae, being more prominent on the right side. Her initial white cell count was 11.3 g/dL with an abnormal renal function-urea of 11.4 and a creatinine of 156. An initial ultrasound scan of the abdomen and pelvis was normal. A computed tomography scan, the following day, revealed a modest amount of free fluid in the abdominal cavity. Free air was shown in the retroperitoneum anterior to the 2nd, 3rd, and 4th parts of the duodenum. A perforation was also noted in a thickened small bowel loop.

A laparotomy demonstrated multiple jejunal diverticular, one of which was perforated locally. This formed an abscess, the wall of which was formed by the mesentery corresponding to the perforated jejunal loop. A segmental resection of the jejunum was performed with a side to side stapled anastomosis. Post operative recovery was unremarkable initially and she was discharged home 8 d post operation. A small subcutaneous abscess was noted on follow-up, which was incised and drained. There were no other post operative problems subsequently.

Case 4: Small bowel obstruction

This 80-year-old gentleman presented with a 2-d history of left iliac fossa pain associated with bilious vomiting. He had not passed a bowel motion since the day before but had had intermittent diarrhoea for the preceding 3 wk. He was known to have sigmoid diverticula, confirmed on colonoscopy. His other medical history included a coronary artery bypass graft, non-insulin dependent diabetes mellitus and an appendicectomy. On examination he was mildly distended with tenderness in his lower abdomen with no associated peritonism. His white cell count was 15.5 cells/mm^3 and his abdominal radiograph showed no obvious abnormality. Computed tomography scan showed dilated small bowel loops with extensive jejunal diverticula.

Due to failure of conservative management, a laparotomy was performed, which revealed dilated proximal bowel with a transition point at the diverticular segment of the jejunum. Multiple large and medium sized inflamed jejunal diverticula were noted. An enterotomy was performed and an obstructing enterolith was removed. One of the inflamed jejunal diverticula was perforated but sealed by omental adhesions. This perforation was subsequently sutured. Post operatively, this patient made a full recovery and was discharged 8 d later. This gentleman's follow- up has been uneventful.

DISCUSSION

Jejunal diverticulosis was first described by Somerling in 1794 and by Sir Astley Cooper in 1807[6]. These false diverticula are acquired outpouchings of mucosa commonly found on the mesenteric border of the jejunum. Jejunal diverticula share similarities with colonic diverticula in that the mucosal herniations occur through gaps in the muscle layers along pathways of the visceral vessels. The sizes of these diverticula vary between a few millimetres to greater than ten centimetres. Jejunal diverticula may be the only site in the gastrointestinal tract. Of these diverticula, 35% are associated with colonic diverticula, 26% with duodenal diverticula and 2% with oesophageal diverticula, respectively[7,8].

Small bowel diverticula are frequently encountered in the elderly and have a slight male predominance[9]. Their presentation is variable from asymptomatic to chronic abdominal symptoms and the complications described in our case series. Their relative clinical rarity and varied presentation may make diagnosis both delayed and difficult. This is exemplified in our third patient whose symptoms were initially attributable to her previous diagnosis of irritable bowel syndrome. The discovery of jejunal diverticula may be incidental in imaging studies or may be found at laparotomy as the cause of clinical deterioration. Radiographic studies, which may incidentally demonstrate jejunal diverticula, are contrast enhanced small bowel follow through studies or computed tomography scans.

Haemorrhage from jejunal diverticula predominantly presents as lower gastrointestinal bleeding although cases of haematemesis have been reported[10]. This bleeding may be acute or chronic with iron deficiency anaemia noted. Gastrointestinal haemorrhage from the jejunum has a similar aetiology to that seen in the large bowel diverticula in that the diverticulum erodes through a perforating artery. Previous literature has presented flow charts to direct the clinician in establishing the difficult diagnosis of local gastrointestinal haemorrhage[11]. If the patient is considered haemodynamically stable, then endoscopic techniques, such as oesophagogastr oduodenoscopy or colonoscopy, may be used. These techniques, however, cannot visualise the jejunum. Small bowel contrast studies and computed tomography scans are able to visualise such regions and thus establish the diagnosis. The most sensitive imaging studies are technetium red cell-tagged scan and/or mesenteric angiogram. In our first patient, the latter modality was the choice of imaging technique and it aided identifying the exact bleeding source. The technique also has the advantage of offering mesenteric embolization. Haemodynamic instability warrants emergency laparotomy, which occurred in both of our patients suffering from a gastrointestinal haemorrhage. In such cases, intense supportive therapy is required adjunct to acute surgical treatment with the purpose of finding the precise bleeding point and ensuring definitive treatment. The preferred approach to acute haemorrhage is intestinal resection of the bleeding jejunal segment with primary anastomoses[12].

Perforation may present as a localised perforation with or without generalized peritonitis. Alternatively, the presentation may be that of a perforation with formation of a walled off abscess[12-14]. Perforation is rare, which may be related to the low intraluminal pressures within the small bowel. Instigating factors for perforation have been shown to be related to a necrotizing inflammatory reaction in 82% of cases, followed by blunt trauma in 12% of cases and foreign body impaction in 6% of cases[15]. Computed tomography is the most useful diagnostic imaging tool in such cases[16]. It has proved to be superior to barium studies in demonstrating the mural, serosal and mesenteric extent of disease[17-19]. The management is surgical with resection of the diseased segment advocated followed by primary jejuno-jejunal or jejuno-ileal anastomoses. Previous studies have shown that a laparoscopic approach is successful[20]. Extensive resection should be avoided as this has the potential to lead to short bowel syndrome. Novak *et al*[21] have demonstrated a few cases where a localised perforation of jejunal diverticula could be treated non-surgically with either intravenous antibiotic therapy or computed tomography-assisted percutaneous drainage of the abscess.

Acute intestinal obstruction is another complication of jejunal diverticula. Obstruction may be related to extrinsic compression from a nearby loop of jejunum containing a large diverticulum or from intussusception[12,22] or may be non-mechanical such as dyskinesia[23]. Obstruction may also be secondary to enterolith formation or gallstone migration. Enteroliths may form inside the diverticulum and consist of choleic acid, either de novo or around a bezoar. The acidic environment within diverticula is ideal for aiding the metabolism of bile salts to choleic acid, hence enterolith obstruction[24]. There are various diagnostic modalities which can be useful in such presentations. Abdominal radiographs may provide evidence of stones in the abdomen external to sites such as the gallbladder or renal tracts. Ultrasound examination may confirm or exclude gallstones. The diagnosis may be confirmed with either barium imaging or computed tomography scanning. Management may be either conservative or surgical. In our case, a trial of conservative treatment was unsuccessful and thus resulted in operative intervention. Strategies, which may be used in laparotomy, are crushing of the enteroliths and milking their fragments into the colon[22,24-28]. If this is unsuccessful, an enterostomy can be performed proximal or distal to the site of obstruction with the enterolith removed. If these two steps are unsuccessful, resection of the involved jejunal segment may have to be considered[27-29].

Though JD is rare, awareness of the wide spectrum of associated complications may be useful in preventing delay in treatment.

REFERENCES

1 **Tsiotos GG**, Farnell MB, Ilstrup DM. Nonmeckelian jejunal or ileal diverticulosis: an analysis of 112 cases. *Surgery* 1994; **116**: 726-731; discussion 731-732
2 **Wilcox RD**, Shatney CH. Surgical implications of jejunal diverticula. *South Med J* 1988; **81**: 1386-1391
3 **Fisher JK**, Fortin D. Partial small bowel obstruction secondary to ileal diverticulitis. *Radiology* 1977; **122**: 321-322
4 **Ross CB**, Richards WO, Sharp KW, Bertram PD, Schaper PW. Diverticular disease of the jejunum and its complications. *Am Surg* 1990; **56**: 319-324
5 **de Bree E**, Grammatikakis J, Christodoulakis M, Tsiftsis D. The clinical significance of acquired jejunoileal diverticula. *Am J Gastroenterol* 1998; **93**: 2523-2528
6 **Williams RA**, Davidson DD, Serota AI, Wilson SE. Surgical problems of diverticula of the small intestine. *Surg Gynecol Obstet* 1981; **152**: 621-626
7 **Baskin RH Jr**, MAYO CW. Jejunal diverticulosis; a clinical study of 87 cases. *Surg Clin North Am* 1952; 1185-1196
8 **Benson RE**, Dixon CF, Waugh JM. Non-Meckelian diverticula of the jejunum and ileum. *Ann Surg* 1943; **118**: 377-393
9 **Akhrass R**, Yaffe MB, Fischer C, Ponsky J, Shuck JM. Small-bowel diverticulosis: perceptions and reality. *J Am Coll Surg* 1997; **184**: 383-388
10 **Altemeier WA**, Bryant LR. The surgical significance of jejunal diverticulosis. *Arch Surg* 1963; **86**: 732-745
11 **Wilcox RD**, Shatney CH. Massive rectal bleeding from jejunal diverticula. *Surg Gynecol Obstet* 1987; **165**: 425-428
12 **Longo WE**, Vernava AM 3rd. Clinical implications of jejunoileal diverticular disease. *Dis Colon Rectum* 1992; **35**: 381-388
13 **Gotian A**, Katz S. Jejunal diverticulitis with localized perforation and intramesenteric abscess. *Am J Gastroenterol* 1998; **93**: 1173-1175
14 **Alvarez OA**, Mejia A, Ostrower VS, Lee M. Jejunal diverticulitis manifesting with abdominal wall abscess. *Am J Gastroenterol* 1995; **90**: 2060-2062

15 **Herrington JL Jr**. Perforation of acuired diverticula of the jejunum and ileum. Analysis of reported cases. *Surgery* 1962; **51**: 426-433
16 **Hibbeln JF**, Gorodetsky AA, Wilbur AC. Perforated jejunal diverticulum: CT diagnosis. *Abdom Imaging* 1995; **20**: 29-30
17 **Goldberg HI**, Gore RM, Margulis AR, Moss AA, Baker EL. Computed tomography in the evaluation of Crohn disease. *AJR Am J Roentgenol* 1983; **140**: 277-282
18 **Kerber GW**, Greenberg M, Rubin JM. Computed tomography evaluation of local and extraintestinal complications of Crohn's disease. *Gastrointest Radiol* 1984; **9**: 143-148
19 **Lieberman JM**, Haaga JR. Computed tomography of diverticulitis. *J Comput Assist Tomogr* 1983; **7**: 431-433
20 **Cross MJ**, Snyder SK. Laparoscopic-directed small bowel resection for jejunal diverticulitis with perforation. *J Laparoendosc Surg* 1993; **3**: 47-49
21 **Novak JS**, Tobias J, Barkin JS. Nonsurgical management of acute jejunal diverticulitis: a review. *Am J Gastroenterol* 1997; **92**: 1929-1931
22 **Steenvoorde P**, Schaardenburgh P, Viersma JH. Enterolith ileus as a complication of jejunal diverticulosis: two case reports and a review of the literature. *Dig Surg* 2003; **20**: 57-60
23 **Krishnamurthy S**, Kelly MM, Rohrmann CA, Schuffler MD. Jejunal diverticulosis. A heterogenous disorder caused by a variety of abnormalities of smooth muscle or myenteric plexus. *Gastroenterology* 1983; **85**: 538-547
24 **Hayee B**, Khan HN, Al-Mishlab T, McPartlin JF. A case of enterolith small bowel obstruction and jejunal diverticulosis. *World J Gastroenterol* 2003; **9**: 883-884
25 **Harris LM**, Volpe CM, Doerr RJ. Small bowel obstruction secondary to enterolith impaction complicating jejunal diverticulitis. *Am J Gastroenterol* 1997; **92**: 1538-1540
26 **Shocket E**, Simon SA. Small bowel obstruction due to enterolith (bezoar) formed in a duodenal diverticulum: a case report and review of the literature. *Am J Gastroenterol* 1982; **77**: 621-624
27 **Lopez PV**, Welch JP. Enterolith intestinal obstruction owing to acquired and congenital diverticulosis. Report of two cases and review of the literature. *Dis Colon Rectum* 1991; **34**: 941-944
28 **Klingler PJ**, Seelig MH, Floch NR, Branton SA, Metzger PP. Small-intestinal enteroliths--unusual cause of small-intestinal obstruction: report of three cases. *Dis Colon Rectum* 1999; **42**: 676-679
29 **Govindan VK**, Balashanmugam. Acute intestinal obstruction due to solitary jejunal diverticulum. *Indian J Gastroenterol* 2002; **21**: 204

S- Editor Zhong XY **L- Editor** Wang XL **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5920

World J Gastroenterol 2008 October 14; 14(38): 5920-5923
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Late hepatic artery pseudoaneurysm: A rare complication after resection of hilar cholangiocarcinoma

Javier Briceño, Álvaro Naranjo, Rubén Ciria, Juan Manuel Sánchez-Hidalgo, Luis Zurera, Pedro López-Cillero

Javier Briceño, Álvaro Naranjo, Rubén Ciria, Juan Manuel Sánchez-Hidalgo, Pedro López-Cillero, Hepatobiliary Surgery and Liver Transplantation Unit, Department of Surgery, Hospital Universitario Reina Sofía, Córdoba 14004, Spain
Luis Zurera, Interventional Radiology Unit, Department of Surgery, Hospital Universitario Reina Sofía, Córdoba 14004, Spain
Author contributions: Briceño J, López-Cillero P and Naranjo A performed surgical procedure; Briceño J and López-Cillero P designed the study; Ciria R and Sánchez-Hidalgo JM prepared the drafts and gave critical revision; Zurera L performed radiological interventions; Briceño J and Ciria R prepared the manuscript.
Supported by Ciberehd (Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas); Ciberehed is funded by the Instituto de Salud Carlos Ⅲ
Correspondence to: Dr. Javier Briceño, Hepatobiliary Surgery and Liver Transplantation Unit, Department of Surgery, Hospital Reina Sofía, Avenida Menéndez Pidal s/n, Córdoba 14004, Spain. javibriceno@hotmail.com
Telephone: +34-957-010132 Fax: +34-957-012876
Received: May 3, 2008 Revised: September 4, 2008
Accepted: September 11, 2008
Published online: October 14, 2008

Abstract

We report an unusual pathological entity of a pseudoaneurysm of the right hepatic artery, which developed two years after the resection of a type Ⅱ hilar cholangiocarcinoma and secondary to an excessive skeletonization for regional lymphadenectomy and neoadjuvant external-beam radiotherapy. After a sudden and massive hematemesis, a multidetector computed tomographic angiography (MDCTA) showed a hepatic artery pseudoaneurysm. Angiography with embolization of the pseudoaneurysm was attempted using microcoils with adequate patency of the hepatic artery and the occlusion of the pseudoaneurysm. A new episode of hematemesis 3 wk later revealed a partial revascularization of the pseudoaneurysm. A definitive interventional radiological treatment consisting of transarterial embolization (TAE) of the right hepatic artery with stainless steel coils and polyvinyl alcohol particles was effective and well-tolerated with normal liver function tests and without signs of liver infarction.

Key words: Hepatic artery pseudoaneurysm; Hilar cholangiocarcinoma; Pseudoaneurysm embolization; Transarterial embolization; Chemoradiotherapy

Peer reviewer: Chao-Long Chen, Professor, Department of Surgery, Chang Gung Memorial Hospital, Kaohsiung Medical Centre, 123, Taipei Rd, Niao Sung Hsiang, Kaohsiung Hsien 83305, Taiwan, China

Briceño J, Naranjo Á, Ciria R, Sánchez-Hidalgo JM, Zurera L, López-Cillero P. Late hepatic artery pseudoaneurysm: A rare complication after resection of hilar cholangiocarcinoma. *World J Gastroenterol* 2008; 14(38): 5920-5923 Available from: URL: http://www.wjgnet.com/1007-9327/14/5920.asp DOI: http://dx.doi.org/10.3748/wjg.14.5920

INTRODUCTION

Bleeding from a pseudoaneurysm of major visceral arteries as a result of an adjacent septic condition or an intraoperative injury may lead to a troublesome emergency. Hepatic artery pseudoaneurysm can result in hemorrhage into the biliary tract when an abnormal communication is established between the vessel and the bile duct. Degeneration and weakness of hepatic arterial wall can be secondary to a trauma (accidental or iatrogenic) or an inflammation of the biliary tract [1,2]. A mechanical injury of the artery during operation, mainly due to lymph node dissection for malignancy, may be a clear predisposing factor for delayed arterial bleeding after hepatobiliary surgery.

We report an unusual pathological entity of a pseudoaneurysm of the right hepatic artery, which developed two years after the resection of a type Ⅱ hilar cholangiocarcinoma. This resulted in catastrophic upper gastrointestinal bleeding, which was ultimately treated by an aggressive combination of interventional radiological procedures.

CASE REPORT

A 78-year-old man with cholangiocarcinoma involving the proximal bile ducts (hilar cholangiocarcinoma or Klatskin tumor) was referred for surgical treatment in July 2005. Abdominal pain, discomfort, anorexia, weight loss, pruritus and jaundice with bilirubin of 18 mg/dL

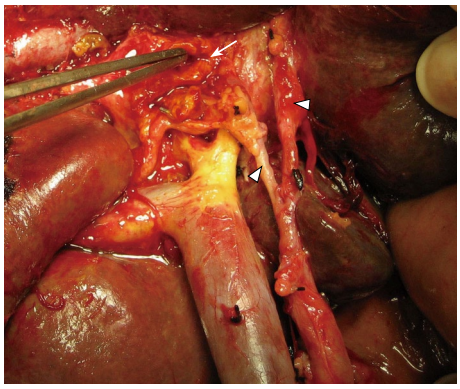


Figure 1 Skeletonization for regional lymphadenectomy with removal of neural and lymphoid tissues from hepatoduodenal ligament and liver hilus in a type Ⅱ hilar cholangiocarcinoma. A too-tight dissection around the right and left hepatic arteries (arrow heads) is shown. Left hepatic duct (arrow) is being prepared for bilio-enteric anastomosis.

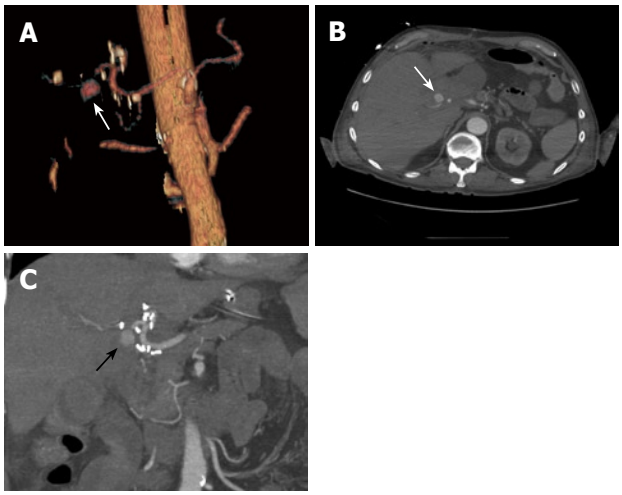


Figure 2 MDCTA. **A**: Arterial phase image precisely depicts a large pseudoaneurysm originating from the right hepatic artery (arrow). **B** and **C**: Jejunum Y-limb did not show a demonstrable communication with the arterial sac in cross-sectional and coronal views, respectively.

were the main symptoms. After a magnetic resonance imaging cholangiography, the patient was diagnosed as having a type Ⅱ cholangiocarcinoma (Bismuth-Corlette modified classification) without invasion of main portal vein, the proper hepatic artery or its branches[3] and a preoperative stage IB, according to AJCC staging system (T2N0M0). Percutaneous transhepatic biliary drainage and subsequent placement of a temporary endoprosthesis were performed prior to resection. A standard extrahepatic biliary tract excision associated with segment I resection was performed through an inverted T-incision. Skeletonization for regional lymphadenectomy with removal of neural and lymphoid tissues from hepatoduodenal ligament and liver hilus was also added (R0 resection) (Figure 1). Bilio-enteric continuity was restored by hepatojejunostomy to a Roux-en-Y loop of jejunum. Three duct orifices (right, left and segment I bile ducts) were anastomosed. The pathological result of the lymphadenectomy revealed metastases to two perihilar lymph nodes (N1). The postoperative stage was IIB (T2N1M0). The patient was discharged 11 d later without complications. Adjuvant therapy consisted of a gemcitabine-based regimen (1000 mg/m^2 as a 30-min infusion every 2 wk until achieving 6 treatment cycles) combined with conventional external-beam radiotherapy (5000 cGy given with an equivalent total dose in a 200 cGy/25 fractions). The time interval between operation and before starting chemo-radiotherapy was 6 wk.

In October 2007, the patient developed a transient minor gastrointestinal bleeding without hemodynamic repercussion and an upper digestive endoscopy disclosed no evidence of gastroduodenal bleeding. Ten days later, the patient was readmitted because of a sudden and massive hematemesis, causing hypovolemia and shock. After an initial resuscitation in ICU, hemodynamic stability was achieved. An upper digestive endoscopy only revealed an hemorrhage from the Y-limb jejunum. A multidetector computed tomographic angiography (MDCTA) was performed with a multidetector 16-row computed tomography scanner. Arterial phase images were obtained after intravenous injection of 150 mL of contrast material at a rate of 4 mL/s using the bolus triggering technique (Figure 2)[4]. This technique precisely detected a large pseudoaneurysm originating from the right hepatic artery. Angiography was carried out immediately with a 4 Fr. catheter. Contrast was seen jetting into the aneurysm, but no communication with the intestine could be proven. Embolization of the pseudoaneurysm was attempted using microcoils to stop the inflow into the pseudoaneurysmal lumen. Patency of the hepatic artery and its branches, and the occlusion of the pseudoaneurysm were confirmed (Figure 3). The patient was discharged in acceptable clinical condition with an uneventfully outcome.

Three weeks later, the patient suffered from a new episode of hematemesis, requiring readmission and supportive treatment. A new angiography displayed a partial revascularization of the pseudoaneurysm (Figure 4A). A definitive interventional radiological treatment was decided, consisting of transarterial embolization (TAE) of the right hepatic artery with stainless steel coils and polyvinyl alcohol particles (Figure 4B). Liver function tests were normal, with a transient elevation of transaminases. Embolization of the complete right hepatic artery was well-tolerated, without signs of liver infarction. The patient is in good clinical condition 5 mo after this last procedure.

DISCUSSION

Three major predisposing factors for delayed arterial bleeding after hepatobiliary surgery have been suggested[1]: (1) digestion of hepatic arterial wall due to infectious bile from anastomotic leakage; (2) arterial irritation by localized abscess in the inferior hepatic space; and (3) a mechanical injury of the artery during operation, mainly due to lymph node dissection for malignancy. The result is the formation of an arterial sac with a possible rupture into the biliary tract, and

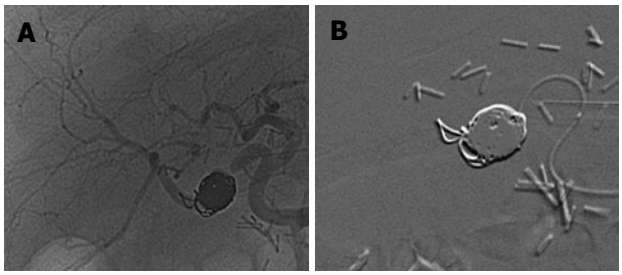


Figure 3 Patency of the hepatic artery and its branches, and the occlusion of the pseudoaneurysm. **A**: Embolization of the pseudoaneurysm using microcoils to stop the inflow into the pseudoaneurysmal lumen with patency of the hepatic artery and its branches. **B**: Subtracted angiogram after right hepatic artery pseudoaneurysm occlusion with stainless steel coils.

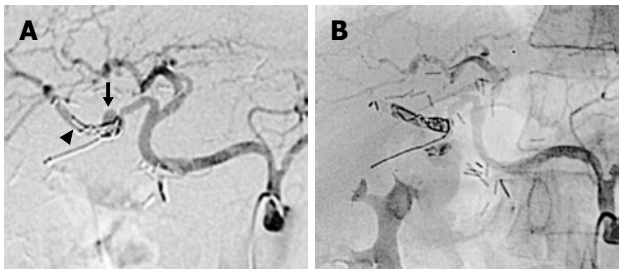


Figure 4 Partial revascularization of the pseudoaneurysm and TAE of the right hepatic artery. **A**: Angiography 3 wk after the embolization of the right hepatic artery pseudoaneurysm with a partial revascularization of the arterial sac (arrow) and a distal migration of coils (arrow head). A partial enteric migration of microcoils after the first and selective embolization suggests an arterio-enteric fistula origin. **B**: TAE of the right hepatic artery with stainless steel coils and polyvinyl alcohol particles. No distal arterial flow can be shown.

consequently, hemobilia. Surgery of the biliary tract may damage the hepatic artery by the dissection or suture, which may result in an arteriobiliary fistula or in a false aneurysm eroding into the extrahepatic bile ducts[5]. When a patient develops bleeding 1 or 2 wk after surgery for hilar cholangiocarcinoma, especially when the event is sudden in onset with hemobilia, arterial injury should be suspected. However, a late catastrophic bleeding requiring life-supporting treatment is unusual after surgery for Klatskin tumor. To our knowledge, there is only one case reported by Siablis *et al*[6] in which a hemobilia secondary to hepatic artery pseudoaneurysm after a resected IIIb Klatskin tumor appeared two years later due to a nearby biloma. In the absence of previous biliary leakage, we propose different explanations for the etiology of artery pseudoaneurysm in the present case report: (1) an excessive skeletonization for regional lymphadenectomy with removal of neural and lymphoid tissues; and (2) postoperative external-beam radiotherapy. Usually, the vasculature is injured by skeletonization for lymphadenectomy during radical surgery and too-tight ligation around the arterial conduit[7,8]. External radiotherapy may cause a degeneration of the arterial media. These two factors, alone or associated, can cause an iatrogenic trauma of the hepatic artery wall and, subsequently, the development of a false arterial sac. Another factor may be the short time interval between operation and before starting chemo-radiotherapy (6 wk), which probably contributed to the pre-existing vessel trauma and affecting optimal wound healing. However, the presence of positive lymph nodes suggested a more advanced disease than planned preoperatively and, consequently; chemo-radiotherapy was administered promptly after surgery in this patient.

MDCTA is a non-invasive modality to select patients who must be treated with angiographic intervention or surgery[4]. This technique is better than conventional contrasted-tomography and shows a good correlation with digital subtraction angiography. A sentinel bleeding as a precursor of vessel erosion after biliary surgery for hilar cholangiocarcinoma may lead to a prompt diagnosis preferentially with MDCTA. We cannot ascertain whether the GI bleeding was coming from an arterio-enteric or an arterio-bilial fistula. However, as shown in Figure 4A, a partial enteric migration of microcoils after the first and selective embolization suggests an arterio-enteric fistula origin.

In the setting of a prior complex bilio-enteric surgery, a new open surgical approach is not recommended because re-entering the previous scarred operative field for repair and hemostasis by suturing hepatic artery or placing a new conduit graft to restore circulation is too difficult and dangerous. Interventional radiology with selective embolization of pseudoanerysm is a safe and a definitive treatment in most of the cases. Angiographic embolization should be performed proximal and distal to the origin of the pseudoaneurysm rather than proceeding with embolization into the pseudoaneurysm cavity[7,9]. Newer technology detachable coils developed for neurovascular procedures may play a role in occluding pseudoaneurysms. These coils are placed into the pseudoaneurysm through microcatheters. Once they are placed in position adequately filling the space needed and stable, they can be released by a low voltage electric charge through the attachment wire. If the radiologist is not satisfied with coil placement or if the distal flow is compromised it can simply be withdrawn without detachment[10].

The best procedure after microcoil-embolization failure is not clear. Some authors suggest good results by placing covered grafts to secure hepatic arterial flow, guiding the blood flow into the proper direction out of the pseudoaneurysmal lumen, avoiding the possibility of interfering with arterial flow to the liver[11-15]. Nonetheless, the dual blood supply to the liver makes it extremely tolerant of arterial occlusion for haemostasis. Indeed, the compensatory arterial circulation to the liver after arterial ligation for the treatment of ruptured hepatic artery has been recognized by Michels *et al*[16]. At least 26 possible routes of collateral arterial blood supply to the liver from the common hepatic trunk were described. The main collateral pathways to the liver after TAE included the right inferior phrenic artery, the aberrant left hepatic artery and the intrahepatic communicating branches. The main vascular collateral pathways in the liver may be reliable in an "undisturbed or virgin liver". In post-resection, where the liver may have undergone extensive

mobilization, these collaterals may have been disrupted and may be unreliable. However, we performed the procedure without this extensive mobilization of the liver, maintaining the native ligaments of the liver and only mobilizing perihilar and hilar tissues. Phrenic arteries and intrahepatic communicating branches were well preserved. In any case, complete hepatic occlusion cannot be a standard approach, and it has only place as an extreme solution for an extremely urgent patient with selective embolization failure and/or a high-risky surgical intervention. In this setting, TAE on the hepatic artery has generally been considered to be relatively safe if the portal blood flow is preserved.

REFERENCES

1 **Okuno A**, Miyazaki M, Ito H, Ambiru S, Yoshidome H, Shimizu H, Nakagawa K, Shimizu Y, Nukui Y, Nakajima N. Nonsurgical management of ruptured pseudoaneurysm in patients with hepatobiliary pancreatic diseases. *Am J Gastroenterol* 2001; **96**: 1067-1071

2 **Green MH**, Duell RM, Johnson CD, Jamieson NV. Haemobilia. *Br J Surg* 2001; **88**: 773-786

3 **Bismuth H**, Nakache R, Diamond T. Management strategies in resection for hilar cholangiocarcinoma. *Ann Surg* 1992; **215**: 31-38

4 **Kayahan Ulu EM**, Coskun M, Ozbek O, Tutar NU, Ozturk A, Aytekin C, Haberal M. Accuracy of multidetector computed tomographic angiography for detecting hepatic artery complications after liver transplantation. *Transplant Proc* 2007; **39**: 3239-3244

5 **Kelley CJ**, Hemingway AP, Mcpherson GA, Allison DJ, Blumgart LH. Non-surgical management of post-cholecystectomy haemobilia. *Br J Surg* 1983; **70**: 502-504

6 **Siablis D**, Papathanassiou ZG, Karnabatidis D, Christeas N, Vagianos C. Hemobilia secondary to hepatic artery pseudoaneurysm: an unusual complication of bile leakage in a patient with a history of a resected IIIb Klatskin tumor. *World J Gastroenterol* 2005; **11**: 5229-5231

7 **Yamashita Y**, Taketomi A, Fukuzawa K, Tsujita E, Harimoto N, Kitagawa D, Kuroda Y, Kayashima H, Wakasugi K, Maehara Y. Risk factors for and management of delayed intraperitoneal hemorrhage after pancreatic and biliary surgery. *Am J Surg* 2007; **193**: 454-459

8 **Abbas MA**, Fowl RJ, Stone WM, Panneton JM, Oldenburg WA, Bower TC, Cherry KJ, Gloviczki P. Hepatic artery aneurysm: factors that predict complications. *J Vasc Surg* 2003; **38**: 41-45

9 **Reber PU**, Baer HU, Patel AG, Wildi S, Triller J, Büchler MW. Superselective microcoil embolization: treatment of choice in high-risk patients with extrahepatic pseudoaneurysms of the hepatic arteries. *J Am Coll Surg* 1998; **186**: 325-330

10 **Amesur NB**, Zajko AB. Interventional radiology in liver transplantation. *Liver Transpl* 2006; **12**: 330-351

11 **Ichihara T**, Sato T, Miyazawa H, Shibata S, Hashimoto M, Ishiyama K, Yamamoto Y. Stent placement is effective on both postoperative hepatic arterial pseudoaneurysm and subsequent portal vein stricture: a case report. *World J Gastroenterol* 2007; **13**: 970-972

12 **Maleux G**, Pirenne J, Aerts R, Nevens F. Case report: hepatic artery pseudoaneurysm after liver transplantation: definitive treatment with a stent-graft after failed coil embolisation. *Br J Radiol* 2005; **78**: 453-456

13 **Sachdev U**, Baril DT, Ellozy SH, Lookstein RA, Silverberg D, Jacobs TS, Carroccio A, Teodorescu VJ, Marin ML. Management of aneurysms involving branches of the celiac and superior mesenteric arteries: a comparison of surgical and endovascular therapy. *J Vasc Surg* 2006; **44**: 718-724

14 **Tulsyan N**, Kashyap VS, Greenberg RK, Sarac TP, Clair DG, Pierce G, Ouriel K. The endovascular management of visceral artery aneurysms and pseudoaneurysms. *J Vasc Surg* 2007; **45**: 276-283; discussion 283

15 **Rami P**, Williams D, Forauer A, Cwikiel W. Stent-graft treatment of patients with acute bleeding from hepatic artery branches. *Cardiovasc Intervent Radiol* 2005; **28**: 153-158

16 **Michels NA**. Collateral arterial pathways to the liver after ligation of the hepatic artery and removal of the celiac axis. *Cancer* 1953; **6**: 708-724

S- Editor Li DL **L- Editor** Ma JY **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5924

World J Gastroenterol 2008 October 14; 14(38): 5924-5926
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Pelvic sepsis after stapled hemorrhoidopexy

Remco JA van Wensen, Maarten H van Leuken, Koop Bosscha

Remco JA van Wensen, Koop Bosscha, Department of Surgery, Jeroen Bosch Hospital, Nieuwstraat 34, 's-Hertogenbosch 5211 NL, Netherlands
Maarten H van Leuken, Department of Radiology; Jeroen Bosch Hospital, Nieuwstraat 34, 's-Hertogenbosch 5211 NL, Netherlands
Author contributions: van Wensen RJA, van Leuken MH and Bosscha K contributed equally to this work; van Wensen RJA did the literature research and wrote the manuscript under the supervision of van Leuken MH and Bosscha K.
Correspondence to: Dr. Koop Bosscha, MD, PhD, Department of Surgery, Jeroen Bosch Hospital, location Groot Ziekengasthuis, Nieuwstraat 34, 's-Hertogenbosch 5211 NL, Netherlands. k.bosscha@jbz.nl
Telephone: +31-73-6992708 Fax: +31-73-6992163
Received: June 24, 2008 Revised: August 26, 2008
Accepted: September 3, 2008
Published online: October 14, 2008

Abstract

Stapled hemorrhoidopexy is a surgical procedure used worldwide for the treatment of grade Ⅲ and Ⅳ hemorrhoids in all age groups. However, life-threatening complications occur occasionally. The following case report describes the development of pelvic sepsis after stapled hemorrhoidopexy. A literature review of techniques used to manage major septic complications after stapled hemorrhoidopexy was performed. There is no standardized treatment currently available. Stapled hemorrhoidopexy is a safe, effective and time-efficient procedure in the hands of experienced colorectal surgeons.

Key words: Hemorrhoids; Hemorrhoids/treatment; Sepsis; Stapled hemorrhoidopexy; Circular mucosectomy

Peer reviewer: Avdyl S Krasniqi, Associate Professor, Department of Abdominal Surgery, University Clinical Centre of Kosova, "Uke Sadiku" 13A, QKUK - Klinika e Kirurgjise, Fakulteti i Mjekesise, Universiteti i Prishtines, Prishtine 10000, Yugoslavia

van Wensen RJA, van Leuken MH, Bosscha K. Pelvic sepsis after stapled hemorrhoidopexy. *World J Gastroenterol* 2008; 14(38): 5924-5926 Available from: URL: http://www.wjgnet.com/1007-9327/14/5924.asp DOI: http://dx.doi.org/10.3748/wjg.14.5924

INTRODUCTION

Stapled hemorrhoidopexy is used worldwide for the treatment of grade Ⅲ and Ⅳ hemorrhoids, because it is a safe, effective and time-efficient procedure for all age groups[1,2]. However, life-threatening complications occur occasionally[3,4]. We carried out a literature search to review the treatment options for managing major septic complications after stapled hemorrhoidopexy. Since there is no standardized treatment, we propose diagnostic and therapeutic guidelines for this complication.

CASE REPORT

A 26-year-old female patient was referred to our hospital with grade Ⅲ-Ⅳ hemorrhoids. She underwent a circular mucosectomy by a stapling device (PPH03, 33 mm, Ethicon EndoSurgery, Cincinnati, OH, USA). Clinical examination showed fully intact doughnuts. The patient recovered well, and left the hospital without any complaints.

After 4 d, she was readmitted with fever and pain over the lower abdomen, without any rebound tenderness. Laboratory tests showed leucocytes count of 16.9×10^9/L (reference 4.0-10 $\times 10^9$/L) and a C-reactive protein of 298 mg/L (reference CRP < 6 mg/L). Computer tomography (CT) scan with rectal contrast showed a presacral fluid collection with gas extending to the retroperitoneal space (Figures 1 and 2). A rectal perforation with a presacral abscess was diagnosed. Laparotomy showed the presence of hematomas in the sigmoid mesentery and in the retroperitoneum. A loop ileostomy was performed. Rectal examination showed a perforation at the right dorsolateral side of the rectum. A Foley catheter was inserted into the presacral space. Postoperatively, the patient received antibiotics, and she was discharged after 19 d.

DISCUSSION

During the last decade, stapled hemorrhoidopexy has become increasingly popular for the treatment of symptomatic grade Ⅲ and Ⅳ hemorrhoids. Stapled hemorrhoidopexy does not remove the hemorrhoids, but rather a strip of mucosa and submucosa at the top of the hemorrhoids is removed. An anastomosis of the proximal and distal mucosa and submucosa is made through the staple line, approximately 4 cm above the dentate line[4,5].

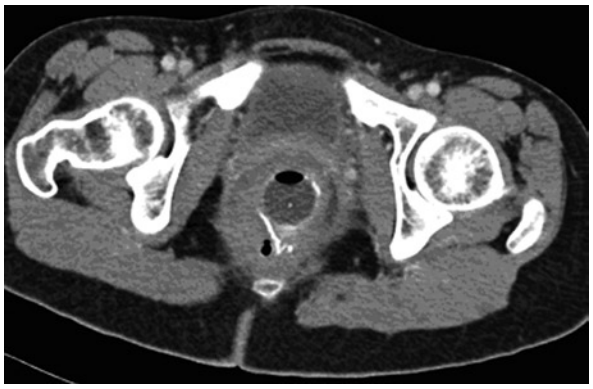


Figure 1 Axial image CT scan: Free air near the staple line.

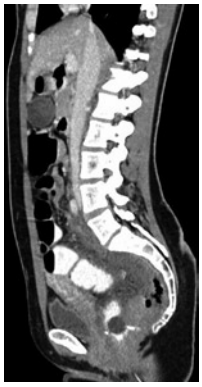


Figure 2 Sagital reconstruction CT scan with rectal contrast: presacral abscess.

Septic complications after stapled hemorrhoidopexy such as in the present case are rare. Brusciano *et al*[5] evaluated the causes of reintervention in patients with complicated or failed stapled hemorrhoidopexy. The reoperation rate was 5.1%. Anorectal sepsis was the indication for reintervention in 16.9% of patients, which is 0.9% of all patients, whereas retro-rectal hematoma occurred in 1.5% patients. An survey of 224 departments of surgery in Germany, involving a total of 4635 stapled hemorrhoidopexies, revealed three cases of rectal perforation, one case of a large retro-rectal hematoma and one patient with lethal sepsis from Fournier's gangrene[6]. The cause of severe sepsis and retroperitoneal sepsis associated with both surgical and non-surgical treatment of hemorrhoids remains uncertain. It has been postulated that stapling itself may allow bacterial entry into the perirectal region. Full-thickness wall stapling may also allow organism to reach the perirectal space. By contrast, the presence of muscle tissue in the excised specimens is not uncommon. Several studies have observed muscle tissue in the doughnuts in 100% of patients[7,8].

There is no standard treatment for sepsis after stapled hemorrhoidopexy, and several different approaches have been used. Molloy and Kingsmore[3] reported a case of retroperitoneal sepsis, which required exploratory laparotomy and an end colostomy. The presacral space was drained. The staple line appeared to be normal and no rectal perforation was identified. Maw *et al*[9] reported a case of retroperitoneal sepsis, which was treated with intravenous antibiotics alone, without any surgical intervention. The staple line appeared to be intact on rectal examination. Wong *et al*[10] described a patient who had an interrupted line and a rectal perforation just above the peritoneal reflection resulting in a fecal peritonitis. The patient was treated with an end colostomy. Pessaux *et al*[11] reported a patient with an incomplete staple line, resulting in pelvic sepsis, that was treated with debridement and a temporary end colostomy.

When the staple line is intact, a conservative approach appears to be sufficient. However, surgery is mandatory when a rectal tear is diagnosed or the staple line is not intact. In our opinion, the latter situation compares with rectal perforation. Several different treatment approaches have been employed for the management of rectal perforations. In a recent review, de Feiter *et al*[12] noted that surgery is not always indicated for rectal perforation occurring after a barium enema. Intramural or a small retroperitoneal perforation can be treated with conservative measures such as bowel rest, total parenteral feeding, intravenous fluids and broad-spectrum antibiotics. Surgical debridement is only required in patients not responding to conservative treatment, and in patients with intramural abscesses. Perirectal abscesses always require drainage. By contrast, conservative treatment has been used even for major rectal perforation occurring after barium enema. This approach can be employed if the perforation was restricted to the retroperitoneum, the patient's general condition was good, the bowel was clean and the rectal tear was minor[13].

Di Venere *et al*[14] have attempted to standardize the treatment of rectal perforation. These workers argued that intraperitoneal rectal perforation should be considered as colonic or sigmoid perforation. Such patients should undergo surgical treatment depending upon the size of the bowel injury, the duration of intestinal spillage, comorbid conditions, and the surgeon's experience. Hartmann procedure was considered as the most appropriate technique if the wound exceeded 50% of the bowel circumference, with extended bowel wall loss and severe peritoneal contamination. Smaller lesions with less peritoneal contamination were considered more suitable for primary closure of the rectal wound followed by a diverting colostomy. A loop colostomy was proposed as the only possible treatment in severely ill and older patients with extraperitoneal rectal perforation. In all other cases of extraperitoneal rectal perforations, these workers recommended primary closure of the rectal wound with a diverting colostomy.

We prefer not to perform a primary closure of such perforations. In patients with intraperitoneal perforation, we recommend resection of the injured rectum, with anastomosis, and with or without a diverting (loop) colostomy or ileostomy. If severe peritonitis is present, we employ the Hartmann procedure. In patients with extraperitoneal perforation, the treatment is based on the extent of the perforation. Antibiotics and bowel rest as sole therapy is used for small perforations. For large perforations and/or severe sepsis, a diverting (loop)

colostomy or ileostomy is performed. Closure of the rectal perforation is not an option, because adequate drainage is the treatment of choice for (presacral) abscesses.

In conclusion, stapled hemorrhoidopexy is a safe, effective and time-efficient procedure. However, life-threatening complications can occur. There is no standard treatment for the management of pelvic sepsis. We recommend that only experienced colorectal surgeons, who are familiar with the technique and its complications, should perform such procedures. Further studies are needed to investigate and classify the best treatment for pelvic sepsis after stapled hemorrhoidopexy.

REFERENCES

1 **Lan P**, Wu X, Zhou X, Wang J, Zhang L. The safety and efficacy of stapled hemorrhoidectomy in the treatment of hemorrhoids: a systematic review and meta-analysis of ten randomized control trials. *Int J Colorectal Dis* 2006; **21**: 172-178

2 **Johnson DB**, DiSiena MR, Fanelli RD. Circumferential mucosectomy with stapled proctopexy is a safe, effective outpatient alternative for the treatment of symptomatic prolapsing hemorrhoids in the elderly. *Surg Endosc* 2003; **17**: 1990-1995

3 **Molloy RG**, Kingsmore D. Life threatening pelvic sepsis after stapled haemorrhoidectomy. *Lancet* 2000; **355**: 810

4 **Stamos MJ**. Stapled hemorrhoidectomy. *J Gastrointest Surg* 2006; **10**: 627-628

5 **Brusciano L**, Ayabaca SM, Pescatori M, Accarpio GM, Dodi G, Cavallari F, Ravo B, Annibali R. Reinterventions after complicated or failed stapled hemorrhoidopexy. *Dis Colon Rectum* 2004; **47**: 1846-1851

6 **Herold A**, Kirsch JJ. Painafter stapled haemorrhoidectomy. *Lancet* 2000; **356**: 2187; author reply 2190

7 **Cheetham MJ**, Mortensen NJ, Nystrom PO, Kamm MA, Phillips RK. Persistent pain and faecal urgency after stapled haemorrhoidectomy. *Lancet* 2000; **356**: 730-733

8 **Fazio VW**. Early promise of stapling technique for haemorrhoidectomy. *Lancet* 2000; **355**: 768-769

9 **Maw A**, Eu KW, Seow-Choen F. Retroperitoneal sepsis complicating stapled hemorrhoidectomy: report of a case and review of the literature. *Dis Colon Rectum* 2002; **45**: 826-828

10 **Wong LY**, Jiang JK, Chang SC, Lin JK. Rectal perforation: a life-threatening complication of stapled hemorrhoidectomy: report of a case. *Dis Colon Rectum* 2003; **46**: 116-117

11 **Pessaux P**, Lermite E, Tuech JJ, Brehant O, Regenet N, Arnaud JP. Pelvic sepsis after stapled hemorrhoidectomy. *J Am Coll Surg* 2004; **199**: 824-825

12 **de Feiter PW**, Soeters PB, Dejong CH. Rectal perforations after barium enema: a review. *Dis Colon Rectum* 2006; **49**: 261-271

13 **Madhala O**, Greif F, Cohen M, Lelcuk S. Major rectal perforations caused by enema: is surgery mandatory? *Dig Surg* 1998; **15**: 270-272

14 **Di Venere B**, Testini M, Miniello S, Piccinni G, Lissidini G, Carbone F, Bonomo GM. Rectal perforations. Personal experience and literature review. *Minerva Chir* 2002; **57**: 357-362

S- Editor Li DL **L- Editor** Anand BS **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5927

World J Gastroenterol 2008 October 14; 14(38): 5927-5929
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Intraperitoneal dedifferentiated liposarcoma: A case report

Ali Karaman, Mehmet Eşref Kabalar, Önder Özcan, Timur Koca, Doğan Nasır Binici

Ali Karaman, Department of Medical Genetics, State Hospital, Erzurum 25240, Turkey
Mehmet Eşref Kabalar, Department of Pathology, State Hospital, Erzurum 25240, Turkey
Önder Özcan, Department of General Surgery, State Hospital, Erzurum 25240, Turkey
Timur Koca, Department of Radiation Oncology, State Hospital, Erzurum 25240, Turkey
Doğan Nasır Binici, Department of Internal Medicine, State Hospital, Erzurum 25240, Turkey
Author contributions: Karaman A performed the PCR analysis of adipose tumor samples and wrote the article; Kabalar ME analyzed the biopsy material; Binici DN performed the physical examination; Özcan Ö performed the surgical operation; Koca T performed the radiation therapy.
Correspondence to: Ali Karaman, MD, Erzurum State Hospital, (Erzurum Numune Hastanesi), Department of Medical Genetics, Erzurum 25240, Turkey. alikaramandr@hotmail.com
Telephone: +90-442-2321139 Fax: +90-442-2321390
Received: June 30, 2008 Revised: August 27, 2008
Accepted: September 3, 2008
Published online: October 14, 2008

Abstract

Dedifferentiated liposarcoma is a variant of liposarcoma with a more aggressive course. Mutations of the *p53* gene have been found in different types of soft tissue sarcoma. It is generally accepted that *p53* mutations in human malignant tumors are often related to a poor prognosis. In our case, analysis of *p53* gene mutation in tumor samples was performed. *p53* gene mutation was observed in dedifferentiated tumor tissue samples but not in well-differentiated tumor tissue samples. It has been reported that *p53* gene mutation occurs most commonly in the retroperitoneum and rarely in other anatomic locations. Herein we report a case of dedifferentiated liposarcoma located at intraperitoneum.

Key words: Dedifferentiated liposarcoma; *p53* gene; Mutation; Intraperitoneum

Peer reviewer: Alastair John Watson, Professor, The Henry Wellcome Laboratory, Nuffield Building, University of Liverpool, Crown St., Liverpool, L69 3GE, United Kingdom

Karaman A, Kabalar ME, Özcan Ö, Koca T, Binici DN. Intraperitoneal dedifferentiated liposarcoma: A case report. *World J Gastroenterol* 2008; 14(38): 5927-5929 Available from: URL: http://www.wjgnet.com/1007-9327/14/5927.asp
DOI: http://dx.doi.org/10.3748/wjg.14.5927

INTRODUCTION

Liposarcoma is one of the most frequent malignant soft tissue tumors and currently classified into five main subgroups: well-differentiated, myxoid, round cell, pleomorphic, and dedifferentiated[1]. It is generally accepted that *p53* mutations in human malignant tumors are often related to a poor prognosis[2,3]. Taubert *et al*[4] reported that patients with nonframeshift mutations have a rather poor prognosis. Moreover, *p53* overexpression alone is associated with a poor clinical outcome[5,6]. Mutations of the *p53* gene have been found in different types of soft tissue sarcoma[7]. Well-differentiated liposarcoma is the most common variant. Dedifferentiated liposarcoma, a variant of liposarcoma with a worse prognosis, has a less frequency and occurs most commonly in retroperitoneum[8,9].

CASE REPORT

A 62-year-old man presented with a 3-mo history of constant right flank discomfort, fatigue, recent weight loss, loss of appetite and abdominal fullness. Physical examination revealed an abdominal mass in the right lower quadrant. T1-weighted magnetic resonance image (MRI) of the abdomen demonstrated a high-intensity mass. The tumor, located at intraperitoneum, was in the inferio-lateral to the cecum (Figure 1). Laboratory tests including tumor markers showed no abnormality. Surgery was performed to expose the tumor through a right lower abdominal incision. Gross examination revealed a 10 cm yellow, fleshy mass. Pathological examination revealed a dedifferentiated liposarcoma (Figure 2). Immunolabeling for S-100 was positive, while CD117 (C-KIT) and CD34 were negative in the well-differentiated tumor tissue samples, thus ruling out a gastrointestinal stromal tumor. Based on the histology and immunoprofile, a diagnosis of dedifferentiated liposarcoma was established. Furthermore, we investigated the occurrence of *p53* gene mutations in the tumor samples.

DNA extraction

Tissue samples were incubated for 15 min at 65℃,

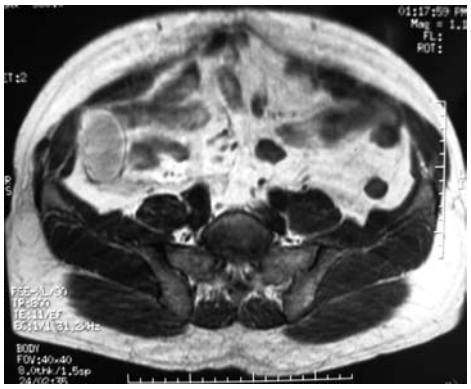


Figure 1 T1-weighted magnetic resonance image (MRI) demonstrating a high-intensity mass.

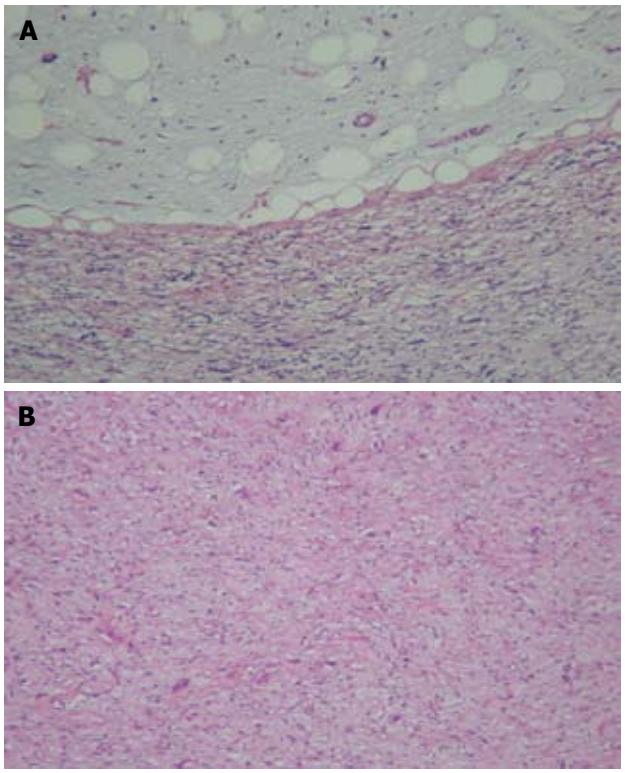


Figure 2 Well-differentiated tumor shows numerous lipoblasts and the dedifferentiated tumor resembles pleomorphic, spindle cells with hyperchromatic nuclei (HE, x 100) in the area revealing an abrupt transition from the well-differentiated to the dedifferentiated tumor (**A**), and dedifferentiated tumor shows low cellularity, fusiform cells with small hyperchromatic nuclei, and abundant collogen in the low grade fibrosarcoma area (**B**) (HE, x 100).

then overnight at 37℃ in a lytic solution containing 1 mg/mL proteinase K, 10 mmol/L Tris/HCl, 10 mmol/L ethylene diamine tetraacetic acid, 150 mmol/L NaCl, 0.4% sodium dodecyl sulfate. DNA was extracted twice with an equal volume of 1:1 SS-phenol/chloroform, and precipitated for 2 h at -20 ℃ after addition of 0.1 volume of 3 mol/L sodium acetate, 20 μg of glycogen as a carrier, and 2.5 volume of 100% ethanol. After centrifugation, the precipitate was washed with 2 mL of 80% ethanol, dried with a Speed Vac concentrator, and reconstituted with 50 μL of TE buffer.

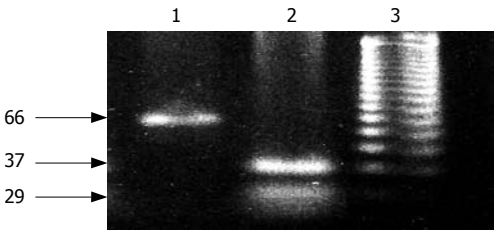


Figure 3 Amplification of the BstU1 within exon 4 produces a 66 bp long segment while cleavage results in 37 bp and 29 bp long fragments. Lane 1: No mutation of *p53* gene in well-differentiated tumor; lane 2: Mutation of *p53* gene in dedifferentiated tumor; lane 3: DNA marker.

PCR analysis

PCR was performed using a thermal cycle (PE 9700) with 50 ng of genomic DNA extracted from biopsy samples, 20 pmol of each primer, four deoxynucleotide triphosphates (dNTPs), reaction buffer and 1 μL (5 units) of fermentase Taq polymerase in a reaction volume of 100 μL. The PCR conditions consisted of an initial cycle at 95℃ for 10 min, at 57℃ for 1 min, and at 72℃ for 10 s followed by 34 cycles at 95℃ for 1 min, at 57℃ for 30 s, and at 72℃ for 10 s. The sequences of primer used are sense: 5'-CAGATGAAGCTCCCAGAA-3' (upstream) and anti-sense: 5'-GTGTAGGAGCTGCTGGTG-3' (downstream). An amplicon of 66 bp was produced, which was cleaved into 29 bp and 37 bp fragments with the enzyme BstU1, if its recognition site (CGCG) was present. The primer amplified a region containing codon 72 in exon 4 of *p53*[10,11]. Mutation of the *p53* gene was only observed in dedifferentiated tumor tissue samples but not in well-differentiated tumor tissue samples (Figure 3).

DISCUSSION

Mutation or allelic deletion of *p53* gene appears to play an important role in the development of human carcinoma[12]. It has been established that accumulation of wild type *p53* protein results in two pathways: cell cycle arrest and programmed cell death, both of which are involved in tumor suppressor functions[13]. Therefore, mutation of *p53* leads to disruption of these pathways, a selective growth advantage for tumor cells, and loss of function may increase proliferation activity and development of tumor[14].

Liposarcoma arising in extremities or retroperitoneum affects middle-aged and old patients, and tend to follow a relatively indolent clinical course with local recurrences after resection and occasional distant metastasis, mainly to the lungs[1]. Dedifferentiated liposarcoma is defined histologically by a transition from well-differentiated liposarcoma to a non-lipogenic sarcoma with variable histological grade[1]. The dedifferentiated tumor can resemble any sarcoma, but often mimics a malignant fibrous histiocytoma (MFH)[9].

Dedifferentiated liposarcoma, despite its high-grade histology, has a less aggressive clinical course than other

types of high grade sarcoma, although the underlying mechanism is unclear[1]. Compared to well-differentiated liposarcoma, dedifferentiated liposarcoma has similar genetic changes, ring or giant marker chromosomes, but a worse prognosis[1]. Approximately 40% of dedifferentiated liposarcomas will recur locally, 17% will metastasize, and 28% of the patients will ultimately die as a result of tumor[1].

It was reported that dedifferentiated liposarcoma occurs most commonly in retroperitoneum but rarely in other anatomic locations[9]. Five cases of dedifferentiated liposarcoma in small bowel mesentery have been described[15]. In addition, a case of dedifferentiated liposarcoma has been documented in the sigmoid mesocolon[16]. The present tumor, located at intraperitoneum.

Immunohistochemically, dedifferentiated liposarcoma is usually negative for CD117 and CD34 in dedifferentiated tissue and positive for S100 protein in well-differentiated tissue. Dedifferentiated liposarcoma needs to be distinguished from other high-grade sarcomas such as MFH because these high-grade sarcomas have a much worse prognosis[17].

We investigated the exon 4 of *p53* gene in adipose tissue tumor by PCR observed mutation of *p53* gene in dedifferentiated liposarcoma samples. Similarly, Taubert *et al*[4] detected mutations of *p53* gene in 5/32 of liposarcomas. It was reported that wild type p53 protein can induce cell apoptosis, whereas intracellular accumulation of mutant p53 protein can inhibit cell apoptosis, promote cell transformation and proliferation, resulting in carcinogenesis[5,14].

The patient underwent adjuvant radiation therapy and was asymptomatic during the 15-mo follow-up period. However, because the recurrence rate of dedifferentiated liposarcoma is very high, it is necessary to follow up carefully for a long term.

REFERENCES

1 **Fletcher CDM**, Unni KK, Mertens F. World Health Organization Classification of Tumors. Pathology and Genetics of Tumours of soft tissue and bone. Lyon: IARC Press, 2002; 40-44, 227-232

2 **Ichikawa A**, Kinoshita T, Watanabe T, Kato H, Nagai H, Tsushita K, Saito H, Hotta T. Mutations of the p53 gene as a prognostic factor in aggressive B-cell lymphoma. *N Engl J Med* 1997; **337**: 529-534

3 **de Anta JM**, Jassem E, Rosell R, Martínez-López E, Jassem J, Monzó M, Sánchez-Hernández JJ, Moreno I, Sánchez-Céspedes M. TP53 mutational pattern in Spanish and Polish non-small cell lung cancer patients: null mutations are associated with poor prognosis. *Oncogene* 1997; **15**: 2951-2958

4 **Taubert H**, Meye A, Wurl P. Prognosis is correlated with p53 mutation type for soft tissue sarcoma patients. *Cancer Res* 1996; **56**: 4134–4136

5 **Drobnjak M**, Latres E, Pollack D, Karpeh M, Dudas M, Woodruff JM, Brennan MF, Cordon-Cardo C. Prognostic implications of p53 nuclear overexpression and high proliferation index of Ki-67 in adult soft-tissue sarcomas. *J Natl Cancer Inst* 1994; **86**: 549-554

6 **Kawai A**, Noguchi M, Beppu Y, Yokoyama R, Mukai K, Hirohashi S, Inoue H, Fukuma H. Nuclear immunoreaction of p53 protein in soft tissue sarcomas. A possible prognostic factor. *Cancer* 1994; **73**: 2499-2505

7 **Diller L**, Sexsmith E, Gottlieb A, Li FP, Malkin D. Germline p53 mutations are frequently detected in young children with rhabdomyosarcoma. *J Clin Invest* 1995; **95**: 1606-1611

8 **Kindblom LG**, Meis-Kindblom JM, Enzinger FM. Variants of liposarcoma. *Am J Surg Pathol* 1995; **19**: 605-606; author reply 606-608

9 **Goldblum JR**, Weiss SW: Enznger and Weiss's Soft Tissue Tumors. 4th ed. St Louis: Mosby, 2001: 641-693

10 **Meltzer SJ**, Yin J, Huang Y, McDaniel TK, Newkirk C, Iseri O, Vogelstein B, Resau JH. Reduction to homozygosity involving p53 in esophageal cancers demonstrated by the polymerase chain reaction. *Proc Natl Acad Sci USA* 1991; **88**: 4976-4980

11 **Fujita M**, Inoue M, Tanizawa O, Iwamoto S, Enomoto T. Alterations of the p53 gene in human primary cervical carcinoma with and without human papillomavirus infection. *Cancer Res* 1992; **52**: 5323-5328

12 **Imazeki F**, Omata M, Nose H, Ohto M, Isono K. p53 gene mutations in gastric and esophageal cancers. *Gastroenterology* 1992; **103**: 892-896

13 **Janus F**, Albrechtsen N, Dornreiter I, Wiesmüller L, Grosse F, Deppert W. The dual role model for p53 in maintaining genomic integrity. *Cell Mol Life Sci* 1999; **55**: 12-27

14 **Zhou X**, Wang XW, Xu L, Hagiwara K, Nagashima M, Wolkowicz R, Zurer I, Rotter V, Harris CC. COOH-terminal domain of p53 modulates p53-mediated transcriptional transactivation, cell growth, and apoptosis. *Cancer Res* 1999; **59**: 843-848

15 **Hasegawa T**, Seki K, Hasegawa F, Matsuno Y, Shimodo T, Hirose T, Sano T, Hirohashi S. Dedifferentiated liposarcoma of retroperitoneum and mesentery: varied growth patterns and histological grades--a clinicopathologic study of 32 cases. *Hum Pathol* 2000; **31**: 717-727

16 **Winn B**, Gao J, Akbari H, Bhattacharya B. Dedifferentiated liposarcoma arising from the sigmoid mesocolon: a case report. *World J Gastroenterol* 2007; **13**: 4147-4148

17 **Henricks WH**, Chu YC, Goldblum JR, Weiss SW. Dedifferentiated liposarcoma: a clinicopathological analysis of 155 cases with a proposal for an expanded definition of dedifferentiation. *Am J Surg Pathol* 1997; **21**: 271-281

S- Editor Xiao LL **L- Editor** Wang XL **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5930

World J Gastroenterol 2008 October 14; 14(38): 5930-5932
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Symptomatic subserosal gastric lipoma successfully treated with enucleation

Avdyl Selmon Krasniqi, Faton Tatil Hoxha, Besnik Xhafer Bicaj, Shemsedin Isuf Hashani, Shpresa Mehmet Hasimja, Sadik Mal Kelmendi, Lumturije Hasan Gashi-Luci

Avdyl Selmon Krasniqi, Faton Tatil Hoxha, Besnik Xhafer Bicaj, Shemsedin Isuf Hashani, Shpresa Mehmet Hasimja, Sadik Mal Kelmendi, Lumturije Hasan Gashi-Luci, University Clinical Center of Kosova, Prishtina 10000, Kosovo
Author contributions: Krasniqi AS and Gashi-Luci LH designed the work; Krasniqi AS and Hoxha FT wrote the paper with contribution from Bicaj BX and Hashani SI; the other authors contributed equally to this work.
Correspondence to: Avdyl Selmon Krasniqi, MD, PhD, University Clinical Center of Kosova, "Uke Sadiku" 13A, Taslixhe Prishtina 10000, Kosovo. advilkrasniqi@gmail.com
Telephone: +377-44-507776 Fax: +381-38-557588
Received: March 23, 2008 Revised: July 19, 2008
Accepted: July 26, 2008
Published online: October 14, 2008

Abstract

Gastric lipomas are rare tumors, accounting for 2%-3% of all benign gastric tumors. They are of submucosal or extremely rare subserosal origin. Although most gastric lipomas are usually detected incidentally, they can cause abdominal pain, dyspeptic disorders, obstruction, invagination, and hemorrhages. Subserosal gastric lipomas are rarely symptomatic. There is no report on treatment of subserosal gastric lipomas in the English literature. We present a case of a 50-year-old male with symptomatic subserosal gastric lipoma which was successfully managed with removal, enucleation of lipoma, explorative gastrotomy and edge resection for histology check of gastric wall. The incidence of gastric lipoma, advanced diagnostic possibilities and their role in treatment modalities are discussed.

Key words: Stomach neoplasm; Subserosal gastric lipoma; Dyspeptic disorders; Surgical treatment/ enucleation

Peer reviewer: María IT López, Professor, Experimental Biology, University of Jaen, araje de las Lagunillas s/n, Jaén 23071, Spain

Krasniqi AS, Hoxha FT, Bicaj BX, Hashani SI, Hasimja SM, Kelmendi SM, Gashi-Luci LH. Symptomatic subserosal gastric lipoma successfully treated with enucleation. *World J Gastroenterol* 2008; 14(38): 5930-5932 Available from: URL: http://www.wjgnet.com/1007-9327/14/5930.asp DOI: http://dx.doi.org/10.3748/wjg.14.5930

INTRODUCTION

Gastrointestinal lipomas are uncommon, slowly growing benign tumors composed of mature adipose tissue that can occur anywhere along the gut. Mostly are located in the colon, ileum and jejunum[1]. Lipomas found in the stomach are even more unusual, accounting for 2%-3% of all benign gastric tumors[2,3]. They are of submucosal or extremely rare subserosal origin, most frequently localized to the antrum[4-7]. Although it is reported that 5%-10% of gastric lipomas are located at subserosal layer[1], so far, there is no report on treatment of subserosal gastric lipomas in the English medical literature. Karadeniz *et al*[8] in 2007 described an exceptional case of subserosal lipoma within an inverted Meckel's diverticulum. In the past, preoperative accurate diagnosis was very difficult even for symptomatic gastric lipomas[9]. The treatment of large symptomatic gastric lipomas has not yet been established[10]. Actual imaging technologies and endoscopy have become new approaches to the management of gastric lipomas[9-12]. We present a case of a large symptomatic subserosal gastric lipoma which was successfully managed with removal, enucleation of lipoma, explorative gastrotomy and edge resection for histology check of gastric wall.

CASE REPORT

A 50 year-old male patient was admitted to the Division of Abdominal Surgery with a 6-mo history of upper abdominal pain, dyspeptic disorders, vomiting mainly after the meal, and tachycardia, without significant weight loss. He was previously medically treated for bronchial asthma and chronic gastritis with pronison, aminophylline, spalmotil, omeprazol, and surgically underwent appendectomy, repair of limb fracture and nose deviation. He stopped smoking twelve years ago.

On admission, physical examination was unremarkable. Abdomen was soft and flat with mild tenderness in epigastrium and no palpable masses. Laboratory examinations including tumor markers, CEA, CA19-9 and AFP, showed no abnormalities. Barium meal study showed

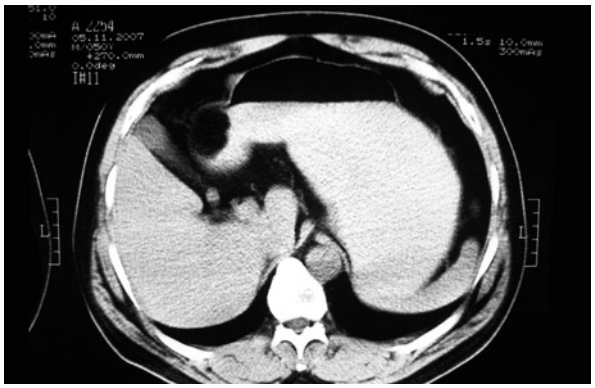


Figure 1 CT scan image showing a well-defined oval mass on the antro-pyloric part of stomach.

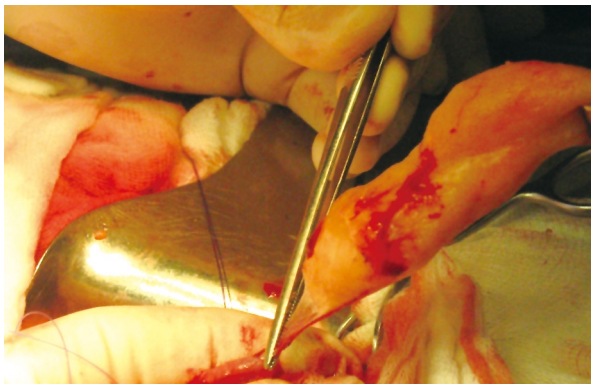


Figure 2 Intraoperative view showing a yellowish subserosal neoplasm on the anterior wall of gastric antrum during extirpation.

a normally shaped stomach without signs of filling defects and motility abnormalities, except for an axial hiatus hernia. Upper gastrointestinal endoscopy which was done two times, revealed a soft submucosal oval-shaped mass with an approximate diameter of 5 cm in gastric antrum. Overlying mucosa had a normal appearance with slight local hyperemia. Multiple gastric mucosa biopsies showed normal epithelium and rare stromal infiltrate with regular mucosa built up by regular tubular glands. Abdominal CT scan showed on the antro-pyloric part of the stomach a homogeneous well-defined oval mass around 6 cm in diameter with negative densitometry values (-50 and -60 Hounsfield units) that corresponds to fatty tissue (Figure 1). The patient underwent midline supraumbilical laparotomy. Intraoperatively, a yellowish, soft surface subserosal, well-defined tumor with a longitudinal diameter of around 5-6 cm, was found in the anterior wall of gastric antrum. The tumor was dissected and totally removed, enucleated, through an incision of gastric serosa without any technical difficulties (Figure 2). Explorative gastrotomy, endoluminal check of mucosa, and edge resection of underlying layers of gastric wall were performed. Gastrotomy was closed with PDS 3.0 using one layer Gambee sutures. Macroscopically, the specimen was capsulated by fatty content in a size of 5.5 cm. Histologicaly, the diagnosis of lipoma containing mature well-differentiated adipocytes (Figure 3), and no pathological lession in resected gastric edges were confirmed. Postoperative course was uneventful and the patient was discharged on the seventh postoperative day. Clinical and endoscopy follow-up 1 and 4 mo later showed that the tumor and clinical symptoms disappeared completely.

DISCUSSION

Lipomas of the stomach are very rare, accounting for less than 3% of all benign tumors of stomach[10,13]. Although generally single, they can be multiple as well[14]. Subserosal gastric lipomas are extremely rarely symptomatic. Probably because of this, there is no report on treatment of subserosal gastric lipomas in the English medical literature. In our case, subserosal lipoma localized in the antrum caused symptoms such as upper

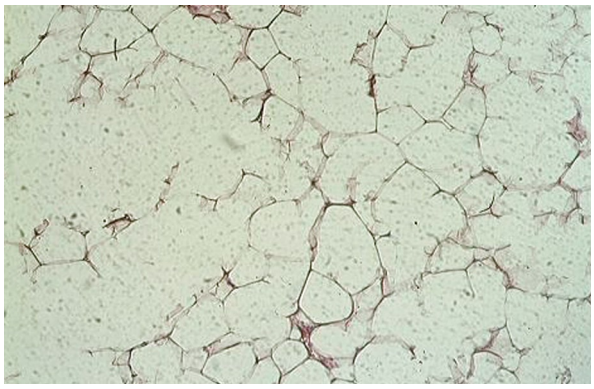


Figure 3 Histopathology confirming the diagnosis of lipoma containing mature fat cells with slight variation in size and shape (HE, x 100).

abdominal pain, dyspeptic disorders, and vomiting. This location is mainly responsible for dyspeptic and obstructive symptomatology[15,16]. The size of the lesion is correlated with the onset of abdominal disorders[7,10].

Diagnosis of gastric lipoma, in the past, before the era of modern diagnostic technology, was generally made after surgery[9,15]. Usually, on barium studies, extra mucosal tumors including lipomas reveal a smooth filling defect with a "bull's eye" appearance that is indistinguishable from other mesenchymal tumors[3]. Computerized tomography is a highly specific imaging diagnostic tool for lipoma[1,3,7,17]. In our case, barium studies did not show any filling defect, whereas abdominal CT scan showed a homogeneous well-defined oval mass with negative densitometry values of -50 and -60 Hounsfield units that corresponds to lipoma (Figure 1). However, CT scan is not accurate in determining the layer of location in the gastric wall. Although endoscopic sonography (EUS) provides more accurate findings of submucosal tumors regarding their shape, size and location inside gastric walls[18], because of limited resources in our hospital, this technique is not available. Upper GI tract endoscopy and biopsies were performed twice for our case. However, they played a complementary role in offering a description of a soft submucosal oval-shaped mass in gastric antrum, normal overlying mucosa and histopathology findings. A clear-cut endoscopic differentiation between gastric lipomas and other submucosal neoplasm is not

feasible, because routine endoscopic gastric biopsies do not reach the submucosal layer[16]. Furthermore, in the subserosal location, it was impossible to reach the neoplasm endoscopically. Despite the fact that CT scan is highly pathognomonic, there are reports suggesting the necessity of verifying specificity and sensibility of non-radiation based imaging systems, such as high resolution transabdominal ultrasound (TAUS)[19] and abdominal magnetic resonance imaging (MRI)[16].

The treatment modalities for gastric lipomas have been changed in parallel with the advances in endoscopic and imaging techniques. The choice of treatment for gastric lipomas is still controversial[16]. It was reported that different surgical and endoscopic procedures have been used in treatment of submucosal lipomas, but more accurate diagnosis preoperatively enable the replacement of previously used resection methods[6,9] by limited procedures such as tumor enucleation, partial resection or other endoscopic and minimally invasive procedures[4,7,10,11,20]. Although there are no malignant transformations of a gastric lipoma, coincidental separated malignant lesions have been reported[18]. As it was discussed by Yamamoto *et al*[18] in 2004, submucosal lipomas that have extended into the gastric lumen may provoke repeat erosions or local inflammation of gastric epithelium, which is thought to promote gastric cancer. Therefore, complete pretreatment diagnostic evaluation is needed. In our patient, preoperative evaluation did not find any morphologic and histological pathology in the gastric epithelium. Since his lipoma was symptomatic and its location in the gastric wall was not diagnosed preoperatively, the patient was treated with open laparotomy. Intraoperatively, a subserosal lipoma was found and enucleated through an incision of gastric serosa. To rule out the underlying concomitant pathology, explorative gastrotomy and edge resection of the gastric wall were carried out.

In summary, we report here a very rare case of subserosal symptomatic gastric lipoma successfully treated with enucleation. Subserosal gastric lipoma, although extremely rare, can be the cause of abdominal pain and dyspeptic disorders that need surgical treatment. Accurate diagnosis of gastric lipoma, which can be reached with a combination of endoscopic and imaging diagnostic techniques, is a very useful precondition in choosing the appropriate, less mutilating procedures of treatment.

REFERENCES

1 **Taylor AJ**, Stewart ET, Dodds WJ. Gastrointestinal lipomas: a radiologic and pathologic review. *AJR Am J Roentgenol* 1990; **155**: 1205-1210

2 **Fernandez MJ**, Davis RP, Nora PF. Gastrointestinal lipomas. *Arch Surg* 1983; **118**: 1081-1083

3 **Park SH**, Han JK, Kim TK, Lee JW, Kim SH, Kim YI, Choi BI, Yeon KM, Han MC. Unusual gastric tumors: radiologic-pathologic correlation. *Radiographics* 1999; **19**: 1435-1446

4 **Zak Y**, Biagini B, Moore H, Desantis M, Ghosh BC. Submucosal resection of giant gastric lipoma. *J Surg Oncol* 2006; **94**: 63-67

5 **Mah YH**, Huang SP, Chen JH, Wang HP, Lin JT. Gastric submucosal tumor removal. *Gastrointest Endosc* 2005; **61**: 290-291

6 **Treska V**, Pesek M, Kreuzberg B, Chudacek Z, Ludvikova M, Topolcan O. Gastric lipoma presenting as upper gastrointestinal obstruction. *J Gastroenterol* 1998; **33**: 716-719

7 **Singh R**, Bawa AS. Lipoma of the stomach. *Indian J Surg* 2004; **66**: 177-179

8 **Karadeniz Cakmak G**, Emre AU, Tascilar O, Bektas S, Ucan BH, Irkorucu O, Karakaya K, Ustundag Y, Comert M. Lipoma within inverted Meckel's diverticulum as a cause of recurrent partial intestinal obstruction and hemorrhage: a case report and review of literature. *World J Gastroenterol* 2007; **13**: 1141-1143

9 **Done HJ**. Lipoma of the stomach. *Br Med J* 1961; **1**: 183

10 **Fukuda S**, Yamagata R, Mikami T, Shimoyama T, Sawaya M, Uno Y, Tanaka M, Munakata A. Gastric lipoma successfully treated by endoscopic unfroofing. *Dig Endosc* 2003; **15**: 228-231

11 **Miki K**. Unroofing technique for the endoscopic resection of an asymptomatic, large gastric lipoma, a new approach to the endoscopic treatment. *Dig Endosc* 2003; **15**: 247-248

12 **Kang JY**, Chan-Wilde C, Wee A, Chew R, Ti TK. Role of computed tomography and endoscopy in the management of alimentary tract lipomas. *Gut* 1990; **31**: 550-553

13 **Chu AG**, Clifton JA. Gastric lipoma presenting as peptic ulcer: case report and review of the literature. *Am J Gastroenterol* 1983; **78**: 615-618

14 **Skinner MS**, Broadaway RK, Grossman P, Seckinger D. Multiple gastric lipomas. *Dig Dis Sci* 1983; **28**: 1147-1149

15 **Regge D**, Lo Bello G, Martincich L, Bianchi G, Cuomo G, Suriani R, Cavuoto F. A case of bleeding gastric lipoma: US, CT and MR findings. *Eur Radiol* 1999; **9**: 256-258

16 **Alberti D**, Grazioli L, Orizio P, Matricardi L, Dughi S, Gheza L, Falchetti D, Caccia G. Asymptomatic giant gastric lipoma: What to do? *Am J Gastroenterol* 1999; **94**: 3634-3637

17 **Thompson WM**, Kende AI, Levy AD. Imaging characteristics of gastric lipomas in 16 adult and pediatric patients. *AJR Am J Roentgenol* 2003; **181**: 981-985

18 **Yamamoto T**, Imakiire K, Hashiguchi S, Matsumoto J, Kadono J, Hamada N, Yoshioka T, Kitajima S. A rare case of gastric lipoma with early gastric cancer. *Intern Med* 2004; **43**: 1039-1041

19 **Tsai TL**, Changchien CS, Hu TH, Hsiaw CM. Demonstration of gastric submucosal lesions by high-resolution transabdominal sonography. *J Clin Ultrasound* 2000; **28**: 125-132

20 **Imasato M**, Yano H, Takiguchi S, Monden T. Gastric Lipoma successfully removed by laparoscopic intragastric surgery. *Dig Endosc* 2005; **17**: 269-272

S- Editor Zhong XY **L- Editor** Wang XL **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5933

World J Gastroenterol 2008 October 14; 14(38): 5933-5937
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Spontaneous necrosis of solid gallbladder adenocarcinoma accompanied with pancreaticobiliary maljunction

Tomohide Hori, Takashi Wagata, Kenji Takemoto, Takanobu Shigeta, Haruko Takuwa, Koichiro Hata, Shinji Uemoto, Naoki Yokoo

Tomohide Hori, Takashi Wagata, Kenji Takemoto, Takanobu Shigeta, Haruko Takuwa, Koichiro Hata, Naoki Yokoo, Department of Surgery, Takayama Red Cross Hospital, 3-11 Tenman-cho, Takayama City, Gifu Prefecture, 506-8550, Japan
Shinji Uemoto, Department of Hepato-pancreato-biliary and Transplant Surgery, Kyoto University Hospital, 54 Shogoinkawara-cho, Sakyo-ku, Kyoto, 606-8507, Japan
Author contributions: Hori T performed the laparoscopic and radical surgery, and wrote the paper; Wagata T, Takemoto K, Shigeta T, Takuwa H, Yokoo N were surgical assistants of the operations; Wagata T and Yokoo N provided the intra-operative suggestive advices for surgical treatment; Uemoto S and Hata K provided the meaningful technical advices for this case.
Correspondence to: Tomohide Hori, Department of Hepato-pancreato-biliary and Transplant Surgery, Kyoto University Hospital, 54 Shogoinkawara-cho, Sakyo-ku, Kyoto, 606-8507, Japan. horit@kuhp.kyoto-u.ac.jp
Telephone: +81-75-7513651 Fax: +81-75-7513106
Received: July 8, 2008 Revised: August 26, 2008
Accepted: September 3, 2008
Published online: October 14, 2008

Abstract

A 71-year-old Japanese man with acute cholecystitis and an incarcerated gallbladder (GB) stone was admitted. Plain ultrasonography (US) incidentally detected a mass-like lesion in the fundus. Doppler US revealed that this elevated lesion had no blood flow. Computed tomography showed a relatively low-density mass, measuring 5 cm x 4 cm in diameter, with no positive enhancement. Magnetic resonance imaging showed a mass in the fundus with a slightly low intensity on T1-weighted images and a slightly high intensity on T2-weighted images. We were agonized in making the qualitative diagnosis of mass-like lesions of the fundus, such as a benign tumor, cancer, or debris. We performed laparoscopic cholecystectomy, because the incarcerated GB stone clearly caused acute cholecystitis. Intra-operative cholangiography clearly revealed pancreaticobiliary maljunction. Amylase levels in the common bile duct and gallbladder were quite high. The elevated lesion in the fundus clearly showed severe necrosis. Although this necrotic nodule included non-viable adenocarcinoma cells, viable cancer cell nests were located in the muscularis propria and subcutaneous layer. Histopathological examination confirmed a solid adenocarcinoma. Thus, we diagnosed it as a gallbladder cancer, based on histopathological analysis of the resected specimen. We therefore undertook radical surgery, including wedge resection of the liver, radical dissection of regional lymph nodes, and resection of the extrahepatic bile duct. Histopathological findings revealed no cancer, hyperplasia or dysplasia in the additionally resected specimens. The patient was finally staged as T2, N0, H0, P0, M(-), stage Ⅱ. We present the first case of spontaneous necrosis of solid gallbladder adenocarcinoma, with a review of previous studies.

Key words: Gallbladder carcinoma; Pancreaticobiliary maljunction; Spontaneous necrosis

Peer reviewer: Fanyin Meng, MD, PhD, Assistant Professor, Department of Internal Medicine, Ohio State University, Room 514A Medical Research Facility, 420 West 12th Avenue, Columbus, Ohio 43210, United States

Hori T, Wagata T, Takemoto K, Shigeta T, Takuwa H, Hata K, Uemoto S, Yokoo N. Spontaneous necrosis of solid gallbladder adenocarcinoma accompanied with pancreaticobiliary maljunction. *World J Gastroenterol* 2008; 14(38): 5933-5937 Available from: URL: http://www.wjgnet.com/1007-9327/14/5933.asp DOI: http://dx.doi.org/10.3748/wjg.14.5933

INTRODUCTION

Traditionally, gallbladder (GB) cancer had a poor prognosis because of the lack of recognized symptoms at early stages. Early-stage GB cancer is often detected in resected specimens after cholecystectomy for benign diseases[1,2]. Moreover, GB cancer is sometimes detected in association with pancreaticobiliary maljunction (PBM), which is the union of the pancreatic and biliary ducts at a site outside the duodenal wall[3].

Some previous studies have described spontaneous necrosis of parathyroid and adrenal gland tumors[4-7]. However, spontaneous necrosis of GB tumors is quite rare. Only one case of spontaneous necrosis of papillary GB adenocarcinoma has been reported[8]. We present

here the first case of spontaneous necrosis of solid GB carcinoma accompanied with PBM, and the etiology of spontaneous necrosis was discussed with a review of previous studies. In the present case, histopathological findings were described, based on the general rules for surgical and pathological studies of cancer of the biliary tract of the Japanese Society of Biliary Surgery[9], according to the TNM classification[10].

CASE REPORT

Clinical course after admission

A 71-year-old Japanese man was referred to Takayama Red Cross Hospital (Gifu, Japan) due to severe right upper quadrant pain and frequent episodes of spiking fever and shivering chills. He had a history of hypertension and atherosclerosis. Ultrasonography (US) showed acute cholecystitis due to an incarcerated GB stone. He underwent drug treatment including sulbactam/cefoperazone (SBT/CPZ) in the first instance, and his symptoms were well controlled, without percutaneous transhepatic bile drainage. Although serum biochemistry showed elevated levels of aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, γ-glutamyltransferase, alkaline phosphatase and C-reactive protein at admission, peripheral blood examination was normal after administration of SBT/CPZ. Thereafter, we performed detailed investigations.

Image studies and tumor markers

Plain US of the GB showed wall thickening of the neck and body (consistent with cholecystitis), a strong echo with acoustic shadow at the neck (consistent with GB stones), and a mass-like lesion of the fundus (Figure 1A). In addition, this mass-like lesion moved according to the patient's position. Doppler US revealed that the elevated lesion of the fundus had no feeding arteries and drainage veins (Figure 1B). Plain computed tomography (CT) showed a relatively low-density mass in the fundus, measuring 5 cm × 4 cm in diameter (Figure 2A). Contrast-enhanced CT revealed that this mass-like lesion had no positive enhancement in the early (Figure 2B) or late (Figure 2C) phase. Magnetic resonance imaging (MRI) showed a mass in the fundus with a slightly low intensity on T1-weighted images (Figure 2D) and a slightly high intensity on T2-weighted images (Figure 2E). Magnetic resonance cholangiography (MRC) showed a round defect due to an incarcerated stone in the neck and irregular defects due to the elevated lesion in the fundus (Figure 3A). Serum carbohydrate antigen (CA) 19-9 level was elevated, although other tumor markers including carcinoembryonic antigen, alpha-fetoprotein, squamous cell carcinoma antigen, CA125 and CA15-3 were all normal. Upper gastrointestinal and colorectal endoscopy did not reveal any abnormal findings. Positron emission tomography with fluorine-18-labeled fluoro-deoxyglucose (FDG-PET) showed no FDG uptake in the GB.

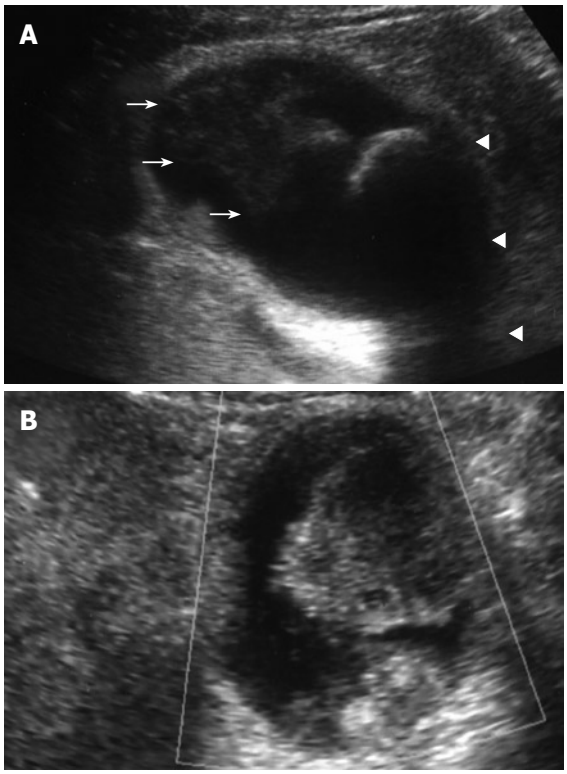


Figure 1 Plain US. (**A**) of the GB showing wall thickening of the neck and body (consistent with cholecystitis), a strong echoic level with acoustic shadow at the neck (consistent with stones), and a mass-like lesion of the fundus, and Doppler US (**B**) revealing no blood flow in the mass-like lesion.

Preoperative diagnosis and surgical treatment

We were agonized in making the qualitative diagnosis of mass-like lesions of the fundus, such as benign tumor, cancer, debris or bile sludge. However, the incarcerated GB stone clearly caused acute cholecystitis. We performed elective surgery (three-port laparoscopic cholecystectomy), with informed consent required for an additional surgery based on histopathological diagnosis. Intraoperative cholangiography clearly revealed PBM (Figure 3B). In addition, amylase levels in the common bile duct (CBD) (bile obtained during cannulation of the CBD) and GB (bile obtained from the resected specimen) were quite high (82 752 IU/L and 69 442 IU/L, respectively). Postoperative course was uneventful, and CA19-9 levels normalized immediately after surgery.

Histopathological diagnosis

Microscopic examination revealed chronic cholecystitis with infiltration of inflammatory cells. In addition, histopathological examinations showed no necrotic changes in walls of the GB neck and body and gangrenous cholecystitis. Moreover, the elevated lesion of the fundus obviously developed necrosis (Figure 4A). This necrotic nodule included non-viable adenocarcinoma cells and remnants of glandular structures and vessel fragments (Figure 4B). Viable cancer cell nests were located in the muscularis propria and the subcutaneous layer beneath the necrotic nodule (Figure 4C). Histopathological examination confirmed a T2 solid

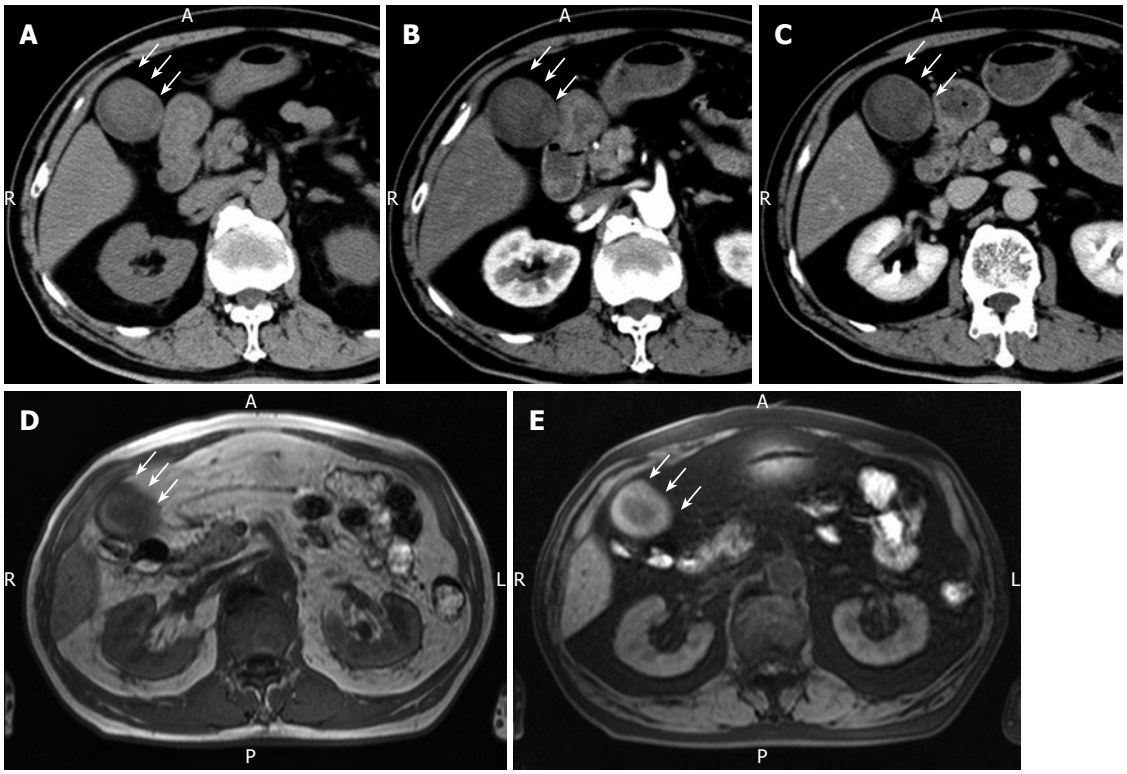


Figure 2 Plain CT. (**A**) showing a relatively low-density mass of the fundus measuring 5 cm x 4 cm in diameter, contrast-enhanced CT revealing no positive enhancement of this mass-like lesion in its early (**B**) and late (**C**) phases, MRI showing a mass in the fundus with a slightly low intensity on T1-weighted images (**D**) and a slightly high intensity on T2-weighted images (**E**). White arrow represents the mass-like lesion of the fundus.

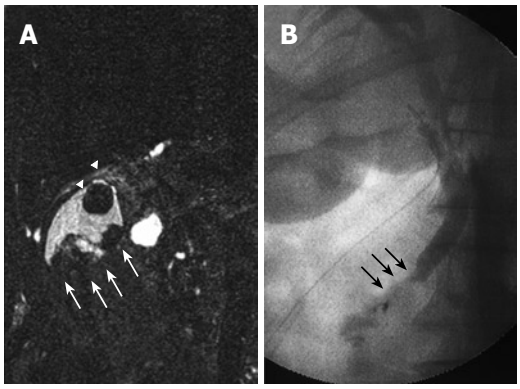


Figure 3 MRC. (**A**) showing irregular defects due to the elevated lesion of the fundus (white arrow) and a round defect due to stone incarceration in the neck (white arrow head), intra-operative cholangiography (**B**) revealing PBM and a 15 mm long common channel (black arrow).

adenocarcinoma [solid adenocarcinoma, ss, INFα, ly0, v0, pn0, hinf0, binf0, pvx, ax, St (+)] (Figure 4D). Regional lymph nodes (LNs) of the cystic duct (LN #12c) were noted without macroscopic metastasis. Thus, we diagnosed it as a GB cancer, based on histopathological analysis of the resected specimen.

Additional radical operation with radical dissection of regional LNs

The viable cells invaded perimuscular connective tissue, but did not penetrate the serosa. We were concerned about the safety margin, regional LN metastasis, and malignant potential of the CBD due to PBM. We therefore performed radical surgery, including wedge resection of the liver, including the GB bed to achieve negative margins, and radical dissection of regional LNs, including para-aortic LNs. Intraoperative histopathological examination of the induration of the mesocolon and peritoneum revealed no cancer dissemination. Furthermore, resection of the bile duct, including intrapancreatic and hilar bile ducts, was carried out. The intrahepatic bile ducts were reconstructed using hepaticojejunostomy in an end-to-side fashion. In addition, we resected the right hepatic artery by *en-bloc* resection with a safety margin, because an elastic, hard induration of the Calot's triangle involved the right hepatic artery showing back flow from the remnant liver, and we did not reconstruct the hepatic artery. Histopathological findings and imaging studies finally staged the patient as T2, N0, H0, P0, M(-), stage Ⅱ. No cancer, hyperplasia or dysplasia was found in the additionally resected specimens, and no regional LNs revealed metastasis (LNs #8a, 8p, 12a, 12b, 12p, 13a, 16a2, 16b1). In addition, no cancer or any precancerous lesions were seen in the extrahepatic bile duct microscopically. Until postoperative day 18, he had a good postoperative course except for gastric ulcer and pneumonitis. However, celiac artery rupture occurred suddenly on postoperative day 19, and he finally died of disseminated intravascular coagulation on postoperative day 20, despite intensive treatments.

DISCUSSION

Occult GB cancer has been incidentally found after

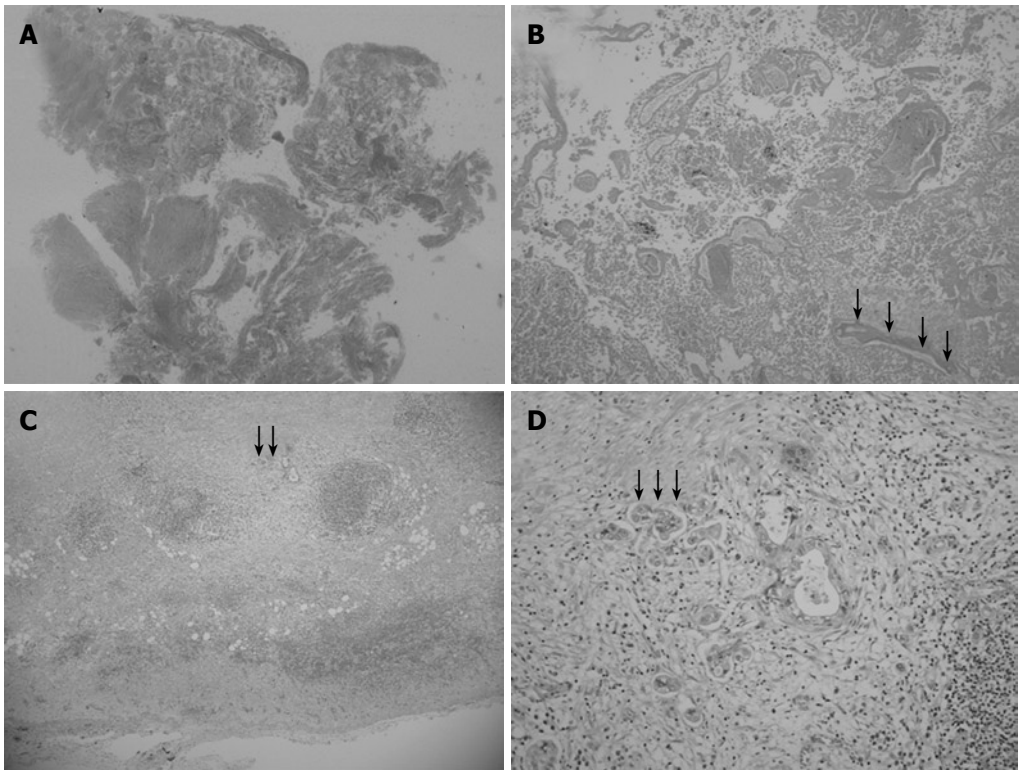


Figure 4 Low magnification view of the elevated lesion of the fundus showing obvious nodule necrosis. (**A**, HE, loupe) including non-viable adenocarcinoma cells and remnants of glandular structures and vessel fragments (**B**, black arrow) (HE, × 40), viable cancer cell nests invading perimuscular connective tissue but not penetrating the serosa in the muscularis propria and subcutaneous layer beneath the necrotic nodule (**C**, black arrow) (HE, × 20), and a solid adenocarcinoma (**D**, black arrow) (HE, × 100).

cholecystectomy (approximate 1%)[1,2]. However, we sometimes have difficulty in making a precise diagnosis of necrotic tumor based on imaging studies[11,12]. In this case, we did not make the qualitative diagnosis before laparoscopic cholecystectomy, although we did not deny the possibility of the existence of cancer. Since cholecystectomy for occult GB cancer may increase the risk of intraperitoneal dissemination and port recurrence[13,14], further precise diagnosis is required before surgery. In this case, we were in a dilemma as to whether we should perform radical surgery in a suspicious case with a risk of postoperative mortality and morbidity. Regional LNs of the cystic duct (LN #12c) are sometimes obtained from resected specimens after cholecystectomy. It was reported that LN #12c may be predictive of metastasis from occult GB cancer after cholecystectomy[15]. In the present case, LNs had no metastasis and the additional resected specimens showed no metastasis microscopically, indicating that this case supports the importance of LN #12c as an indication for additional surgery in cases of occult GB cancer after cholecystectomy. Even if radical resection for inapparent carcinoma after cholecystectomy is warranted, based on the excellent prognosis after additional radical surgery[1,2,13,15,16], it is still controversial about the optimal radical surgery for T2 GB cancer of the fundus[1,2,13-17]. Some investigators have recommended several surgical methods for T2 GB cancer, such as extended right hepatectomy, segmental resection of 4a/5 or 4b/5, and radical cholecystectomy with a 2-cm margin of liver around the GB bed[17-21]. Especially in cases of T2 carcinoma of the fundus after cholecystectomy, we still have some concerns about the determination of optimal surgical treatment. The establishment of optimal surgical treatment for occult T2 cancer after cholecystectomy is necessary.

Sakurai *et al*[8] reported a rare case of spontaneous necrosis of GB papillary adenocarcinoma of the fundus with PBM and suggested that the causes of spontaneous necrosis are disturbance of blood flow, gangrenous cholecystitis, necrotic debris, initial wall thickening due to cancer itself, PBM and increased intraluminal pressure in the GB. We could not clearly explain the cause of spontaneous necrosis, even after reviewing the literature. Although some of our findings are consistent with previously reported findings, such as cancer in the fundus with cholecystitis and PBM[8], some factors such as initial wall thickening due to cancer itself, intravascular thrombosis, gangrenous cholecystitis and necrotic debris were not seen in our case. We speculate that the cause of spontaneous necrosis of GB cancer is the increased intraluminal pressure in GB due to stone incarceration or the activated pancreatic enzymes consequently stimulating mutagenicity due to PBM[22,23]. In particular, our case clearly demonstrated a significantly elevated level of amylase in GB, suggesting that PBM participates in the process of necrosis. The case presented may show that overlapping of increased intraluminal pressure and activated pancreatic enzyme causes spontaneous necrosis.

Bile duct carcinoma including GB cancer is one of the malignant gastroenterological tumors which are most difficult to be cured and curative resection is essential for the long-term survival of such patients. In the surgical treatment for these cancers, systematic extended lymph node dissection is frequently required for curative resection. However, even with margin-negative resection, its prognosis after curative resection remains poor. The possible reason for the poor outcome is existence of occult lymph node metastasis that cannot be detected by conventional hematoxylin and eosin staining at surgical resection[24,25]. Recently, immunohistochemical and molecular techniques have made it possible to identify lymph node micrometastasis missed by traditional methods, and Taniguchi *et al*[24,25] clearly demonstrated that occult lymph node metastasis is crucial for the prognosis of bile duct carcinomas including GB cancer. At present, we are still troubled about the additional treatment for occult T2 GB carcinoma after cholecystectomy. For the time being, we will recommend the radical surgery including systematic lymph node dissection for this cancer, because of the excellent prognosis after additional radical surgery[1,2,13,15,16] and the possibility of lymph node micrometastasis[24,25].

This is the first case of spontaneous necrosis of solid GB adenocarcinoma. As GB carcinoma is rarely accompanied with spontaneous necrosis, more cases should be studied in order to analyze the mechanism of spontaneous necrosis, to make a precise preoperative diagnosis, and to perform optimal radical surgery based on the adequate indication.

ACKNOWLEDGMENTS

The authors are grateful to Kenichi Nonaka (Department of Oncologic Surgery, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu City, Gifu Prefecture, 501-1194, Japan) for his expert assistance.

REFERENCES

1 **Fong Y**. Aggressive therapy is warranted for gallbladder cancer. *Cancer Invest* 1998; **16**: 64-65
2 **Fong Y**, Heffernan N, Blumgart LH. Gallbladder carcinoma discovered during laparoscopic cholecystectomy: aggressive reresection is beneficial. *Cancer* 1998; **83**: 423-427
3 **Suda K**, Miyano T, Konuma I, Matsumoto M. An abnormal pancreatico-choledocho-ductal junction in cases of biliary tract carcinoma. *Cancer* 1983; **52**: 2086-2088
4 **Chan WB**, Chow CC, King AD, Yeung VT, Li JK, So WY, Cockram CS. Spontaneous necrosis of parathyroid adenoma: biochemical and imaging follow-up for two years. *Postgrad Med J* 2000; **76**: 96-98
5 **Le Nestour E**, Abecassis JP, Bertagna X, Bonnin A, Luton JP. Silent necrosis of a pituitary corticotroph adenoma revealed by timely magnetic resonance imaging: a cause of spontaneous remission of Cushing's disease. *Eur J Endocrinol* 1994; **130**: 469-471
6 **Vasatko T**, Cap J, Ryska A, Kohout A. [Spontaneous necrosis of parathyroid adenoma] *Vnitr Lek* 2004; **50**: 781-785
7 **Howard JE**, Follis RH Jr, Yendt ER, Connor TB. Hyperparathyroidism; case report illustrating spontaneous remission due to necrosis of adenoma, and a study of the incidence of necroses in parathyroid adenomas. *J Clin Endocrinol Metab* 1953; **13**: 997-1008
8 **Sakurai Y**, Shoji M, Matsubara T, Suganuma M, Hasegawa S, Imazu H, Ochiai M, Funabiki T, Urano M, Mizoguchi Y, Kuroda M. Spontaneous necrosis of gallbladder carcinoma in patient with pancreaticobiliary maljunction. *J Hepatobiliary Pancreat Surg* 2001; **8**: 95-100
9 **Japanese Society of Biliary Surgery**. General rules for surgical and pathological studies on cancer of biliary tract. 5th ed. Tokyo: Kanehara Pub, 2003
10 **Sobin LH**, Wittekind Ch. TNM classification of marlignant tumours. 5th ed. New York: Wiley-Liss, 1997
11 **Grazi GL**, Mazziotti A, Gruttadauria S, Jovine E, Principe A, Cavallari A. Solitary necrotic nodules of the liver. *Am Surg* 1998; **64**: 764-767
12 **Zoeller G**, Pekrun A, Lakomek M, Ringert RH. Staging problems in the pre-operative chemotherapy of Wilms' tumour. *Br J Urol* 1995; **76**: 501-503
13 **Wakai T**, Shirai Y, Hatakeyama K. Radical second resection provides survival benefit for patients with T2 gallbladder carcinoma first discovered after laparoscopic cholecystectomy. *World J Surg* 2002; **26**: 867-871
14 **Shirai Y**, Ohtani T, Hatakeyama K. Tumor dissemination during laparoscopic cholecystectomy for gallbladder carcinoma. *Surg Endosc* 1997; **11**: 1224-1225
15 **Shirai Y**, Yoshida K, Tsukada K, Muto T. Inapparent carcinoma of the gallbladder. An appraisal of a radical second operation after simple cholecystectomy. *Ann Surg* 1992; **215**: 326-331
16 **Wakai T**, Shirai Y, Yokoyama N, Ajioka Y, Watanabe H, Hatakeyama K. Depth of subserosal invasion predicts long-term survival after resection in patients with T2 gallbladder carcinoma. *Ann Surg Oncol* 2003; **10**: 447-454
17 **Reddy SK**, Marroquin CE, Kuo PC, Pappas TN, Clary BM. Extended hepatic resection for gallbladder cancer. *Am J Surg* 2007; **194**: 355-361
18 **Ohtsuka M**, Miyazaki M, Itoh H, Nakagawa K, Ambiru S, Shimizu H, Nakajima N, Akikusa B, Kondo Y. Routes of hepatic metastasis of gallbladder carcinoma. *Am J Clin Pathol* 1998; **109**: 62-68
19 **Suzuki M**, Yamamoto K, Unno M, Katayose Y, Endo K, Oikawa M, Matsuno S. Detection of perfusion areas of the gallbladder vein on computed tomography during arterial portography (CTAP)--the background for dual S4a. S5 hepatic subsegmentectomy in advanced gallbladder carcinoma. *Hepatogastroenterology* 2000; **47**: 631-635
20 **Yoshimitsu K**, Honda H, Kuroiwa T, Irie H, Aibe H, Tajima T, Chijiiwa K, Shimada M, Masuda K. Liver metastasis from gallbladder carcinoma: anatomic correlation with cholecystic venous drainage demonstrated by helical computed tomography during injection of contrast medium in the cholecystic artery. *Cancer* 2001; **92**: 340-348
21 **Mekeel KL**, Hemming AW. Surgical management of gallbladder carcinoma: a review. *J Gastrointest Surg* 2007; **11**: 1188-1193
22 **Komi N**, Tamura T, Miyoshi Y, Kunitomo K, Udaka H, Takehara H. Nationwide survey of cases of choledochal cyst. Analysis of coexistent anomalies, complications and surgical treatment in 645 cases. *Surg Gastroenterol* 1984; **3**: 69-73
23 **Matsubara T**, Tsuji T, Miyama A, Yamaguchi H, Funabiki T. Mutagenicity of bile and pancreatic juice from patients with pancreatico-biliary maljunction. *Hepatogastroenterology* 1995; **42**: 113-116
24 **Taniguchi K**, Iida T, Hori T, Yagi S, Imai H, Shiraishi T, Uemoto S. Impact of lymph node micrometastasis in hilar bile duct carcinoma patients. *World J Gastroenterol* 2006; **12**: 2549-2555
25 **Taniguchi K**, Tabata M, Iida T, Hori T, Yagi S, Uemoto S. Significance of lymph node micrometastasis in pN0 hilar bile duct carcinoma. *Eur J Surg Oncol* 2006; **32**: 208-212

S- Editor Xiao LL **L- Editor** Wang XL **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5938

World J Gastroenterol 2008 October 14; 14(38): 5938-5939
World Journal of Gastroenterology ISSN 1007-9327

LETTERS TO THE EDITOR

Pharmacopoeia of acute pancreatitis: Is the roster complete?

Maxim S Petrov

Maxim S Petrov, Department of Surgery, Nizhny Novgorod State Medical Academy, Nizhny Novgorod, Russia
Author contributions: Petrov MS contributed all to this paper.
Correspondence to: Dr. Maxim S Petrov, MD, MPH, Department of Surgery, Nizhny Novgorod State Medical Academy, PO Box 568, Nizhny Novgorod 603000, Russia. max.petrov@gmail.com
Telephone: +7-910-3833963 Fax: +7-831-4339721
Received: June 4, 2008 Revised: September 16, 2008
Accepted: September 24, 2008
Published online: October 14, 2008

Abstract

Acute pancreatitis is one of the most common diseases in the everyday's practise of gastroenterologists and surgeons. However, the physicians' therapeutic armamentarium is very limited. The present letter to the editor briefly describes the recent evidence from the literature with the aim to optimize a conservative management of patients with acute pancreatitis.

Key words: Acute pancreatitis; Conservative treatment; Enteral nutrition

Peer reviewer: Marko Duvnjak, MD, Department of Gastroenterology and Hepatology, Sestre Milosrdnice University Hospital, Vinogradska Cesta 29, 10000 Zagreb, Croatia

Petrov MS. Pharmacopoeia of acute pancreatitis: Is the roster complete? *World J Gastroenterol* 2008; 14(38): 5938-5939 Available from: URL: http://www.wjgnet.com/1007-9327/14/5938.asp DOI: http://dx.doi.org/10.3748/wjg.14.5938

TO THE EDITOR

In a recent issue of the Journal, I read with interest the article by Bang and co-authors[1], who reviewed the current possibilities of pharmacological prevention and treatment of patients with acute pancreatitis. Obviously, the chosen topic is of contemporary interest from both scientific and practical points of view, as the therapeutic armamentarium for this disease is fairly scarce. Herewith, I would like to make a brief comment on the list of options considered in the referred review.

A substantial part of the article by Bang *et al*[1] is devoted to the pharmacological prevention of post-endoscopic retrograde cholangiopancreatography (ERCP) acute pancreatitis. Unfortunately, the promising potential of many drugs in the experimental studies has never been confirmed in the clinical trials and the data from many reports are inconsistent. At the same time, as a recent meta-analysis of randomised controlled trials[2] revealed no difference in outcomes between patients with acute pancreatitis but without coexisting acute cholangitis who received either early ERCP or conservative care, I would argue that the best preventive strategy for post-ERCP acute pancreatitis is to obviate the unnecessary ERCP, i.e. to use it only as a therapeutic but not as a diagnostic modality. Thus, it should be mainly reserved for patients with acute cholangitis, the incidence of which is marginal. In contrast, the majority of patients without cholangitis should be diagnosed noninvasively by endoscopic ultrasonography or by magnetic resonance tomography, which are virtually riskless. Adherence to this strategy may drastically diminish the need for pharmacological prevention of post-ERCP acute pancreatitis.

Another matter of concern is that Bang *et al*[1] did not consider a favorable effect of enteral nutrition in patients with (severe) acute pancreatitis. Probably, this is because enteral nutrition has been viewed as a supportive care for years. Indeed, in the majority of clinical settings, it should be considered so, because it is possible to demonstrate a beneficial effect of enteral nutrition only on nutritional parameters (body weight, nitrogen balance *etc*), which are essentially surrogate endpoints. However, the use of enteral nutrition in acute pancreatitis, unlike many other pathologies, is associated with a significant reduction in the incidence of clinically meaningful outcomes (infectious complications, need for surgery, mortality) coherently derived from nearly a dozen of randomized controlled trials. The findings from only high quality of these studies have been statistically aggregated in a recent meta-analysis[3], showing a significantly reduced risk of total infectious complications [relative risk (RR) = 0.47, 95% confidence interval (CI) = 0.28-0.77, $P < 0.001$], pancreatic infectious complications (RR = 0.48, 95% CI = 0.26-0.91; $P = 0.02$), need for surgery (RR = 0.37, 95% CI = 0.21-0.65, $P = 0.001$), and mortality (RR = 0.32, 95% CI = 0.11-0.98, $P = 0.03$) with the use of enteral nutrition in patients with severe acute pancreatitis.

Furthermore, as Dorland's medical dictionary[4] defines a drug as "any chemical compound that may be used in or administered to humans or animals as an aid in the diagnosis, treatment, or prevention of diseases or other abnormal conditions, for the relief of pain or suffering, or to control or improve any physiologic or pathologic condition", it seems to me that it is time now to consider enteral nutrition as a full-fledged drug in patients with acute pancreatitis.

REFERENCES

1 **Bang UC**, Semb S, Nojgaard C, Bendtsen F. Pharmacological approach to acute pancreatitis. *World J Gastroenterol* 2008; **14**: 2968-2976

2 **Petrov MS**, van Santvoort HC, Besselink MG, van der Heijden GJ, van Erpecum KJ, Gooszen HG. Early endoscopic retrograde cholangiopancreatography versus conservative management in acute biliary pancreatitis without cholangitis: a meta-analysis of randomized trials. *Ann Surg* 2008; **247**: 250-257

3 **Petrov MS**, van Santvoort HC, Besselink MG, van der Heijden GJ, Windsor JA, Gooszen HG. Enteral nutrition and the risk of mortality and infectious complications in patients with severe acute pancreatitis: a meta-analysis of randomized trials. *Arch Surg* 2008; **143:** 1111-1117

4 **Dorland's Illustrated Medical Dictionary**. 28th ed. Philadelphia: WB Saunders Co., 1994: 1859

S- Editor Zhong XY **L- Editor** Wang XL **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2008 October 14; 14(38): 5940
World Journal of Gastroenterology ISSN 1007-9327

ACKNOWLEDGMENTS

Acknowledgments to Reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

Robert J Korst, MD
Department of Cardiothoracic Surgery, Weill Medical College of Cornell University, Room M404, 525 East 68th Street, New York 10032, United States

Katsutoshi Yoshizato, PhD
Academic Advisor , Phoenix Bio Co., Ltd., 3-4-1 Kagamiyama, Higashihiroshima, 739-0046, Japan

Jian Wu, Associate Professor of Medicine
Internal Medicine/Transplant Research Program, University of California, Davis Medical Center, 4635 2nd Ave. Suite 1001, Sacramento CA 95817, United States

Dr. Philip Abraham, Professor
Consultant Gastroenterologist & Hepatologist, P. D. Hinduja National Hospital & Medical Research Centre, Veer Savarkar Marg, Mahim, Mumbai 400 016, India

Michael A Zimmerman, MD, Assistant Professor
Division of Transplant Surgery, University of Colorado Health Sciences Center, 1635 N. Ursula St, Po Box 6510, 7th floor, Aurora, Colorado 80045, United States

Silvana Zanlungo, Professor
Departamento de Gastroenterología, Pontificia Universidad Católica de Chile, Marcoleta 367, Casilla 114-D, Santiago, Chile

Anna S Gukovskaya, Professor
VA Greater Los Angeles Health Care System ,University of California, Los Angeles, 11301 Wilshire Blvd, Los Angeles 91301, United States

Mercedes Susan Mandell, MD, PhD
Department of Anesthesiology, University of Colorado Health Sciences Ctr., 12401 E. 17th Ave, B113 Aurora, CO 80045, United States

Wing-Kin Syn, MD
Division of Gastroenterology, GSRB-1, Suite 1073, DUMC 3256, 595 LaSalle Street, Durham, NC27710, United States

Laura Lladóa, PhD
Department of Surgery, Liver Transplant Unit, Hospital Universitari de Bellvitge, IDIBELL, 08907 Barcelona, Spain

Tom H Karlsen, MD
Institute of Immunology, Rikshospitalet University Hospital, N-0027 Oslo, Norway

Minna Nyström, PhD
Department of Biological and Environmental Sciences, PO Box 56 (Viikinkaari 5 D), University of Helsinki, FI-00014 Helsinki, Finland

Sri P Misra, Professor
Gastroenterology, Moti Lal Nehru Medical College, Allahabad 211001, India

Alyssa M Krasinskas, MD, Assistant Professor
Department of Pathology, University of Pittsburgh Medical Center, Presbyterian Hospital, A610, 200 Lothrop Street, Pittsburgh, PA 15213-2546, United States

Robert D Odze, MD, FRCPc, Chief
Gastrointestinal Pathology Service, Associate Professor of Pathology , Brigham and Women's Hospital , Harvard Medical School, Boston MA, United States

Giulio Marchesini, Professor
Department of Internal Medicine and Gastroenterology, "Alma Mater Studiorum" University of Bologna, Policlinico S. Orsola, Via Massarenti9, Bologna 40138, Italy

Dr. Mitsuhiro Fujishiro
Department of Gastroenterology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan

Kyoichi Adachi, MD
Department of Gastroenterology and Hepatology, Shimane University, School of Medicine Shimane, 89-1 Enya-cho, Izumo-shi Shimane 693-8501, Japan

Dr. Serdar Karakose, Professor
Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey

World J Gastroenterol 2008 October 14; 14(38): 5941
World Journal of Gastroenterology ISSN 1007-9327

Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
January 24-25, Frankfurt, Germany
Falk Workshop: Perspectives in Liver
Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
E-mail: education@asge.org

June 4-7, Helsinki, Finland
The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
ESGAR 2008 19th Annual Meeting and Postgraduate Course
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden
16th International Congress of the European Association for Endoscopic Surgery
E-mail: info@eaes-eur.org

June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
10th World Congress on Gastrointestinal Cancer
Imedex and ESMO
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland
Joint Meeting of the European Pancreatic Club (EPC)
and the International Association of Pancreatology (IAP)
E-mail: office@epc-iap2008.org
www.e-p-c.org
www.pancreatology.org

June 26-28, Bratislava, Slovakia
5th Central European Gastroenterology Meeting
www.ceurgem2008.cz

July 9-12, Paris, France
ILTS 14th Annual International Congress
www.ilts.org

September 10-13, Budapest, Hungary
11th World Congress of the International Society for Diseases of the Esophagus
E-mail: isde@isde.net

September 13-16, New Delhi, India
Asia Pacific Digestive Week
E-mail: apdw@apdw2008.net

APDW 2008
September 13-16, New Delhi, Indian
Organized: Indian Society of Gastroenterology

III FALK GASTRO-CONFERENCE
September 17, Mainz, Germany
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
Falk Symposium 166:
GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
Falk Symposium 167:
Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
Third Annual Meeting
European Society of Coloproctology
www.escp.eu.com

October 8-11, Istanbul, Turkey
18th World Congress of the International Association of Surgeons,
Gastroenterologists and Oncologists
E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria
16th United European Gastroenterology Week
www.negf.org
www.acv.at

October 22-25, Minnesota, USA
Anstralian Gastroenterology Week 2008
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia
71st Annual Colon and Rectal Surgery Conference
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA
59th AASLD Annual Meeting and Postgraduate Course
The Liver Meeting
Information: www.aasld.org

November 6-9, Lucerne, Switzerland
Neurogastroenterology & Motility Joint International Meeting 2008
E-mail: ngm2008@mci-group.com
www.ngm2008.com

November 12, Santiago de Chile, Chile
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt
1st Hepatology and Gastroenterology Post Graduate Course
www.egyptgastrohep.com

December 7-9, Seoul, Korea
6th International Meeting
Hepatocellular Carcinoma: Eastern and Western Experiences
E-mail: sglee@amc.seoul.kr

INFORMATION FOR ALL
FALK FOUNDATION e.V.
E-mail: symposia@falkfoundation.de
www.falkfoundation.de

Advanced Courses - European Institute of Telesurgery EITS - 2008
Strasbourg, France
January 18-19, March 28-29, June 6-7, October 3-4

N.O.T.E.S
April 3-5, November 27-29
Laparoscopic Digestive Surgery

June 27-28, November 7-8
Laparoscopic Colorectal Surgery

July 3-5
Interventional GI Endoscopy Techniques
Contact address for all courses:
E-mail: info@eits.fr

International Gastroenterological Congresses 2009
March 23-26, Glasgow, Scotland
Meeting of the British Society of Gastroenterology (BSG)
E-mail: bsg@mailbox.ulcc.ac.uk

May 17-20, Denver, Colorado, USA
Digestive Disease Week 2009

November 21-25, London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2008 October 14; 14(38): 5942-5944
World Journal of Gastroenterology ISSN 1007-9327

Instructions to authors

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol* ISSN 1007-9327 CN 14-1219/R) is a weekly open access peer-reviewed journal supported by an editorial board consisting of 1208 experts in gastroenterology and hepatology from 60 countries. The aim of the journal is to deliver the most clinically relevant original and commentary articles to readers, and to make the full text publicly available to all clinicians, scientists, patients and biomedical students on an unrestricted platform, so that they can access and learn about the most recent key advances in the field.

In addition to the open access nature, another key characteristic of *WJG* is its reading guidance for each article which includes background, research frontier, related reports, breakthroughs, applications, terminology, and comments of peer reviewers for the general readers.

WJG publishes articles on esophageal, gastrointestinal, hepatobiliary and pancreatic tumors, and other esophageal, gastrointestinal, hepatic-biliary and pancreatic diseases in relation to epidermiology, immunology, microbiology, motility & nerve-gut interaction, endocrinology, nutrition & obesity, endoscopy, imaging and advanced hi-technology.

The main goal of *WJG* is to publish high quality commentary articles contributed by leading experts in gastroenterology and hepatology and original articles that combine the clinical practice and advanced basic research, to provide an interactive platform for clinicians and researchers in internal medicine, surgery, infectious diseases, traditional Chinese medicine, oncology, integrated Chinese and Western medicine, imaging, endoscopy, interventional therapy, pathology and other basic medical specialities, and thus eventually improving the clinical practice and healthcare for patients.

Indexed and abstracted in

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, Index Medicus, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology* and *Hepatology*, CAB Abstracts and Global Health. ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

Published by

The WJG Press

SUBMISSION OF MANUSCRIPTS

Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of The WJG Press, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the International Committee of Medical Journal Editors to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now available, to our knowledge, is http://www. clinicaltrials.gov sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

Authors should retain one copy of the text, tables, photographs and illustrations because rejected manuscripts will not be returned to the author(s) and the editors will not be responsible for loss or damage to photographs and illustrations sustained during mailing.

Online submissions

Manuscripts should be submitted through the Online Submission System at: http://wjg.wjgnet.com/wjg. Authors are highly recommended to consult the ONLINE INSTRUCTIONS TO AUTHORS (http://www.wjgnet.com/wjg/help/instructions.jsp) before attempting to submit online. For assistance, authors encountering problems with the Online Submission System may send an email describing the problem to submission@wjgnet.com, or by telephone: +86-10-85381892. If you submit your manuscript online, do not make a postal contribution. Repeated online submission for the same manuscript is strictly prohibited.

MANUSCRIPT PREPARATION

All contributions should be written in English. All articles must be submitted using word-processing software. All submissions must be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Style should conform to our house format. Required information for each of the manuscript sections is as follows:

Title page

Full manuscript title, running title, all author(s) name(s), affiliations, institution(s) and/or department(s) where the work was carried out; author contributions; disclosure of any financial support for the research; and the name, full address, telephone and fax numbers and email address of the corresponding author should be included. Titles should be concise and informative (remove all unnecessary words), emphasize what is new, and avoid abbreviations. A short running title of less than 40 letters should be provided. List the author(s)' name(s) as follows: initial and/or first name, middle name or initial(s), and full family name.

Author controbutions: The format of this section should be like this: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed research; Wang CL, Zou CC, Hong F and Wu XM performed research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed data; and Wang CL, Liang L and Fu JF wrote the paper.

Peer reviewers: All articles received are subject to peer review. Normally, three experts are invited for each article. Decision for acceptance is made only when at least two experts recommend an article for publication. Reviewers for accepted manuscripts are acknowledged in each manuscript, and reviewers of articles which were not accepted will be acknowledged at the end of each issue. To ensure the quality of the articles published in *WJG*, reviewers of accepted manuscripts will be announced by publishing the name, title/position and institution of the reviewer in the footnote accompanying the printed article. For example, reviewers: Professor Jing-Yuan Fang, Shanghai Institute of Digestive Disease, Shanghai, Affiliated Renji Hospital, Medical Faculty, Shanghai Jiaotong University, Shanghai, China; Professor Xin-Wei Han, Department of Radiology, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan Province, China; and Professor Anren Kuang, Department of Nuclear Medicine, Huaxi Hospital, Sichuan University, Chengdu, Sichuan Province, China.

Abstract

An informative, structured abstract of no more than 350 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections: AIM: Only the purpose should be included. METHODS: The materials, techniques, instruments and equipment, and the experimental procedures should be included. RESULTS: The observed and experimental results, including data, effects, outcome, *etc.* should be included. Authors should present *P* value where necessary, and also include any significant data. CONCLUSION: Accurate view and the value of the results should be included.

The format for structured abstracts can be found at: http://www.wjgnet.com/wjg/help/11.doc.

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles, rapid communication

and case reports, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the body text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, should be found at: http://www.wjgnet.com/wjg/help/instructions.jsp.

Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: …*etc.* It is our principle to publish high resolution-figures for the printed and E-versions.

Tables

Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of P values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscripts and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

Coding system

The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability[1,2]". If references are cited directly in the text, they should be put together within the text, for example, "From references[19,22-24], we know that..."

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

PMID requirement

PMID roots in the abstract serial number indexed by PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed). The author should supply the PMID for journal citation. For those references that have not been indexed by PubMed, a printed copy of the first page of the full reference should be submitted.

The accuracy of the information for journal citations is very important. Using the reference testing system, the authors and editor should check the authors name, title, journal title, publication date, volume number, start page, and end page. We will interlink all references with PubMed in an ASP file so that the readers can immediately access the abstract of the citations online.

DOI requirement

A CrossRef DOI® (Digital Object Identifier) name is a unique string created to identify a piece of scholarly content in the online environment. The author should supply the DOIs for journal citation(doi:10.3748/wjg.13.6458). This link (http://www.crossref.org/SimpleTextQuery/) allows you to retrieve Digital Object Identifiers (DOIs) for journal articles, books, and chapters by simply cutting and pasting the reference list into the box. You may use the form with any reference style, although the tool works most reliably if references are formatted in a standard style such as shown in this example: Assimakopoulos SF, Scopa CD, Vagianos CE. Pathophysiology of increased intestinal permeability in obstructive jaundice. *World J Gastroenterol* 2007; 13(48): 6458-6464

The accuracy of the information of journal citations is very important. We will interlink all references with DOI in ASP file so that readers can access the abstracts of cited articles online immediately.

Style for journal references

Authors: the name of the first author should be typed in bold-faced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

Style for book references

Authors: the name of the first author should be typed in bold-faced letters. The surname of all authors should be typed with the initial letter capitalized, followed by their abbreviated middle and first initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR) Book title. Publication number. Publication place: Publication press, Year: start page and end page.

Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Inappropriate references

Authors should always cite references that are relevant to their article, and avoid any inappropriate references. Inappropriate references include those linked with a hyphen when the difference between the two numbers is greater than five. For example, [1-6], [2-14] and [1, 3, 4-10, 22] are all considered inappropriate references. Authors should not cite their own unrelated published articles.

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/wjg/help/15.doc.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H pylori*, *E coli*, *etc.*

SUBMISSION OF THE REVISED MANUSCRIPTS AFTER ACCEPTED

Please revise your article according to the revision policies of *WJG*. The revised version including manuscript and high-resolution image figures (if any) should be copied on a floppy or compact disk. The author should send the revised manuscript, along with printed high-resolution color or black and white photos, copyright transfer letter, and responses to the reviewers by courier (such as EMS/DHL).

Editorial Office

World Journal of Gastroenterology
Editorial Department: Room 903
Ocean International Center, Building D
No. 62 Dongsihuan Zhonglu
Chaoyang District, Beijing 100025, China
E-mail: wjg@wjgnet.com
http://www.wjgnet.com
Telephone: +86-10-59080039
Fax: +86-10-85381893

Language evaluation

The language of a manuscript will be graded before it is sent for revision. (1) Grade A: priority publishing; (2) Grade B: minor language polishing; (3) Grade C: a great deal of language polishing needed; (4) Grade D: rejected. Revised articles should reach Grade A or B.

Copyright assignment form

Please download a Copyright assignment form from http://www.wjgnet.com/wjg/help/9.doc.

Responses to reviewers

Please revise your article according to the comments/suggestions provided by the reviewers. The format for responses to the reviewers' comments can be found at: http://www.wjgnet.com/wjg/help/10.doc.

Proof of financial support

For paper supported by a foundation, authors should provide a copy of the document and serial number of the foundation.

Links to documents related to the manuscript

WJG will be initiating a platform to promote dynamic interactions between the editors, peer reviewers, readers and authors. After a manuscript is published online, links to the PDF version of the submitted manuscript, the peer-reviewers' report and the revised manuscript will be put on-line. Readers can make comments on the peer reviewer's report, authors' responses to peer reviewers, and the revised manuscript. We hope that authors will benefit from this feedback and be able to revise the manuscript accordingly in a timely manner.

Science news releases

Authors of accepted manuscripts are suggested to write a science news item to promote their articles. The news will be released rapidly at EurekAlert/AAAS (http://www.eurekalert.org). The title for news items should be less than 90 characters; the summary should be less than 75 words; and main body less than 500 words. Science news items should be lawful, ethical, and strictly based on your original content with an attractive title and interesting pictures.

Publication fee

Authors of accepted articles must pay a publication fee.
EDITORIAL, TOPIC HIGHLIGHTS, BOOK REVIEWS and LETTERS TO THE EDITOR are published free of charge.

ISSN 1007-9327
CN 14-1219/R

World Journal of Gastroenterology®

Indexed and Abstracted in:

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, *Index Medicus*, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology and Hepatology*, CAB Abstracts and Global Health.
ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

Volume 14 Number 39
October 21, 2008
World J Gastroenterol
2008 October 21; 14(39): 5945-6108

Online Submissions
wjg.wjgnet.com
www.wjgnet.com
Printed on Acid-free Paper
世界胃肠病学杂志

A Weekly Journal of Gastroenterology and Hepatology

World Journal of Gastroenterology®

Editorial Board

2007-2009

Published by The WJG Press and Baishideng
Room 903, Ocean International Center, Building D
No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Fax: +86-10-8538-1893 E-mail: wjg@wjgnet.com http://www.wjgnet.com

The World Journal of Gastroenterology Editorial Board consists of 1208 members, representing a team of worldwide experts in gastroenterology and hepatology. They are from 60 countries, including Albania (1), Argentina (4), Australia (39), Austria (10), Belarus (1), Belgium (15), Brazil (2), Bulgaria (1), Canada (28), Chile (1), China (60), Croatia (2), Cuba (1), Czech (2), Denmark (7), Egypt (4), Estonia (1), Finland (4), France (44), Germany (108), Greece (9), Hungary (2), Iceland (1), India (12), Iran (3), Ireland (4), Israel (8), Italy (96), Japan (176), Lebanon (3), Lithuania (1), Macedonia (1), Malaysia (3), Mexico (6), Monaco (1), Morocco (1), The Netherlands (26), New Zealand (1), Nigeria (1), Norway (3), Pakistan (2), Peru (1), Poland (6), Portugal (1), Russia (3), Saudi Arabia (2), Serbia (1), Singapore (4), Slovakia (2), Slovenia (1), South Africa (2), South Korea (14), Spain (38), Sweden (15), Switzerland (13), Turkey (8), United Arab Emirates (1), United Kingdom (83), United States (316) and Uruguay (2).

Andrew V Biankin, *Sydney*
Filip Braet, *Sydney*
Andrew D Clouston, *Sydney*
Graham Cooksley, *Queensland*
Darrell HG Crawford, *Brisbane*
Adrian G Cummins, *Woodville South*
Guy D Eslick, *Sydney*
Michael A Fink, *Melbourne*
Robert JL Fraser, *Daw Park*
Peter Raymond Gibson, *Victoria*
Jacob George, *Westmead*
Mark D Gorrell, *Sydney*
Yik-Hong Ho, *Townsville*
Gerald J Holtmann, *Adelaide*
Michael Horowitz, *Adelaide*
John E Kellow, *Sydney*
Rupert Leong, *Concord*
Geoffrey W McCaughan, *Sydney*
Finlay A Macrae, *Victoria*
Daniel Markovich, *Brisbane*
Phillip S Oates, *Perth*
Jacqui Richmond, *Victoria*
Stephen M Riordan, *Sydney*
Ian C Roberts-Thomson, *Adelaide*
Devanshi Seth, *Camperdown*
Arthur Shulkes, *Melbourne*
Ross C Smith, *Sydney*
Kevin J Spring, *Brisbane*
Huy A Tran, *New South Wales*
Debbie Trinder, *Fremantle*
Martin J Veysey, *Gosford*
Daniel L Worthley, *Bedford*

Austria

Peter Ferenci, *Vienna*
Valentin Fuhrmann, *Vienna*
Alfred Gangl, *Vienna*
Christoph Gasche, *Vienna*
Kurt Lenz, *Linz*
Markus Peck-Radosavljevic, *Vienna*
Rudolf E Stauber, *Auenbruggerplatz*
Herbert Tilg, *Innsbruck*
Michael Trauner, *Graz*
Harald Vogelsang, *Vienna*
Guenter Weiss, *Innsbruck*

Belarus

Yury K Marakhouski, *Minsk*

Belgium

Rudi Beyaert, *Gent*
Bart Rik De Geest, *Leuven*
Inge I Depoortere, *Leuven*
Olivier Detry, *Liège*
Benedicte Y De Winter, *Antwerp*
Karel Geboes, *Leuven*
Thierry Gustot, *Brussels*
Yves J Horsmans, *Brussels*
Geert G Leroux-Roels, *Ghent*
Louis Libbrecht, *Leuven*
Etienne M Sokal, *Brussels*
Marc Peeters, *De Pintelaan*
Gert A Van Assche, *Leuven*
Yvan Vandenplas, *Brussels*
Eddie Wisse, *Keerbergen*

Brazil

Heitor Rosa, *Goiania*
Ana Cristina Simões e Silva, *Belo Horizonte*

Bulgaria

Zahariy Krastev, *Sofia*

Canada

Fernando Alvarez, *Québec*
David Armstrong, *Ontario*
Jeffrey P Baker, *Toronto*
Olivier Barbier, *Québec*
Nancy Baxter, *Toronto*
Matthew Bjerknes, *Toronto*
Frank J Burczynski, *Manitoba*
Michael F Byrne, *Vancouver*
Wang-Xue Chen, *Ottawa*
Chantal Guillemette, *Québec*
Samuel S Lee, *Calgary*
Gary A Levy, *Toronto*
Andrew L Mason, *Alberta*
John K Marshall, *Ontario*
Donna-Marie McCafferty, *Calgary*
Thomas I Michalak, *St. John's*
Gerald Y Minuk, *Manitoba*
Paul Moayyedi, *Hamilton*
Kostas Pantopoulos, *Québec*
William G Paterson, *Kingston*
Eldon Shaffer, *Calgary*
Morris Sherman, *Toronto*
Martin Storr, *Calgary*
Elena F Verdu, *Ontario*
John L Wallace, *Calgary*
Eric M Yoshida, *Vancouver*

Chile

Silvana Zanlungo, *Santiago*

China

Henry LY Chan, *Hongkong*
Xiao-Ping Chen, *Wuhan*
Zong-Jie Cui, *Beijing*
Da-Jun Deng, *Beijing*
Er-Dan Dong, *Beijing*
Sheung-Tat Fan, *Hong Kong*
Jin Gu, *Beijing*
Xin-Yuan Guan, *Pokfulam*
De-Wu Han, *Taiyuan*
Ming-Liang He, *Hong Kong*
Wayne HC Hu, *Hong Kong*
Chee-Kin Hui, *Hong Kong*
Ching-Lung Lai, *Hong Kong*
Kam Chuen Lai, *Hong Kong*
James YW Lau, *Hong Kong*
Yuk-Tong Lee, *Hong Kong*
Suet-Yi Leung, *Hong Kong*
Wai-Keung Leung, *Hong Kong*
John M Luk, *Pokfulam*
Chung-Mau Lo, *Hong Kong*
Jing-Yun Ma, *Beijing*
Ronnie Tung Ping Poon, *Hong Kong*
Lun-Xiu Qin, *Shanghai*
Yu-Gang Song, *Guangzhou*
Qin Su, *Beijing*
Wai-Man Wong, *Hong Kong*
Hong Xiao, *Shanghai*
Dong-Liang Yang, *Wuhan*
Winnie Yeo, *Hong Kong*
Yuan Yuan, *Shenyang*
Man-Fung Yuen, *Hong Kong*
Jian-Zhong Zhang, *Beijing*
Xin-Xin Zhang, *Shanghai*
Bo-Jian Zheng, *Hong Kong*
Shu Zheng, *Hangzhou*

Croatia

Tamara Cacev, *Zagreb*
Marko Duvnjak, *Zagreb*

Cuba

Damian C Rodriguez, *Havana*

Czech

Milan Jirsa, *Praha*
Pavel Trunečka, *Prague*

Denmark

Peter Bytzer, *Copenhagen*
Asbjørn M Drewes, *Aalborg*
Hans Gregersen, *Aalborg*
Jens H Henriksen, *Hvidovre*
Claus P Hovendal, *Odense*
Fin S Larsen, *Copenhagen*
Søren Møller, *Hvidovre*

Egypt

Abdel-Rahman El-Zayadi, *Giza*
Amr M Helmy, *Cairo*
Ayman Yosry, *Cairo*

Estonia

Riina Salupere, *Tartu*

Finland

Irma E Jarvela, *Helsinki*
Katri M Kaukinen, *Tampere*
Minna Nyström, *Helsinki*
Pentti Sipponen, *Espoo*

France

Bettaieb Ali, *Dijon*
Corlu Anne, *Rennes*
Denis Ardid, *Clermont-Ferrand*
Charles P Balabaud, *Bordeaux*
Soumeya Bekri, *Rouen*
Jacques Belghiti, *Clichy*
Jacques Bernuau, *Clichy Cedex*
Pierre Brissot, *Rennes*
Patrice P Cacoub, *Paris*
Franck Carbonnel, *Besancon*
Laurent Castera, *Pessac*
Bruno Clément, *Rennes*
Benoit Coffin, *Colombes*
Jacques Cosnes, *Paris*
Thomas Decaens, *Cedex*

Francoise L Fabiani, *Angers*
Gérard Feldmann, *Paris*
Jean Fioramonti, *Toulouse*
Jean-Noël Freund, *Strasbourg*
Jean-Paul Galmiche, *Nantes*
Catherine Guettier, *Villejuif*
Chantal Housset, *Paris*
Juan L Iovanna, *Marseille*
Rene Lambert, *Lyon*
Patrick Marcellin, *Paris*
Philippe Mathurin, *Lille*
Tamara Matysiak–Budnik, *Paris*
Francis Mégraud, *Bordeaux*
Richard Moreau, *Clichy*
Thierry Piche, *Nice*
Raoul Poupon, *Paris*
Jean Rosenbaum, *Bordeaux*
Dominique Marie Roulot, *Bobigny*
Thierry Poynard, *Paris*
Jean-Philippe Salier, *Rouen*
Didier Samuel, *Villejuif*
Jean-Yves Scoazec, *Lyon*
Khalid A Tazi, *Clichy*
Emmanuel Tiret, *Paris*
Baumert F Thomas, *Strasbourg*
Marie-Catherine Vozenin-brotons, *Villejuif*
Jean-Pierre H Zarski, *Grenoble*
Jessica Zucman-Rossi, *Paris*

Germany

Hans-Dieter Allescher, *G-Partenkirchen*
Martin Anlauf, *Kiel*
Rudolf Arnold, *Marburg*
Max G Bachem, *Ulm*
Thomas F Baumert, *Freiburg*
Daniel C Baumgart, *Berlin*
Hubert Blum, *Freiburg*
Thomas Bock, *Tuebingen*
Katja Breitkopf, *Mannheim*
Dunja Bruder, *Braunschweig*
Markus W Büchler, *Heidelberg*
Christa Buechler, *Regensburg*
Reinhard Buettner, *Bonn*
Elke Cario, *Essen*
Uta Dahmen, *Essen*
Christoph F Dietrich, *Bad Mergentheim*
Arno J Dormann, *Koeln*
Rainer J Duchmann, *Berlin*
Volker F Eckardt, *Wiesbaden*
Paul Enck, *Tuebingen*
Fred Fändrich, *Kiel*
Ulrich R Fölsch, *Kiel*
Helmut Friess, *Heidelberg*
Peter R Galle, *Mainz*
Nikolaus Gassler, *Aachen*
Andreas Geier, *Aachen*
Markus Gerhard, *Munich*
Wolfram H Gerlich, *Giessen*
Dieter Glebe, *Giessen*
Burkhard Göke, *Munich*
Florian Graepler, *Tuebingen*
Axel M Gressner, *Aachen*
Veit Gülberg, *Munich*
Rainer Haas, *Munich*
Eckhart G Hahn, *Erlangen*
Stephan Hellmig, *Kiel*
Martin Hennenberg, *Bonn*
Johannes Herkel, *Hamburg*
Klaus R Herrlinger, *Stuttgart*
Eva Herrmann, *Homburg/Saar*
Eberhard Hildt, *Berlin*
Joerg C Hoffmann, *Berlin*
Ferdinand Hofstaedter, *Regensburg*
Werner Hohenberger, *Erlangen*
Jörg C Kalff, *Bonn*
Ralf Jakobs, *Ludwigshafen*
Jutta Keller, *Hamburg*
Andrej Khandoga, *Munich*
Sibylle Koletzko, *München*
Stefan Kubicka, *Hannover*
Joachim Labenz, *Siegen*
Frank Lammert, *Bonn*
Thomas Langmann, *Regensburg*
Christian Liedtke, *Aachen*
Matthias Löhr, *Mannheim*
Christian Maaser, *Muenster*
Ahmed Madisch, *Dresden*
Peter Malfertheiner, *Magdeburg*
Michael P Manns, *Hannover*
Helmut Messmann, *Augsburg*
Stephan Miehlke, *Dresden*
Sabine Mihm, *Göttingen*
Silvio Nadalin, *Essen*
Markus F Neurath, *Mainz*
Johann Ockenga, *Berlin*
Florian Obermeier, *Regensburg*
Gustav Paumgartner, *Munich*
Ulrich KS Peitz, *Magdeburg*
Markus Reiser, *Bochum*
Emil C Reisinger, *Rostock*
Steffen Rickes, *Magdeburg*
Tilman Sauerbruch, *Bonn*
Dieter Saur, *Munich*
Hans Scherubl, *Berlin*
Joerg Schirra, *Munich*
Roland M Schmid, *München*
Volker Schmitz, *Bonn*
Andreas G Schreyer, *Regensburg*
Tobias Schroeder, *Essen*
Henning Schulze-Bergkamen, *Mainz*
Hans Seifert, *Oldenburg*
Norbert Senninger, *Muenster*
Manfred V Singer, *Mannheim*
Gisela Sparmann, *Rostock*
Christian J Steib, *München*
Jurgen M Stein, *Frankfurt*
Ulrike S Stein, *Berlin*
Manfred Stolte, *Bayreuth*
Christian P Strassburg, *Hannover*
Wolfgang R Stremmel, *Heidelberg*
Harald F Teutsch, *Ulm*
Robert Thimme, *Freiburg*
Hans L Tillmann, *Leipzig*
Tung-Yu Tsui, *Regensburg*
Axel Ulsenheimer, *Munich*
Patrick Veit-Haibach, *Essen*
Claudia Veltkamp, *Heidelberg*
Siegfried Wagner, *Deggendorf*
Henning Walczak, *Heidelberg*
Heiner Wedemeyer, *Hannover*
Fritz von Weizsacker, *Berlin*
Jens Werner, *Heidelberg*
Bertram Wiedenmann, *Berlin*
Reiner Wiest, *Regensburg*
Stefan Wirth, *Wuppertal*
Stefan JP Zeuzem, *Homburg*

Greece

Alexandra A Alexopoulou, *Athens*
George N Dalekos, *Larissa*
Christos Dervenis, *Athens*
Melanie Maria Deutsch, *Athens*
Tsianos Epameinondas, *Ioannina*
Elias A Kouroumalis, *Heraklion*
George Papatheodoridis, *Athens*
Spiros Sgouros, *Athens*

Hungary

Peter L Lakatos, *Budapest*
Zsuzsa Szondy, *Debrecen*

Iceland

Hallgrimur Gudjonsson, *Reykjavik*

India

Philip Abraham, *Mumbai*
Rakesh Aggarwal, *Lucknow*
Kunissery A Balasubramanian, *Vellore*
Deepak Kumar Bhasin, *Chandigarh*
Sujit K Bhattacharya, *Kolkata*
Yogesh K Chawla, *Chandigarh*
Radha K Dhiman, *Chandigarh*
Sri Prakash Misra, *Allahabad*
Ramesh Roop Rai, *Jaipur*
Nageshwar D Reddy, *Hyderabad*
Rakesh Kumar Tandon, *New Delhi*

Iran

Seyed-Moayed Alavian, *Tehran*
Reza Malekzadeh, *Tehran*
Seyed A Taghavi, *Shiraz*

Ireland

Billy Bourke, *Dublin*
Ronan A Cahill, *Cork*
Anthony P Moran, *Galway*

Israel

Simon Bar-Meir, *Hashomer*
Abraham R Eliakim, *Haifa*
Zvi Fireman, *Hadera*
Yaron Ilan, *Jerusalem*
Avidan U Neumann, *Ramat-Gan*
Yaron Niv, *Pardesia*
Ran Oren, *Tel Aviv*
Ami D Sperber, *Beer-Sheva*

Italy

Giovanni Addolorato, *Roma*
Luigi E Adinolfi, *Naples*
Domenico Alvaro, *Rome*
Mario Angelico, *Rome*
Vito Annese, *San Giovanni Rotond*
Filippo Ansaldi, *Genoa*
Adolfo F Attili, *Roma*
Giovanni Barbara, *Bologna*
Claudio Bassi, *Verona*
Gabrio Bassotti, *Perugia*
Pier M Battezzati, *Milan*
Stefano Bellentani, *Carpi*
Antonio Benedetti, *Ancona*
Mauro Bernardi, *Bologna*
Livia Biancone, *Rome*
Luigi Bonavina, *Milano*
Flavia Bortolotti, *Padova*
Giuseppe Brisinda, *Rome*
Elisabetta Buscarini, *Crema*
Giovanni Cammarota, *Roma*

Antonino Cavallari, *Bologna*
Giuseppe Chiarioni, *Valeggio*
Michele Cicala, *Rome*
Massimo Colombo, *Milan*
Amedeo Columbano, *Cagliari*
Massimo Conio, *Sanremo*
Dario Conte, *Milano*
Gino R Corazza, *Pavia*
Francesco Costa, *Pisa*
Antonio Craxi, *Palermo*
Silvio Danese, *Milan*
Roberto de Franchis, *Milano*
Roberto De Giorgio, *Bologna*
Maria Stella De Mitri, *Bologna*
Giovanni D De Palma, *Naples*
Fabio Farinati, *Padua*
Giammarco Fava, *Ancona*
Francesco Feo, *Sassari*
Fiorucci Stefano, *Perugia*
Andrea Galli, *Firenze*
Valeria Ghisett, *Turin*
Gianluigi Giannelli, *Bari*
Edoardo G Giannini, *Genoa*
Paolo Gionchetti, *Bologna*
Fabio Grizzi, *Milan*
Salvatore Gruttadauria, *Palermo*
Mario Guslandi, *Milano*
Pietro Invernizzi, *Milan*
Ezio Laconi, *Cagliari*
Giacomo Laffi, *Firenze*
Giovanni Maconi, *Milan*
Lucia Malaguarnera, *Catania*
Emanuele D Mangoni, *Napoli*
Paolo Manzoni, *Torino*
Giulio Marchesini, *Bologna*
Fabio Marra, *Florence*
Marco Marzioni, *Ancona*
Giuseppe Mazzella, *Bologna*
Mario U Mondelli, *Pavia*
Giuseppe Montalto, *Palermo*
Giovanni Monteleone, *Rome*
Giovanni Musso, *Torino*
Gerardo Nardone, *Napoli*
Valerio Nobili, *Rome*
Fabio Pace, *Milano*
Luisi Pagliaro, *Palermo*
Francesco Pallone, *Rome*
Fabrizio R Parente, *Milan*
Maurizio Parola, *Torino*
Francesco Perri, *San Giovanni Rotondo*
Raffaele Pezzilli, *Bologna*
Alberto Pilotto, *San Giovanni Rotondo*
Alberto Piperno, *Monza*
Mario Pirisi, *Novara*
Anna C Piscaglia, *Roma*
Paolo Del Poggio, *Treviglio*
Gabriele B Porro, *Milano*
Piero Portincasa, *Bari*
Cosimo Prantera, *Roma*
Bernardino Rampone, *Siena*
Oliviero Riggio, *Rome*
Claudio Romano, *Messina*
Marco Romano, *Napoli*
Gerardo Rosati, *Potenza*
Mario Del Tacca, *Pisa*
Gloria Taliani, *Rome*
Pier A Testoni, *Milan*
Enrico Roda, *Bologna*
Domenico Sansonno, *Bari*
Vincenzo Savarino, *Genova*
Vincenzo Stanghellini, *Bologna*
Giovanni Tarantino, *Naples*
Roberto Testa, *Genoa*
Dino Vaira, *Bologna*
Anna Linda Zignego, *Florence*

Japan

Kyoichi Adachi, *Izumo*
Yasushi Adachi, *Sapporo*
Taiji Akamatsu, *Matsumoto*
Sk Md Fazle Akbar, *Ehime*
Takafumi Ando, *Nagoya*
Akira Andoh, *Otsu*
Taku Aoki, *Tokyo*
Masahiro Arai, *Tokyo*
Tetsuo Arakawa, *Osaka*
Yasuji Arase, *Tokyo*
Masahiro Asaka, *Sapporo*
Hitoshi Asakura, *Tokyo*
Takeshi Azuma, *Fukui*
Yoichi Chida, *Fukuoka*
Takahiro Fujimori, *Tochigi*
Jiro Fujimoto, *Hyogo*
Kazuma Fujimoto, *Saga*
Mitsuhiro Fujishiro, *Tokyo*
Yoshihide Fujiyama, *Otsu*
Hiroyuki Fukui, *Tochigi*
Hiroyuki Hanai, *Hamamatsu*
Naohiko Harada, *Fukuoka*
Makoto Hashizume, *Fukuoka*
Tetsuo Hayakawa, *Nagoya*
Toru Hiyama, *Higashihiroshima*
Kazuhide Higuchi, *Osaka*
Keisuke Hino, *Ube*
Keiji Hirata, *Kitakyushu*
Yuji Iimuro, *Nishinomiya*
Kenji Ikeda, *Tokyo*
Toru Ikegami, *Fukuoka*
Kenichi Ikejima, *Bunkyo-ku*
Fumio Imazeki, *Chiba*
Yutaka Inagaki, *Kanagawa*
Yasuhiro Inokuchi, *Yokohama*
Haruhiro Inoue, *Yokohama*
Masayasu Inoue, *Osaka*
Hiromi Ishibashi, *Nagasaki*
Shunji Ishihara, *Izumo*
Toru Ishikawa, *Niigata*
Kei Ito, *Sendai*
Masayoshi Ito, *Tokyo*
Hiroaki Itoh, *Akita*
Ryuichi Iwakiri, *Saga*
Yoshiaki Iwasaki, *Okayama*
Terumi Kamisawa, *Tokyo*
Hiroshi Kaneko, *Aichi-Gun*
Shuichi Kaneko, *Kanazawa*
Takashi Kanematsu, *Nagasaki*
Mitsuo Katano, *Fukuoka*
Junji Kato, *Sapporo*
Mototsugu Kato, *Sapporo*
Shinzo Kato, *Tokyo*
Norifumi Kawada, *Osaka*
Sunao Kawano, *Osaka*
Mitsuhiro Kida, *Kanagawa*
Yoshikazu Kinoshita, *Izumo*
Tsuneo Kitamura, *Chiba*
Seigo Kitano, *Oita*
Kazuhiko Koike, *Tokyo*
Norihiro Kokudo, *Tokyo*
Satoshi Kondo, *Sapporo*
Shoji Kubo, *Osaka*
Shigeki Kuriyama, *Kagawa*[2]
Katsunori Iijima, *Sendai*
Masato Kusunoki, *Tsu Mie*
Shin Maeda, *Tokyo*
Shigeru Marubashi, *Suita*
Masatoshi Makuuchi, *Tokyo*
Osamu Matsui, *Kanazawa*
Yasuhiro Matsumura, *Chiba*
Yasushi Matsuzaki, *Tsukuba*
Kiyoshi Migita, *Omura*
Kenji Miki, *Tokyo*
Tetsuya Mine, *Kanagawa*
Hiroto Miwa, *Hyogo*
Masashi Mizokami, *Nagoya*
Yoshiaki Mizuguchi, *Tokyo*
Motowo Mizuno, *Hiroshima*
Morito Monden, *Suita*
Hisataka S Moriwaki, *Gifu*
Yasuaki Motomura, *Iizuka*
Yoshiharu Motoo, *Kanazawa*
Naofumi Mukaida, *Kanazawa*
Kazunari Murakami, *Oita*
Kunihiko Murase, *Tusima*
Hiroaki Nagano, *Suita*
Masahito Nagaki, *Gifu*
Masaki Nagaya, *Kawasaki*
Yujl Naito, *Kyoto*
Atsushi Nakajima, *Yokohama*
Hisato Nakajima, *Tokyo*
Hiroki Nakamura, *Yamaguchi*
Shotaro Nakamura, *Fukuoka*
Mikio Nishioka, *Niihama*
Shuji Nomoto, *Nagoya*
Susumu Ohmada, *Maebashi*
Hirohide Ohnishi, *Akita*
Masayuki Ohta, *Oita*
Tetsuo Ohta, *Kanazawa*
Kazuichi Okazaki, *Osaka*
Katsuhisa Omagari, *Nagasaki*
Saburo Onishi, *Nankoku*
Morikazu Onji, *Ehime*
Satoshi Osawa, *Hamamatsu*
Masanobu Oshima, *Kanazawa*
Hiromitsu Saisho, *Chiba*
Hidetsugu Saito, *Tokyo*
Yutaka Saito, *Tokyo*
Isao Sakaida, *Yamaguchi*
Michiie Sakamoto, *Tokyo*
Yasushi Sano, *Chiba*
Hiroki Sasaki, *Tokyo*
Iwao Sasaki, *Sendai*
Motoko Sasaki, *Kanazawa*
Chifumi Sato, *Tokyo*
Shuichi Seki, *Osaka*
Hiroshi Shimada, *Yokohama*
Mitsuo Shimada, *Tokushima*
Tomohiko Shimatan, *Hiroshima*
Hiroaki Shimizu, *Chiba*
Ichiro Shimizu, *Tokushima*
Yukihiro Shimizu, *Kyoto*
Shinji Shimoda, *Fukuoka*
Tooru Shimosegawa, *Sendai*
Tadashi Shimoyama, *Hirosaki*
Ken Shirabe, *Iizuka City*
Yoshio Shirai, *Niigata*
Katsuya Shiraki, *Mie*
Yasushi Shiratori, *Okayama*
Masayuki Sho, *Nara*
Yasuhiko Sugawara, *Tokyo*
Hidekazu Suzuki, *Tokyo*
Minoru Tada, *Tokyo*
Tadatoshi Takayama, *Tokyo*
Tadashi Takeda, *Osaka*
Koji Takeuchi, *Kyoto*
Kiichi Tamada, *Tochigi*
Akira Tanaka, *Kyoto*
Eiji Tanaka, *Matsumoto*
Noriaki Tanaka, *Okayama*
Shinji Tanaka, *Hiroshima*
Hideki Taniguchi, *Yokohama*
Kyuichi Tanikawa, *Kurume*
Akira Terano, *Shimotsugagun*
Hitoshi Togash, *Yamagata*
Shinji Togo, *Yokohama*
Kazunari Tominaga, *Osaka*
Takuji Torimura, *Fukuoka*

Minoru Toyota, *Sapporo*
Akihito Tsubota, *Chiba*
Takato Ueno, *Kurume*
Naomi Uemura, *Tokyo*
Shinichi Wada, *Tochigi*
Hiroyuki Watanabe, *Kanazawa*
Toshio Watanabe, *Osaka*
Yuji Watanabe, *Ehime*
Toshiaki Watanabe, *Tokyo*
Chun-Yang Wen, *Nagasaki*
Satoshi Yamagiwa, *Niigata*
Koji Yamaguchi, *Fukuoka*
Takayuki Yamamoto, *Yokkaichi*
Takashi Yao, *Fukuoka*
Masashi Yoneda, *Tochigi*
Hiroshi Yoshida, *Tokyo*
Masashi Yoshida, *Tokyo*
Norimasa Yoshida, *Kyoto*
Hitoshi Yoshiji, *Nara*
Kentaro Yoshika, *Toyoake*
Yasunobu Yoshikai, *Fukuoka*
Masahide Yoshikawa, *Kashihara*
Katsutoshi Yoshizato, *Higashihiroshima*

Lebanon

Bassam N Abboud, *Beirut*
Ala I Sharara, *Beirut*
Joseph D Boujaoude, *Beirut*

Lithuania

Limas Kupcinskas, *Kaunas*

Macedonia

Vladimir C Serafimoski, *Skopje*

Malaysia

Andrew Seng Boon Chua, *Ipoh*
Khean-Lee Goh, Kuala *Lumpur*
Jayaram Menon, *Sabah*

Mexico

Diego Garcia-Compean, *Monterrey*
Eduardo R Marin-Lopez, *Jesús García*
Nahum Méndez-Sánchez, *Mexico*
Saúl Villa-Treviño, *México*

Monaco

Patrick Rampal, *Monaco*

Morocco

Abdellah Essaid, *Rabat*

The Netherlands

Ulrich Beuers, *Amsterdam*
Gerd Bouma, *Amsterdam*
Lee Bouwman, *Leiden*
J Bart A Crusius, *Amsterdam*
NKH de Boer, *Amsterdam*
Koert P de Jong, *Groningen*
Henrike Hamer, *Maastricht*
Frank Hoentjen, *Haarlem*
Janine K Kruit, *Groningen*
Ernst J Kuipers, *Rotterdam*
CBHW Lamers, *Leiden*
Ton Lisman, *Utrecht*
Yi Liu, *Amsterdam*
Jeroen Maljaars, *Maastricht*
Servaas Morré, *Amsterdam*
Chris JJ Mulder, *Amsterdam*
Michael Müller, *Wageningen*
Amado S Peña, *Amsterdam*
Robert J Porte, *Groningen*
Ingrid B Renes, *Rotterdam*
Andreas Smout, *Utrecht*
Paul E Sijens, *Groningen*
Reinhold W Stockbrugger, *Maastricht*
Luc JW van der Laan, *Rotterdam*
Karel van Erpecum, *Utrecht*
Gerard P VanBerge-Henegouwen,*Utrecht*

New Zealand

Ian D Wallace, *Auckland*

Nigeria

Samuel B Olaleye, *Ibadan*

Norway

Trond Berg, *Oslo*
Tom H Karlsen, *Oslo*
Helge L Waldum, *Trondheim*

Pakistan

Muhammad S Khokhar, *Lahore*
Syed MW Jafri, *Karachi*

Peru

Hector H Garcia, *Lima*

Poland

Tomasz Brzozowski, *Cracow*
Robert Flisiak, *Bialystok*
Hanna Gregorek, *Warsaw*
Dariusz M Lebensztejn, *Bialystok*
Wojciech G Polak, *Wroclaw*
Marek Hartleb, *Katowice*

Portugal

Miguel C De Moura, *Lisbon*

Russia

Vladimir T Ivashkin, *Moscow*
Leonid Lazebnik, *Moscow*
Vasiliy I Reshetnyak, *Moscow*

Saudi Arabia

Ibrahim A Al Mofleh, *Riyadh*
Ahmed Helmy, *Riyadh*

Serbia

Dusan M Jovanovic, *Sremska Kamenica*

Singapore

Bow Ho, *Singapore*
Khek-Yu Ho, *Singapore*
Fock Kwong Ming, *Singapore*
Francis Seow-Choen, *Singapore*

Slovakia

Silvia Pastorekova, *Bratislava*
Anton Vavrecka, *Bratislava*

Slovenia

Sasa Markovic, *Ljubljana*

South Africa

Rosemar Joyce Burnett, *Pretoria*
Michael C Kew, *Parktown*

South Korea

Byung Ihn Choi, *Seoul*
Ho Soon Choi, *Seoul*
Marie Yeo, *Suwon*
Sun Pyo Hong, *Gyeonggi-do*
Jae J Kim, *Seoul*
Jin-Hong Kim, *Suwon*
Myung-Hwan Kim, *Seoul*
Chang Hong Lee, *Seoul*
Jong Kyun Lee, *Seoul*
Eun-Yi Moon, *Seoul*
Jae-Gahb Park, *Seoul*
Dong Wan Seo, *Seoul*
Dong Jin Suh, *Seoul*
Byung Chul Yoo, *Seoul*

Spain

Juan G Abraldes, *Barcelona*
Agustin Albillos, *Madrid*
Raul J Andrade, *Málaga*
Luis Aparisi, *Valencia*
Fernando Azpiroz, *Barcelona*
Ramon Bataller, *Barcelona*
Josep M Bordas, *Barcelona*
Xavier Calvet, *Sabadell*
Jordi Camps, *Catalunya*
Andres Cardenas, *Barcelona*
Vicente Carreño, *Madrid*
Jose Castellote, *Barcelona*
Antoni Castells, *Barcelona*
Vicente Felipo, *Valencia*
Juan C Garcia-Pagán, *Barcelona*
Jaime B Genover, *Barcelona*
Javier P Gisbert, *Madrid*
Jaime Guardia, *Barcelona*
Isabel Fabregat, *Barcelona*
Mercedes Fernandez, *Barcelona*
Angel Lanas, *Zaragoza*
Juan-Ramón Larrubia, *Guadalajara*
Laura Lladóa, *Barcelona*
María IT López, *Jaén*
Juan R Malagelada, *Barcelona*
José M Mato, *Derio*
Juan F Medina, *Pamplona*
Miguel A Muñoz-Navas, *Pamplona*
Julian Panes, *Barcelona*
Miguel M Perez, *Valencia*
Miguel Perez-Mateo, *Alicante*

Josep M Pique, *Barcelona*
Jesús M Prieto, *Pamplona*
Sabino Riestra, *Pola De Siero*
Luis Rodrigo, *Oviedo*
Manuel Romero-Gómez, *Sevilla*
Joan Roselló-Catafau, *Barcelona*

Sweden

Einar S Björnsson, *Gothenburg*
Curt Einarsson, *Huddinge*
Per M Hellström, *Stockholm*
Ulf Hindorf, *Lund*
Elisabeth Hultgren-Hörnquist, *Örebro*
Anders E Lehmann, *Mölndal*
Hanns-Ulrich Marschall, *Stockholm*
Lars C Olbe, *Molndal*
Lars A Pahlman, *Uppsala*
Matti Sallberg, *Stockholm*
Magnus Simrén, *Göteborg*
Xiao-Feng Sun, *Linköping*
Ervin Tóth, *Malmö*
Weimin Ye, *Stockholm*
Christer S von Holstein, *Lund*

Switzerland

Chrish Beglinger, *Basel*
Pierre A Clavien, *Zurich*
Jean-Francois Dufour, *Bern*
Franco Fortunato, *Zürich*
Jean L Frossard, *Geneva*
Gerd A Kullak-Ublick, *Zurich*
Pierre Michetti, *Lausanne*
Francesco Negro, *Genève*
Bruno Stieger, *Zurich*
Radu Tutuian, *Zurich*
Stephan R Vavricka, *Zurich*
Gerhard Rogler, *Zurich*
Arthur Zimmermann, *Berne*

Turkey

Yusuf Bayraktar, *Ankara*
Figen Gurakan, *Ankara*
Aydin Karabacakoglu, *Konya*
Serdar Karakose, *Konya*
Hizir Kurtel, *Istanbul*
Osman C Ozdogan, *Istanbul*
Özlem Yilmaz, *Izmir*
Cihan Yurdaydin, *Ankara*

United Arab Emirates

Sherif M Karam, *Al-Ain*

United Kingdom

David H Adams, *Birmingham*
Simon Afford, *Birmingham*
Navneet K Ahluwalia, *Stockport*
Ahmed Alzaraa, *Manchester*
Lesley A Anderson, *Belfast*
Charalambos G Antoniades, *London*
Anthony TR Axon, *Leeds*
Qasim Aziz, *Manchester*
Nicholas M Barnes, *Birmingham*
Jim D Bell, *London*
Mairi Brittan, *London*
Alastair D Burt, *Newcastle*
Simon S Campbell, *Manchester*
Simon R Carding, *Leeds*
Paul J Ciclitira, *London*
Eithne Costello, *Liverpool*
Tatjana Crnogorac-Jurcevic, *London*
Harry Dalton, *Truro*
Amar P Dhillon, *London*
William Dickey, *Londonderry*
James E East, *London*
Emad M El-Omar, *Aberdeen*
Ahmed M Elsharkawy, *Newcastle Upon Tyne*
Annette Fristscher-Ravens, *London*
Elizabeth Furrie, *Dundee*
Daniel R Gaya, *Edinburgh*
Subrata Ghosh, *London*
William Greenhalf, *Liverpool*
Indra N Guha, *Southampton*
Peter C Hayes, *Edinburgh*
Gwo-Tzer Ho, *Edinburgh*
Anthony R Hobson, *Salford*
Lesley A Houghton, *Manchester*
Stefan G Hübscher, *Birmingham*
Robin Hughes, *London*
Pali Hungin, *Stockton*
David P Hurlstone, *Sheffield*
Rajiv Jalan, *London*
Janusz AZ Jankowski, *Oxford*
Brian T Johnston, *Belfast*
David EJ Jones, *Newcastle*
Roger Jones, *London*
Michael A Kamm, *Harrow*
Peter Karayiannis, *London*
Laurens Kruidenier, *Harlow*
Patricia F Lalor, *Birmingham*
Chee Hooi Lim, *Midlands*
Hong-Xiang Liu, *Cambridge*
Yun Ma, *London*
Kenneth E L McColl, *Glasgow*
Stuart AC McDonald, *London*
Dermot P Mcgovern, *Oxford*
Giorgina Mieli-Vergani, *London*
Nikolai V Naoumov, *London*
John P Neoptolemos, *Liverpool*
James Neuberger, *Birmingham*
Philip Noel Newsome, *Birmingham*
Mark S Pearce, *Newcastle Upon Tyne*
Stephen P Pereira, *London*
D Mark Pritchard, *Liverpool*
Sakhawat Rahman, *London*
Stephen E Roberts, *Swansea*
Marco Senzolo, *Padova*
Soraya Shirazi-Beechey, *Liverpool*
Robert Sutton, *Liverpool*
Simon D Taylor-Robinson, *London*
Paris P Tekkis, *London*
Ulrich Thalheimer, *London*
David G Thompson, *Salford*
Nick P Thompson, *Newcastle*
David Tosh, *Bath*
Frank I Tovey, *London*
Chris Tselepis, *Birmingham*
Diego Vergani, *London*
Geoffrey Warhurst, *Salford*
Alastair John Watson, *Liverpool*
Peter J Whorwell, *Manchester*
Roger Williams, *London*
Karen L Wright, *Bath*
Min Zhao, *Foresterhill*

United States

Manal F Abdelmalek, *Durham*
Gary A Abrams, *Birmingham*
Maria T Abreu, *New York*
Reid B Adams, *Virginia*
Golo Ahlenstiel, *Bethesda*
BS Anand, *Houston*
Frank A Anania, *Atlanta*
M Ananthanarayanan, *New York*
Gavin E Arteel, *Louisville*
Jasmohan S Bajaj, *Milwaukee*
Subhas Banerjee, *Palo Alto*
Peter A Banks, *Boston*
Jamie S Barkin, *Miami Beach*
Kim E Barrett, *San Diego*
Marc D Basson, *Detroit*
Anthony J Bauer, *Pittsburgh*
Wallace F Berman, *Durham*
Timothy R Billiar, *Pittsburgh*
Edmund J Bini, *New York*
David G Binion, *Milwaukee*
Jennifer D Black, *Buffalo*
Herbert L Bonkovsky, *Charlotte*
Carla W Brady, *Durham*
Andrea D Branch, *New York*
Robert S Bresalier, *Houston*
Alan L Buchman, *Chicago*
Ronald W Busuttil, *Los Angeles*
Alan Cahill, *Philadelphia*
John M Carethers, *San Diego*
David L Carr-Locke, *Boston*
Maurice A Cerulli, *New York*
Ravi S Chari, *Nashville*
Jiande Chen, *Galveston*
Xian-Ming Chen, *Omaha*
Xin Chen, *San Francisco*
Ramsey Chi-man Cheung, *Palo Alto*
William D Chey, *Ann Arbor*
John Y Chiang, *Rootstown*
Parimal Chowdhury, *Arkansas*
Raymond T Chung, *Boston*
James M Church, *Cleveland*
Ram Chuttani, *Boston*
Mark G Clemens, *Charlotte*
Ana J Coito, *Los Angeles*
Vincent Coghlan, *Beaverton*
David Cronin II, *New Haven*
John Cuppoletti, *Cincinnati*
Mark J Czaja, *New York*
Peter V Danenberg, *Los Angeles*
Kiron M Das, New *Brunswick*
Conor P Delaney, *Cleveland*
Jose L del Pozo, *Rochester*
Sharon DeMorrow, *Temple*
Deborah L Diamond, *Seattle*
Douglas A Drossman, *Chapel Hill*
Katerina Dvorak, *Tucson*
Bijan Eghtesad, *Cleveland*
Hala El-Zimaity, *Houston*
Michelle Embree-Ku, *Providence*
Sukru Emre, New *Haven*
Douglas G Farmer, *Los Angeles*
Alessio Fasano, *Baltimore*
Mark A Feitelson, *Philadelphia*
Ariel E Feldstein, *Cleveland*
Alessandro Fichera, *Chicago*
Robert L Fine, *New York*
Magali Fontaine, *Stanford*
Chris E Forsmark, *Gainesville*
Glenn T Furuta, *Aurora*
Chandrashekhar R Gandhi, *Pittsburgh*
Susan L Gearhart, *Baltimore*
Xupeng Ge, *Boston*
Xin Geng, *New Brunswick*
M Eric Gershwin, *Suite*
Jean-Francois Geschwind, *Baltimore*
Ignacio Gil-Bazo, *New York*
Shannon S Glaser, *Temple*
Ajay Goel, *Dallas*
Richard M Green, *Chicago*
Julia B Greer, *Pittsburgh*

James H Grendell, *New York*
David R Gretch, *Seattle*
Stefano Guandalini, *Chicago*
Anna S Gukovskaya, *Los Angeles*
Sanjeev Gupta, *Bronx*
David J Hackam, *Pittsburgh*
Stephen B Hanauer, *Chicago*
Gavin Harewood, *Rochester*
Margaret M Heitkemper, *Washington*
Alan W Hemming, *Gainesville*
Samuel B Ho, *San Diego*
Peter R Holt, *New York*
Colin W Howden, *Chicago*
Hongjin Huang, *Alameda*
Jamal A Ibdah, *Columbia*
Atif Iqbal, *Omaha*
Hajime Isomoto, *Rochester*
Hartmut Jaeschke, *Tucson*
Dennis M Jensen, *Los Angeles*
Cheng Ji, *Los Angeles*
Leonard R Johnson, *Memphis*
Michael P Jones, *Chicago*
Peter J Kahrilas, *Chicago*
Anthony N Kalloo, *Baltimore*
Marshall M Kaplan, *Boston*
Neil Kaplowitz, *Los Angeles*
Serhan Karvar, *Los Angeles*
Rashmi Kaul, *Tulsa*
Jonathan D Kaunitz, *Los Angeles*
Ali Keshavarzian, *Chicago*
Miran Kim, *Providence*
Joseph B Kirsner, *Chicago*
Leonidas G Koniaris, *Miami*
Burton I Korelitz, *New York*
Robert J Korst, *New York*
Richard A Kozarek, *Seattle*
Alyssa M Krasinskas, *Pittsburgh*
Michael Kremer, *Chapel Hill*
Shiu-Ming Kuo, *Buffalo*
Paul Y Kwo, *Indianapolis*
Daryl Tan Yeung Lau, *Galvesto*
Stephen J Lanspa, *Omaha*
Joel E Lavine, *San Diego*
Bret Lashner, *Cleveland*
Dirk J van Leeuwen, *Lebanon*
Glen A Lehman, *Indianapolis*
Alex B Lentsch, *Cincinnati*
Andreas Leodolter, *La Jolla*
Gene LeSage, *Houston*
Josh Levitsky, *Chicago*
Cynthia Levy, *Gainesville*
Ming Li, *New Orleans*
Zhiping Li, *Baltimore*
Zhe-Xiong Lian, *Davis*
Lenard M Lichtenberger, *Houston*
Gary R Lichtenstein, *Philadelphia*
Otto Schiueh-Tzang Lin, *Seattle*
Martin Lipkin, *New York*
Chen Liu, *Gainesville*
Edward V Loftus, *Rocheste*
Robin G Lorenz, *Birmingham*
Michael R Lucey, *Madison*
James D Luketich, *Pittsburgh*
Guangbin Luo, *Cheveland*
Henry T Lynch, *Omaha*
Patrick M Lynch, *Houston*
John S Macdonald, *New York*
Bruce V MacFadyen, *Augusta*
Willis C Maddrey, *Dallas*
Ashok Malani, *Los Angeles*
Mercedes Susan Mandell, *Aurora*
Peter J Mannon, *Bethesda*
Charles M Mansbach, *Tennessee*
John F Di Mari, *Texas*
John M Mariadason, *Bronx*
Jorge A Marrero, *Ann Arbor*
Paul Martin, *New York*
Paulo Ney Aguiar Martins, *Boston*
Wendy M Mars, *Pittsburgh*
Laura E Matarese, *Pittsburgh*
Richard W McCallum, *Kansas*
Beth A McCormick, *Charlestown*
Lynne V McFarland, *Washington*
Kevin McGrath, *Pittsburgh*
Harihara Mehendale, *Monroe*
Ali Mencin, *New York*
Fanyin Meng, *Ohio*
Stephan Menne, *New York*
Didier Merlin, *Atlanta*
Howard Mertz, *Nashville*
George W Meyer, *Sacramento*
George Michalopoulos, *Pittsburgh*
James M Millis, *Chicago*
Fabrizio Michelassi, *New York*
Albert D Min, *New York*
Pramod K Mistry, *New Haven*
Emiko Mizoguchi, *Boston*
Smruti R Mohanty, *Chicago*
Satdarshan S Monga, *Pittsburgh*
Timothy H Moran, *Baltimore*
Peter L Moses, *Burlington*
Steven F Moss, *Providence*
Andrew J Muir, *Durham*
Milton G Mutchnick, *Detroit*
Masaki Nagaya, *Boston*
Victor Navarro, *Philadelphia*
Laura E Nagy, *Cleveland*
Hiroshi Nakagawa, *Philadelphia*
Douglas B Nelson, *Minneapolis*
Justin H Nguyen, *Florida*
Patrick G Northup, *Charlottesville*
Christopher O'Brien, *Miami*
Robert D Odze, *Boston*
Brant K Oelschlager, *Washington*
Curtis T Okamoto, *Los Angeles*
Stephen JD O'Keefe, *Pittsburgh*
Dimitry Oleynikov, *Omaha*
Stephen J Pandol, *Los Angeles*
Georgios Papachristou, *Pittsburgh*
Pankaj J Pasricha, *Galveston*
Zhiheng Pei, *New York*
Michael A Pezzone, *Pittsburgh*
CS Pitchumoni, *New Brunswiuc*
Paul J Pockros, *La Jolla*
Jay Pravda, *Gainesville*
Massimo Raimondo, *Jacksonville*
GS Raju, *Galveston*
Raymund R Razonable, *Minnesota*
Murray B Resnick, *Providence*
Adrian Reuben, *Charleston*
Douglas K Rex, *Indianapolis*
Victor E Reyes, *Galveston*
Basil Rigas, *New York*
Yehuda Ringel, *Chapel Hill*
Richard A Rippe, *Chapel Hill*
Maribel Rodriguez-Torres, *Santurce*
Marcos Rojkind, *Washington*
Philip Rosenthal, *San Francisco*
Barry Rosser, *Jacksonville Florida*
Hemant K Roy, *Evanston*
Sammy Saab, *Los Angeles*
Shawn D Safford, *Norfolk*
Dushyant V Sahani, *Boston*
Bruce E Sands, *Boston*
James M Scheiman, *Ann Arbor*
Eugene R Schiff, *Miami*
Nicholas J Shaheen, *Chapel Hill*
Vanessa M Shami, *Charlottesville*
Prateek Sharma, *Kansas City*
Harvey L Sharp, *Minneapolis*
Stuart Sherman, *Indianapolis*
Shivendra Shukla, *Columbia*
Alphonse E Sirica, *Virginia*
Shanthi V Sitaraman, *Atlanta*
Stuart J Spechler, *Dallas*
Shanthi Srinivasan, *Atlanta*
Michael Steer, *Boston*
Peter D Stevens, *New York*
Charmaine A Stewart, *Rochester*
Christian D Stone, *Saint Louis*
Gary D Stoner, *Columbus*
R Todd Stravitz, *Richmond*
Liping Su, *Chicago*
Christina Surawicz, *Seattle*
Robert W Summers, *Iowa City*
Wing-Kin Syn, *Durham*
Gyongyi Szabo, *Worcester*
Yvette Taché, *Los Angeles*
Seng-Lai Tan, *Seattle*
Andrzej S Tarnawski, *Orange*
K-M Tchou-Wong, *New York*
Jonathan P Terdiman, *San Francisco*
Neil D Theise, *New York*
Christopher C Thompson, *Boston*
Swan N Thung, *New York*
Michael Torbenson, *Baltimore*
Natalie J Torok, *Sacramento*
RA Travagli, *Baton Rouge*
George Triadafilopoulos, *Stanford*
Chung-Jyi Tsai, *Lexington*
Janet Elizabeth Tuttle-Newhall, *Durham*
Andrew Ukleja, *Florida*
Michael F Vaezi, *Nashville*
Hugo E Vargas, *Scottsdale*
Arnold Wald, *Wisconsin*
Scott A Waldman, *Philadelphia*
Jian-Ying Wang, *Baltimore*
Timothy C Wang, *New York*
Irving Waxman, *Chicago*
Steven A Weinman, *Galveston*
Steven D Wexner, *Weston*
Keith T Wilson, *Baltimore*
Jacqueline L Wolf, *Boston*
Jackie Wood, *Ohio*
George Y Wu, *Farmington*
Jian Wu, *Sacramento*
Samuel Wyllie, *Houston*
Wen Xie, *Pittsburgh*
Vijay Yajnik, *Boston*
Vincent W Yang, *Atlanta*
Francis Y Yao, *San Francisco*
Hal F Yee, *San Francisco*
Xiao-Ming Yin, *Pittsburgh*
Min You, *Tampa*
Zobair M Younossi, *Virginia*
Liqing Yu, *Winston-Salem*
David Yule, *Rochester*
Ruben Zamora, *Pittsburgh*
Michael E Zenilman, *New York*
Zhi Zhong, *Chapel Hill*
Michael A Zimmerman, *Colorado*
Stephen D Zucker, *Cincinnati*

Uruguay

Henry Cohen, *Montevideo*

[1]Passed away on October 20, 2007
[2]Passed away on June 11, 2007

National Journal Award 2005

World Journal of Gastroenterology®

Weekly Established in October 1995

Volume 14 Number 39
October 21, 2008

Contents

Contents

World Journal of Gastroenterology
Volume 14 **Number** 39 **October** 21, 2008

World Journal of Gastroenterology
Volume 14 **Number** 39 **October** 21, 2008

Contents

NAME OF JOURNAL
World Journal of Gastroenterology

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

EDITING
Editorial Board of *World Journal of Gastroenterology,* Room 903, Ocean International Center, Building D, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

PUBLISHING
The WJG Press and Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Ocean International Center, Building D, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

PRINTING
Beijing Kexin Printing House

OVERSEAS DISTRIBUTOR
Beijing Bureau for Distribution of Newspapers and Journals
(Code No. 82-261)
China International Book Trading Corporation PO Box 399, Beijing, China (Code No. M4481)

PUBLICATION DATE
October 21, 2008

EDITOR-IN-CHIEF
Lian-Sheng Ma, *Beijing*

SUBSCRIPTION
RMB 50 Yuan for each issue, RMB 2400 Yuan for one year

CSSN
ISSN 1007-9327
CN 14-1219/R

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/wjg/help/instructions.jsp. If you do not have web access please contact the editorial office.

ONLINE SUBMISSION
http://wjg.wjgnet.com

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5945

World J Gastroenterol 2008 October 21; 14(39): 5945-5961
World Journal of Gastroenterology ISSN 1007-9327

REVIEW

Role of sex steroid receptors in pathobiology of hepatocellular carcinoma

Mamta Kalra, Jary Mayes, Senait Assefa, Anil K Kaul, Rashmi Kaul

Mamta Kalra, Jary Mayes, Senait Assefa, Anil K Kaul, Rashmi Kaul, Department of Biochemistry and Microbiology, Oklahoma State University Center for Health Sciences, Tulsa, Oklahoma 74107, United States
Correspondence to: Rashmi Kaul, PhD, Associate Professor of Immunology, Department of Biochemistry and Microbiology, Oklahoma State University Center for Health Sciences, 1111 W 17th street, Tulsa, Oklahoma 74107, United States. rashmi.kaul10@okstate.edu
Telephone: +1-918-5611231 Fax: +1-918-5615798
Received: May 8, 2008 Revised: September 16, 2008
Accepted: September 23, 2008
Published online: October 21, 2008

Abstract

The striking gender disparity observed in the incidence of hepatocellular carcinoma (HCC) suggests an important role of sex hormones in HCC pathogenesis. Though the studies began as early as in 1980s, the precise role of sex hormones and the significance of their receptors in HCC still remain poorly understood and perhaps contribute to current controversies about the potential use of hormonal therapy in HCC. A comprehensive review of the existing literature revealed several shortcomings associated with the studies on estrogen receptor (ER) and androgen receptor (AR) in normal liver and HCC. These shortcomings include the use of less sensitive receptor ligand binding assays and immunohistochemistry studies for ERα alone until 1996 when ERβ isoform was identified. The animal models of HCC utilized for studies were primarily based on chemical-induced hepatocarcinogenesis with less similarity to virus-induced HCC pathogenesis. However, recent *in vitro* studies in hepatoma cells provide newer insights for hormonal regulation of key cellular processes including interaction of ER and AR with viral proteins. In light of the above facts, there is an urgent need for a detailed investigation of sex hormones and their receptors in normal liver and HCC. In this review, we systematically present the information currently available on androgens, estrogens and their receptors in normal liver and HCC obtained from *in vitro*, *in vivo* experimental models and clinical studies. This information will direct future basic and clinical research to bridge the gap in knowledge to explore the therapeutic potential of hormonal therapy in HCC.

Key words: Sex hormones; Estrogen receptor; Androgen receptor; Hepatocellular carcinoma; Hepatocarcinogenesis

Peer reviewers: Dr. Henning Schulze-Bergkamen, First Medical Department, University of Mainz, Langenbeckstrasse 1, Mainz 55101, Germany; Dr. Ursula M Gehling, Department of Hepatobiliary Surgery and Visc, University Hospital Hamburg-Eppendorf, Martinistrasse 52, Hamburg 20246, Germany

Kalra M, Mayes J, Assefa S, Kaul AK, Kaul R. Role of sex steroid receptors in pathobiology of hepatocellular carcinoma. *World J Gastroenterol* 2008; 14(39): 5945-5961 Available from: URL: http://www.wjgnet.com/1007-9327/14/5945.asp DOI: http://dx.doi.org/10.3748/wjg.14.5945

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most lethal malignancies with limited treatment options. The major risk factors for HCC are chronic liver diseases with cirrhosis that include hepatitis B, hepatitis C, alcoholic liver disease and non-alcoholic steatohepatitis. Epidemiological reports indicate that regardless of etiologies, the incidence of HCC is higher in males than in females with the male: female ratio usually averaging between 2:1 and 4:1[1]. This male predominance is further supported by the clinical observations that chronic liver disease progresses more rapidly to cirrhosis in males than females and therefore cirrhosis that leads to HCC development is largely considered to be the disease of men and postmenopausal women[2]. In view of this remarkable gender disparity, various *in vitro* as well as *in vivo* studies have been initiated from time to time to explore the importance of sex hormones in HCC. However, the precise role of male and female sex hormones and their receptors in HCC remains still poorly understood. Androgens have been suggested to induce and promote HCC[3] and altered androgen metabolism has been reported to be associated with HCC[4]. In contrast, the role of estrogen in HCC has been controversial with evidence suggesting both carcinogenic and protective effects in the liver[3,5-9]. Very limited information is currently available regarding the mechanism of estrogen and androgen action in normal liver as well as in HCC.

It is well known that estrogen and androgen mediate their biological functions by binding with a high affinity to specific receptors, the estrogen receptor (ER) and the androgen receptor (AR). Both ER and AR belong to the family of nuclear receptors that act as transcription factors and regulate the expression of several genes. Our present day knowledge of structure and function of these receptors is primarily attributed to the extensive research on ER and AR in cancer of reproductive organs. However, recent advances in molecular research reveal that sex hormones do play a significant role in normal physiology of various organs other than the organs of the reproductive system. Both androgens and estrogens regulate transcriptional activation of various molecules involved in key cellular processes such as generation of immune responses, cell proliferation and apoptosis through functional receptors localized in various sub-cellular organelles[10-12].

The expression and functional status of AR and ER appear to play a significant role in the carcinogenesis of all hormone sensitive organs. However, liver has remained a less studied organ in the context of sex hormones and their receptors. Differential expression of wild type and variant forms of ER and AR has been reported in normal liver and HCC, indicating a strong link between sex hormones and pathogenesis of HCC[13-19]. Recent *in vitro* studies also provide further evidence in support of AR and ER involvement in various cellular events as well as interaction with viral proteins in hepatitis B virus (HBV) and hepatitis C virus (HCV)-induced HCC[20-25]. This review is focused on the compilation of the information so far available on the significance of AR and ERs in HCC and brings forth wide gaps in the existing knowledge to the notice of scientific world for future research.

SEX HORMONE RECEPTORS IN NORMAL HUMAN LIVER AND HCC

Estrogen receptor

The role of estrogen in modulating morphological and physiological features of liver became evident in early 1970s when a possible correlation between occurrence of hepatic neoplasms and use of oral contraceptives was suggested[26,27]. In the reproductive system, estrogen is known to act by binding to specific cytoplasmic and nuclear receptors. Hence, search began to identify such a receptor in the liver. In 1978, Duffy and Duffy first reported the presence of ER in normal human liver[28]. Subsequently, the presence of ERs in human HCC was demonstrated by Molteni *et al*[29] followed by Friedman *et al*[30] and Iqbal *et al*[31]. Since then, a number of studies have been reported addressing the expression of ERs in normal as well as neoplastic liver tissues. The early studies used indirect methods of receptor detection based on ligand binding assays. Table 1 gives the details of these studies[13,15,17,30-37]. These assays were quantitative and measured the amount of receptor in the samples as well as its affinity for the ligand in terms of dissociation constants. The percentage positivity for ER expression varied significantly among different studies. These variations may be attributed to the differences in sample size, methodologies, ethnicity of the population studied, stage of the disease and underlying etiologies. Earlier studies by Friedman *et al*[30] and Iqbal *et al*[31] showed that ER content is similar in HCC and normal liver. In contrast, later studies consistently showed that the expression of ER is decreased in HCC tissue specimens as compared to normal liver tissue specimens or the non-tumor part of the liver[17,32,38]. However, Eagon *et al*[33] documented elevated levels of cytosolic ER in 3 of the 9 tumors as compared to non-cancerous tissues. Nuclear ER expression was found to be suppressed in all HCC samples as compared to normal samples[33]. The major drawback of these studies was the use of binding assays for detection that do not provide any information on the subtype of ER, i.e. ERα and ERβ as known today. It is important to study the relative expression of both isoforms of ER since ERα and ERβ are known to have overlapping but quite distinct functions. There are few reports on direct detection of ER using specific antibodies. Table 1 gives the details of these studies[32,39-47]. However, all these studies have employed either immunohistochemistry (IHC) or enzyme-immuno assays (EIA) using antibodies specific for only ERα isoform. The ERβ isoform was identified later in 1996[48] and information on the expression of ERβ protein in HCC is lacking though few studies at mRNA levels have been documented.

Since the molecular characterization and cloning of ERα in the mid 1980s[49,50], attempts have been made to determine the expression of ER in liver tumors at mRNA level. Table 2 gives the details of these studies[18,19,40,45,51,52]. *In situ* hybridization using ER specific oligonucleotide sequence probe revealed that 11 out of 15 HCC tissue samples expressed ER mRNA[40]. Interestingly, the same samples were found to be negative for ER protein by IHC, suggesting the use of more sensitive methods and more specific antibodies for detecting ER at protein level. Subsequently, the mRNA expression of ER in HCC tissues was studied in different populations by a highly sensitive method of reverse transcriptase-polymerase chain reaction (RT-PCR). Villa *et al*[51] were the first to demonstrate the presence of wild type ERα in peritumoral and tumoral tissue of HCC patients using this technique. The use of RT-PCR further enabled the authors to detect a splice variant of ERα lacking exon 5 in the hormone binding domain[18,51]. A similar splice variant has been described in breast cancer tissues to be associated with tumor pathogenesis[53]. The significance of the variant ERα (vERα) in pathology, prognosis and treatment of HCC has also been studied. The presence of vER receptor is able to influence the natural history of patients with HCC by regulating tumor growth as well as patient survival. The presence of the liver vERα transcript in the tumor has been described to be the strongest negative predictor of survival in operable HCC patients[54,55]. Furthermore, the presence of vERα was found to correlate with a higher

Table 1 Estrogen receptor expression in liver tissue samples from HCC patients

Subcellular localization	Method	ER subtype/Antibody source	*n*	Subjects	Positive cases	Country and area	Yr	Reference
Cytosolic	BA	NA	5	5 M	5 M	United States	1982	[30]
Cytosolic and nuclear	BA	NA	5	3 M, 2 F	3 M, 2 F	United Kingdom	1983	[31]
Cytosolic	IHC	NA, Anti ER Ab Hypolabs, Switzerland	10	NA	1	Singapore	1984	[39]
Cytosolic	BA	NA	30	29 M, 1 F	12	Japan	1986	[13]
Cytosolic and nuclear	BA	NA	8	6 M, 2 F	1 M	Japan	1986	[17]
Cytosolic	BA	NA	13	9 M, 4 F	1 F	Japan	1987	[32]
Cytosolic	EIA	Abbot ER-EIA monoclonal kit	13	9 M, 4 F	3 M, 2 F	Japan	1987	[32]
Cytosolic	BA	NA	19	19 F	7 F	Japan	1989	[15]
Cytosolic	BA	NA	66	52 M, 14 F	23 M, 3 F	Japan	1990	[34]
Cytosolic	BA	NA	6	4 M, 2 F	1 F	Japan	1990	[35]
Cytosolic	BA	NA	21	18 M, 3 F	9 M, 1 F	Japan	1991	[36]
Cytosolic and nuclear	BA	NA	9	6 M, 3 F	6 M, 2 F	Italy, United States	1991	[37]
Cytosolic and nuclear	BA	NA	9	6 M, 3 F	6 M, 2 F	Italy, United States	1991	[33]
NA	IHC	NA, Abott, ER-ICA	15	12 M, 3 F	0	Italy	1993	[40]
Cytosolic	EIA	NA, Abbot anti ER	26	18 M, 8 F	4	Spain	1993	[41]
Cytosolic	EIA	NA, Abbot anti ER	33	20 M, 13 F	8 M, 5 F	Germany	1997	[42]
Cytosolic	IHC	NA, ER monoclonal Ab, Dako	71	59 M, 12 F	15 M, 2 F	Hong Kong	1997	[43]
Cytosolic and nuclear	IHC	ERα, Santacruz	45	37 M, 8 F	21 (cytosolic) 11 (nuclear)	United States, Korea[1]	2004	[44]
NA	IHC	NA	28	NA	11	China	2004	[45]
NA	IHC	NA	66		3	Mexico	2007	[46]
Nuclear	IHC	ERα, Dako (ID5)	31	26 M, 5 F	12 M, 4 F	Spain	2007	[47]

ER: Estrogen receptor; BA: Binding assay; EIA: Enzyme immunoassay; IHC: Immunohistochemistry; *n*: Sample size; M: Male; F: Female; NA: Information not available; Ab: Antibody. [1]Study conducted in USA on patient population from Korea.

Table 2 Messenger RNA (mRNA) expression of estrogen receptor in liver tissue samples from HCC patients

Method	ER subtype	*n*	Subjects	Positive cases	Country	Yr	Reference
ISH	ERα Wt	15	12 M, 3 F	11	Italy	1993	[40]
RT-PCR	ERα Wt	14	7 M, 7 F	1 M, 7 F	Italy	1995	[51]
RT-PCR	ERα delta5 variant	14	7 M, 7 F	7 M, 3 F	Italy	1995	[51]
RT-PCR	ERα Wt	40	25 M, 15 F	16 M, 12 F	Italy	1998	[18]
RT-PCR	ERα delta5 variant	40	25 M, 15 F	20 M, 10 F	Italy	1998	[18]
RT-PCR	ERα Wt	42	35 M, 7 F	20 M, 5 F	Italy	2003	[19]
RT-PCR	ERα delta5 variant	42	35 M, 7 F	37	Italy	2003	[19]
RT-PCR	ERβ Wt	42	35 M, 7 F	12 M, 4 F	Italy	2003	[19]
RT-PCR	ERα Wt	28	NA	25	China	2004	[45]
RT-PCR	ERα delta5 variant	28	NA	27	China	2004	[45]
RT-PCR	ERα Wt	32	23 M, 9 F	23 M, 9 F	Korea	2006	[52]
RT-PCR	ERα delta5 variant	32	23 M, 9 F	21 M, 9 F	Korea	2006	[52]
RT-PCR	ERβ Wt	32	23 M, 9 F	26	Korea	2006	[52]

ER: Estrogen receptor; ISH: *In situ* hybridization; RT-PCR: Reverse transcriptase-polymerase chain reaction; Wt: Wild type; *n*: Sample size; M: Male; F: Female; NA: Information not available.

clinical aggressiveness of the tumor in comparison with the tumors characterized by wild-type ERα transcript. These tumors were responsive to megestrol and unresponsive to anti estrogen tamoxifen. High rates of vERα expression have been shown to be present in men at high risk of HCC development[56,57]. In patients with chronic hepatitis and cirrhosis, the expression of vERα has been associated with higher oxidative stress-induced DNA damage and c-myc mRNA expression, a factor indicating increased genomic instability, augmented cytoproliferation and carcinogenesis[5].

Using RT-PCR, in addition to ERα, the expression of the lately identified isoform of ER, i.e. ERβ, has also been studied in HCC patients. Iavarone *et al*[19] report that both ERβ and ERα wild type receptors either alone or together with vER are co-expressed more frequently in patients with chronic liver disease than in those with HCC. However, both ERs are similarly expressed in tumoral and extratumoral tissues of HCC patients[19]. In this study, HBV-related tumors either expressed wild type ERα and ERβ or expressed variant ER and ER delta 5 more often than HCV-related tumor, and HBV-related tumors showed a tendency towards loss of ERβ expression as the disease progressed from chronic inflammatory liver disease to HCC[19].

Breast cancer studies suggest that ERα:ERβ expression ratio changes during carcinogenesis and is believed to play a role in tumor development[58]. Recently, Wang *et al*[52] studied the expression of ERα and ERβ in HCC tissues of Korean population using RT-PCR, and assessed 32 tumoral and peritumoral tissues from HCC patients with underlying chronic HBV or HCV

Table 3 Estrogen receptor and androgen receptor expression in normal and non-cancerous liver tissue samples

Receptor protein/mRNA	Type of liver tissue	Subjects	Positive cases	Method	Country	Yr	Reference
Estrogen Receptor (ER)							
ER protein (cytosolic)	Normal liver tissue	4 F	4 F	BA	United Kingdom	1978	[28]
ER protein	Normal	3 F	3 F	BA	Germany	1978	[59]
ER protein	Normal	2 M	2 M	BA	United States	1982	[30]
ER protein	Normal	1 M, 5 F	1 M, 5 F	BA	Germany	1982	[60]
ER protein	Normal	3 M, 3 F	3 M, 3 F	BA	United States	1983	[61]
ER protein (cytosolic & nuclear)	Normal	2 M, 2 F	2 M, 2 F	BA	United Kingdom	1983	[31]
ER protein (cytosolic)	Surrounding liver tissue	30	13	BA	Japan	1986	[13]
ER protein (cytosolic & nuclear)	Non-cancerous tissue	7	3	BA	Japan	1986	[17]
ER protein (cytosolic & nuclear)	Normal	NA	NA	BA	United States	1987	[62]
ER protein (cytosolic)	Non-cirrhotic liver	5 M, 7 F	5 M, 7 F	BA, EIA	Japan	1987	[32]
ER protein	Normal	2	2		Japan	1988	[63]
ER protein (cytosolic)	Surrounding liver tissue	17	11	BA	Japan	1989	[15]
ER protein	Surrounding non-cancerous tissue	22	14	NA	Japan	1989	[64]
ER protein (cytosolic)	Surrounding normal liver	4 M, 1 F	4 M, 1 F	BA	Japan	1990	[35]
ER protein	Adjacent normal tissue	6 M, 3 F	6 M, 3 F	BA	Italy, United States	1991	[33]
ER protein (cytosolic)	Non-tumoral liver	18 M, 8 F	9 M, 2 F	BA	Spain	1993	[41]
ER mRNA	Non-tumorous liver tissue	13	7	ISH	Italy	1993	[40]
ER mRNA	Peri-tumor tissue	32	28	RT-PCR	Korea	2006	[52]
Androgen Receptor (AR)							
AR protein (cytosolic & nuclear)	Normal	2 M, 2 F	0	BA	United Kingdom	1983	[31]
AR protein (cytosolic & nuclear)	Non-cancerous tissue	6	1	BA	Japan	1986	[17]
AR protein (cytosolic)	Non-neoplastic liver tissues	17	11	BA	Japan	1989	[15]
AR protein	Surrounding non-cancerous tissues	21	7	NA	Japan	1989	[64]
AR protein (cytosolic)	Surrounding liver	9 M, 1 F	7 M, 1 F	BA	Japan	1990	[35]
AR protein	Adjacent normal tissue	6 M, 3 F	6 M, 3 F	NA	Italy, United States	1991	[33]
AR mRNA	Peri-tumor tissue	23 M, 9 F	23 M, 9 F	RT-PCR	Korea	2006	[52]

BA: Binding assay; EIA: Enzyme immunoassay; IHC: Immunohistochemistry; M: Male; F: Female; NA: Information not available.

infection and observed that wild type and variant ERα are expressed in all the samples. However, the expression of vERα is stronger in tumor than in peritumor tissues. Interestingly, ERβ was found to be significantly over-expressed in HCV-infected HCC tissues as compared to HBV-infected HCC tissues. The differences in ER expression in HCV-infected HCC tissues compared to HBV-infected HCC tissues suggest different pathogenetic mechanisms. Overall from these studies there appears to be a change in co-expression pattern of ERα and ERβ from cirrhosis to HCC development in both HBV- and HCV- related tumors. Thus, these studies provide further evidence in support of importance of wild type and variant ERs in HCC, suggesting detailed investigations in this area.

Androgen receptor

Like estrogens, androgens have also been reported to play an important role in liver carcinogenesis. Iqbal *et al*[31] showed the presence of androgen receptors (AR) in HCC in 1983. However, it was not until 1985 that normal human liver was believed to express AR. In 1985, Nagasue and colleagues[14] demonstrated the presence of AR in normal human liver as well as in tumor and non-tumor parts of HCC tissues. Since then, several reports showing protein and mRNA expression of AR in liver have been published. Table 3[13,15,17,28,30-33,35,40,41,52,59-64] and Table 4[14-17,31,33-36,39,41,47,52,65-71] give the details of these studies. In general, AR is found to be over expressed in liver tumor compared to the adjacent normal tissue[15,17,33,36,38]. However, like ER expression studies, majority of the early studies employed indirect binding assays to detect AR in the liver tissues. More precise quantitative methods of direct detection of AR protein are needed. Reports using antibody-based detection of AR in liver tissues are very sparse.

The mRNA levels of AR have been assessed in the non-cancerous and HCC tissues primarily by RT-PCR (Tables 3 and 4). In 1994, Negro and colleagues[16] developed a non-radioisotopic *in situ* hybridization assay specific for human AR mRNA and found that 73% of HCC tissues could express variable amount of AR mRNA. However, normal hepatocytes were stained weakly in 42% of the non-neoplastic tissues. Though initial binding studies demonstrated higher AR levels in tumor tissues than in respective peri-tumoral part[15,17,33,36,38], more recent observations based on mRNA expression do not reveal any significant differences in tumor and peri-tumor tissues. Tavian *et al*[71] found higher AR mRNA levels in tumor than in the corresponding peri-tumoral tissue in a relatively small percentage of HCC samples, suggesting that AR mRNA levels are associated with the histological tumor differentiation showing a lower AR expression in poorly-differentiated HCC than in well-differentiated tumors. In contrast, AR levels in Korean population with HCC do not show differences between tumor and peri-tumor tissues using RT-PCR[52]. Due to these conflicting reports on AR expression in HCC, there is a need for detailed investigation of AR mRNA as well as protein levels using more sensitive and accurate detecting quantitative methods.

Table 4 Androgen receptor protein and mRNA expression in liver tissue samples from HCC patients

	Subcellular Organelle	Method	*n*	Subjects	Positive	Country	Yr	Reference
AR protein	Cytosolic and nuclear	BA	5	3 M, 2 F	3 M, 2 F	United Kingdom	1983	[31]
	Cytosolic	IHC	10	NA	5	Singapore	1984	[39]
	Cytosolic	BA	19	19 M	14 M	Japan	1985	[14]
	Cytosolic and nuclear	BA	5	3 M, 2 F	3 M, 2 F	United Kingdom	1985	[65]
	Cytosolic and nuclear	BA	8	6 M, 2 F	2 M, 2 F	Japan	1986	[17]
	Cytosolic	BA	13	8 M, 5 F	8 M, 5 F	United Kingdom	1988	[66]
	Cytosolic	BA	19	19 F	7 F	Japan	1989	[15]
	Cytosolic	BA	45	31 M, 14 F	25 M, 6 F	Japan	1989	[67]
	Cytosolic	BA	11	9 M, 2 F	6 M, 1 F	Japan	1990	[35]
	Cytosolic	BA	21	18 M, 3 F	18	Japan	1991	[36]
	Cytosolic and nuclear	BA	9	6 M, 3 F	6 M, 3 F	Italy, United States	1991	[33]
	Cytosolic	BA	5	3 M, 2 F	3 M, 2F	Japan	1992	[68]
	Cytosolic	BA	26	18 M, 8 F	14	Spain	1993	[41]
	Cytosolic	BA	43	30 M, 13 F	28	Spain	1995	[69]
	NA	BA	32			China	1998	[70]
	Nuclear	IHC	31	26 M, 5 F	18 M, 3 F	Spain	2007	[47]
AR mRNA		ISH	22	16 M, 6 F	13 M, 3 F	Italy	1994	[16]
		RT-PCR	38	24 M, 14 F	21 M, 13 F	Italy	2002	[71]
		RT-PCR	32	23 M, 9 F	23 M, 9 F	Korea	2006	[52]

AR: Androgen receptor; BA: Binding assay; IHC: Immunohistochemistry; ISH: *In situ* hybridization; RT-PCR: Reverse transcriptase-polymerase chain reaction; *n*: Sample size; M: Male; F: Female; NA: Information not available.

CLINICAL AND PATHOLOGICAL SIGNIFICANCE OF ESTROGEN AND ANDROGEN RECEPTORS IN HCC

Despite a wide variability observed in studies of ER and AR expression in HCC, attempts have been made to determine the significance of these receptors by correlating their levels with clinical and pathological parameters. Table 5[13,15,34,42,55,69,70,72] and Table 6[30,54,73-77] present the salient findings of such clinical studies.

In few earlier studies using binding assays, no correlation was found between ER protein expression and sex, age, alcohol abuse, serum alpha-feto protein, carcinoembryonic antigen, HBV markers or tumor histology[15,34]. However, in subsequent reports, ER mRNA levels were shown to be associated with sex and viral etiology[19,51,52]. Increased vERα expression has been demonstrated more often in males than in females with HCC in Italian population, suggesting a strong link of ER with a higher incidence of HCC in males[19,51]. On the other hand, in Korean subjects no correlation has been found between the expression of vERα and HCC prevalence in males[52]. Interestingly, a distinct difference in ER expression pattern was observed in HBV- and HCV-infected HCC patients. Delta 5 deletion variants of ERα (vERα) and ERβ were found to be more often expressed in HBV-related tumors than in HCV-related tumors (67% *vs* 15%, $P < 0.0007$)[19]. In contrast, Wang *et al*[52] showed no remarkable difference in vERα levels in HCV- and HBV-infected HCC tissues (91.3% *vs* 100%). Nevertheless, a predominant expression of ERβ has been reported in HCV-infected than in HBV-infected patients with HCC (95.7% *vs* 44.4%, $P < 0.05$), suggesting that ERβ may play an important role in HCV-induced liver disease[52].

In addition to gender and etiological factors, tumor size, histopathology, operative mortality, tumor recurrence and survival after curative resection have also been studied in relation to ER expression in HCC[34,42,55]. Nagasue *et al*[34] showed that the large tumors are more commonly found in ER negative HCC patients and therefore the incidence of major hepatic resection is significantly higher in this group than in ER positive HCC patients. However, they did not report significant differences in histopathology of ER positive and ER negative tumors. Rates of mortality, tumor recurrence and long-term survival were also found to be similar in the two groups. In contrast to these observations, Jonas *et al*[42] showed that in patients undergoing curative resection, the 1- and 2-year survival rates in ER positive group are substantially lower than in ER negative group[42], suggesting that ER positive status has a negative effect on patient survival after curative resection of advanced HCC. Further, significantly longer survival rates have been reported in HCC patients with wild type ERs than in those expressing variant ERs[55].

In contrast with ER expression, AR levels are strongly associated with intra hepatic recurrence of tumors. The 5-year survival of recurrence free HCC patients was shown to be 55% for AR negative, 24% for ER negative, 10% for ER positive and 0% for AR positive tumors[67]. Similar findings have been reported by other researchers, suggesting a negative impact of AR positivity on tumor recurrence[69]. It was reported that AR negative patients show significantly better survival than AR positive patients[70]. Considering the tumor size, variable results have been documented in relation with AR expression. Boix *et al*[69] showed that AR expression is significantly related to smaller tumor size while Zhang *et al*[70] found that AR levels are positively correlated to tumor size.

Table 5 Studies on correlation of estrogen receptor and androgen receptor expression with clinical and pathological parameters in HCC patients

Receptor protein/ mRNA expression	Clinical parameter	*n*	Country	Salient findings	Yr	Reference
ER protein	Serum alpha-fetoprotein, carcinoembryonic antigen, HBV profile, tumor histology	30	Japan	No correlation with any parameter	1986	[13]
ER and AR protein	Serum alpha fetoprotein, HBV markers, histopathology	19	Japan	No correlation	1989	[15]
ER protein	Sex, age, alcohol abuse, underlying liver disease, hepatic functions	66	Japan	No correlation	1990	[34]
	Tumor size, hepatic resection Histopathology			Large tumor size and higher rate of resection in ER- No differences in ER+ and ER-		
	Operative mortality, tumor recurrence, long-term survival rate			Similar in ER+ and ER-		
ER and AR protein	Intrahepatic recurrence	78	Japan	AR expression strongly associated with intrahepatic recurrence. Weak association with ER expression	1995	[72]
ER protein	Survival after curative resection	28	Germany	Negative effect of an ER+ tumor on patient survival after curative resection	1997	[42]
Wild type and variant ER mRNA	Survival	96	Italy	Significantly long survival in patients with wild type ERs than variant ERs	2000	[55]
AR protein	Recurrence rate	45	Japan	Significantly higher recurrence rates in AR+ group than AR-	1989	[15]
	Survival rate			Significantly better survival rates in AR- patients than in AR+		
AR protein	Tumor size	43	Spain	AR expression was significantly related to smaller tumor size	1995	[69]
	Tumor recurrence			Higher tumor recurrence rates in surrounding tissues of AR+ than AR-		
AR protein	Tumor size and survival time	32	China	Survival rate correlated inversely with the levels of AR expression	1998	[70]
				AR levels had positive correlation with the tumor size		

CURRENT STATUS OF HORMONAL TREATMENT IN HCC

The association of estrogens and androgens in HCC observed in basic and clinical studies has led to initiation of various clinical trials on hormonal treatment of HCC. Differential clinical outcome was reported in these trials that have resulted in continued debate about the use of hormonal therapy in HCC. Table 6 presents a list of few clinical studies that utilized hormonal therapy in HCC patients. A systematic review of these clinical trials on therapeutic evaluation of anti-estrogen and anti-androgen agents in liver cancer has been recently compiled by Di Maio *et al*[78]. The authors conclude that hormonal treatment should not be a part of the current management of HCC patients[78,79]. However, in most of these clinical studies, various inherent factors may have contributed to the observed inconclusive results. Few of these may include faulty patient subset selection criteria, no monitoring of tumor ER and AR expression at the time of recruitment and also during treatment of these patients and lastly the type of hormonal treatment given to the patient. Therefore, the debatable potential of hormone therapy in HCC may finally be attributed to the lack of complete understanding of ER and AR expression and hormonal responsiveness in the liver and their involvement in development of HCC.

Currently, limited information is available on the functional significance of ER and AR in HCC. In the following sections, we review the *in vivo* animal studies on liver carcinogenesis and *in vitro* studies on cell lines that have been conducted to understand the role of ER and AR in the liver.

ESTROGEN RECEPTOR AND ANDROGEN RECEPTOR STUDIES IN ANIMAL MODELS OF EXPERIMENTAL LIVER CARCINOGENESIS

Estrogen receptor studies

Several attempts have been made to establish the role of estrogen and its receptors in hepatocarcinogenesis using animal models. Rat is the most extensively used model to study liver carcinogenesis. Rat hepatocytes are known to express ERs. ERα is the predominant isoform expressed in rat hepatocytes while cholangiocytes express both ERα and ERβ[80]. However, Inoue *et al*[81] showed that the levels of ERβ are higher than those of ER in cultured rat hepatocytes[81]. Hepatic stellate cells from rats appear to contain mainly ERβ[82]. Due to the lack of information about the existence of various ER isoforms, in most of the earlier studies, hepatic stellate cells did not differentiate ER into ERα and ERβ. In addition, majority of the *in vivo* studies have been conducted in animal models of chemical carcinogenesis. Diethylnitrosamine (DEN) is the most commonly used carcinogen in rat and mouse models of HCC. The pathogenesis of HCC in DEN-induced carcinogenesis in

Table 6 Clinical outcome of hormonal therapeutic trials in HCC patients

Receptor protein/ mRNA expression	Clinical parameter	Treatment	*n*	Country	Salient Findings	Yr	Reference
ER protein	Tumor growth	Progestin	5	United States	Tumor regression in 2	1982	[30]
NA	Anti-tumor response	Tamoxifen 20 mg twice daily	33	United States	No complete or partial antitumor response	1990	[73]
	Survival time				Long term survival (18+ to 39+ mo) in 4 patients		
NA	Anti-tumoral effect	Tamoxifen 20 mg daily	120 (placebo = 62)	Spain	No-antitumor effect	1995	[74]
	Survival time				No significant differences in survival rate of placebo and treated groups		
Wild type and variant ER mRNA	Tumor size and growth rate	Tamoxifen 80 mg daily or Megestrol 160 mg daily	8	Italy	Growth rate 4 times higher in tumors expression variant ER than wild type ERs. Tumor regression to half size in patients with wild type ER following tamoxifen treatment. Megestrol slowed down tumor growth in tumors with variant ERs	1996	[54]
ER protein	Mortality rates	Tamoxifen	119 (placebo = 58)	China	No difference in 1 mo mortality rates and median survival in treated and control groups	2000	[75]
	Survival				No effect of ER expression on survival		
Variant ER mRNA	Tumor growth, survival	Megestrol 160 mg daily	24 placebo, 21 treated	Italy	Significantly slowed down tumor growth and improved survival in treated patients than placebo group	2001	[76]
NA	Survival rates	Tamoxifen 120 mg daily or 60 mg daily	329	Singapore	No positive effect on survival and increasingly negative impact with increasing doses	2005	[77]

NA: Information not available.

animal models differs from that in humans and therefore may not be directly comparable to human HCC[83]. Nevertheless, the histology and genetic signatures are similar to human HCC and a striking gender disparity with male predominance is also observed in these animal models as seen in humans[84]. In addition to DEN, acetylaminofluorene (AAF), di(2-ethylhexyl) phthalate (DEHP), peroxisome proliferator, arsenic and carbon tetrachloride have been used to induce HCC in various animal models[85-90].

Use of oral contraceptives and synthetic estrogens in women is reported to be a major risk factor for the development of hepatocellular adenoma, a benign liver tumor with malignant potential[26,27]. Shimomura *et al*[91] studied the role of ethinyl estradiol (EE) in inducing HCC in female rats, following EE treatment for 12 mo, 8% of rats developed HCC, revealing that EE causes mutations in hepatocytes leading to DNA adduct formation and induces HCC development in affected cells. The initial events in HCC, i.e. DNA adduct formation by EE, appear to be carried out in an ER independent manner since tamoxifen, a known selective estrogen receptor modulator (SERM), inhibited ER expression and suppressed HCC, but did not affect DNA adduct formation. Exogenous estrogens have also been shown to promote hepatocarcinogenesis induced by other agents[92,93]. Campen *et al*[92] documented that administration of 17-alpha ethinylestradiol in ovariectomized rats promotes DEN-induced carcinogenesis in a dose dependent manner. Further, it was reported that synthetic female hormones act synergistically with ethanol to increase HCC incidence[93]. Alcohol could affect HCC development due to EE by promoting changes in ER kinetics and expression as well as in DNA adduct formation.

Liver is the major site of estrogen metabolism[94]. Alterations in sex hormone metabolism are also considered a critical factor determining the significance of sex hormones in the process of liver carcinogenesis. Eagon *et al*[86,87] reported that the activity of male estrogen-metabolizing enzyme, estrogen 2-hydroxylase and male specific estrogen sequestering protein is reduced in liver, which explains the raised serum estradiol levels but the decreased hepatic activity of cytosolic and nuclear ER observed in DEHP-induced HCC in male Fischer 344 rats. The expression of cytochrome P450 enzymes that play an important role in estrogen metabolism have also been shown to be affected during hepatocarcinogenesis. Waalkes and colleagues[88] have described the feminized pattern of P450 genes in male mice with HCC induced by exposure to arsenic in utero. The expression of female dominant CYP2A4 and CYP2B9 is increased whereas levels of male dominant CYP7B1 gene gets reduced in arsenic treated animals. Recent findings suggest that cytochrome P450 (CYP) is regulated by estrogen itself through the involvement of estrogen receptors[94]. Nonetheless, elucidation of the exact mechanism of regulation of CYP isoforms by estrogen in liver needs further investigation.

In contrast with earlier animal studies that support

estrogens in promoting and inducing carcinogenesis, recent studies highlighted the protective role of estrogens in HCC development. Shimizu *et al*[8] showed that estrogen can suppress chemical hepatocarcinogenesis induced by dimethylnitrosamine (DEN)-2-acetylaminofluorene (AAF) in partial hepatectomy (PH) model of hepatocarcinogenesis. In addition, estrogen has been shown to prevent the progression of liver disease to HCC. Estradiol treatment could reduce hepatic steatosis and restore the impairment in mitochondrial and peroxisomal fatty acid β-oxidation in aromatase-deficient mice which lack intrinsic ability to produce estrogen[95]. Furthermore, estradiol treatment was also shown to result in a dose dependent suppression of hepatic fibrosis in hepatic fibrosis models of male rats[96,97]. The mechanism of protective action of estrogens against progression of chronic liver disease has been recently reviewed by Shimizu and Ito[7].

Recently, using a mouse model of DEN-induced hepatic carcinogenesis, Naugler *et al*[83] described a molecular mechanism explaining the lower HCC susceptibility in females and the anti-inflammatory role of estrogen in preventing HCC development. The authors investigated the relationship between HCC development and gender dependent expression of interleukin-6 (IL-6). IL-6 is a proinflammatory cytokine that plays an important role in chronic hepatitis, the prerequisite for progression to cirrhosis and HCC. The serum IL-6 levels were higher in male mice than in female mice after administration of DEN, leading to a higher rate of liver cell proliferation in male mice. This effect can be further mediated by ERα, suggesting that ERβ plays a little role in modulating the expression of IL-6.

Androgen receptor studies

The role of AR has also been studied in animal models of chemical induced carcinogenesis[87,98-100]. In DEN treated Wistar rats, a 20-fold increase in hepatic AR concentration was reported in females, suggesting that increased hepatic AR concentration is correlated with accelerated tumor development in these animals, in which male rats showed a slower tumor development with no change in AR concentrations[98]. Subsequent studies in the same model revealed that removal of ovary increases AR levels in the liver of female rats but testosterone treatment does not further enhance AR levels[99]. On the other hand, normal adult males with intact testis or testosterone treatment maintain high levels of AR but in castrated rats estrogen treatment reduces AR expression[99]. Animal studies demonstrated that the expression of both AR and ER increases during preneoplastic stages and that progression towards cancer development can suppress ER and maintain AR expression levels[94,100].

Interestingly, anti-androgen treatment has been shown to reduce AR levels in liver as well as the size and number of tumors in male Spargue Dawley rat model of hepatocarcinogenesis[101]. It has been shown that inhibition of AR positive HCC with anti-androgen cypertone acetate in male mice involves cell cycle arrest and to some extent induction of apoptosis due to increased synthesis of transforming growth factor-β 1 (TGF-β1)[102]. In another model of chemical-induced liver carcinogenesis, inhibition of androgens using 5-alpha reductase inhibitors significantly suppressed HCC development in rats[103]. Recent studies in a xenograft model of hepatocarcinogenesis in nude mice suggested that AR expression remains elevated until development of tumor and starts declining as the size of tumor increases[104]. It is therefore proposed that androgen therapy may be ineffective after establishment of the tumor. Nevertheless, for better understanding and rationale design of hormone-based therapies, it is mandatory to study the role of ERs and AR in animal models mimicking the natural course of disease progression to HCC development as in humans. Currently available HBV and HCV transgenic mice depicting features close to human HCC pathogenesis, appear to be promising models for future *in vivo* studies.

SEX HORMONE RECEPTORS: GENOMIC AND NON-GENOMIC ACTIONS IN LIVER CELLS MEDIATING HEPATOCARCINOGENESIS

Estrogen receptor

Estrogen action and the role of ERs in carcinogenesis have been well documented in mammary carcinoma and the studies have revealed the involvement of estrogens in key cellular processes such as apoptosis, cell cycle, proliferation, oxidative stress and inflammation. The progress in understanding the role of estrogen in regulating various cellular events in liver carcinogenesis has been rather slow. However, the research conducted over recent years provides key insights in this direction.

The classical mode of estrogen action is the genomic mechanism in which ERs function as ligand-activated transcription factors[105]. Activated ERs translocate to the nuclei and regulate the expression of specific target genes. These transcriptional regulations are achieved through interaction with estrogen responsive element (ERE) sequences located in the promoter region of the target gene[106]. However, one third of the genes regulated by ERs in humans do not contain ERE-like sequences[107]. ERs can also regulate the transcription of such genes without binding to DNA through protein-protein interactions with other transcription factors, such as AP-1 and Sp-1 in the nuclei[108]. In addition, this transcriptional control at alternate response elements is also facilitated by non-genomic actions of estrogen. The non-genomic functions of estrogen are initiated by membrane-localized ERs and are associated with activation of various signaling pathways especially protein kinases[109]. The functions of many transcription factors are regulated through protein kinase-mediated phosphorylation including CREB, NF-κB and AP-1 and these transcription factors may thus be targets

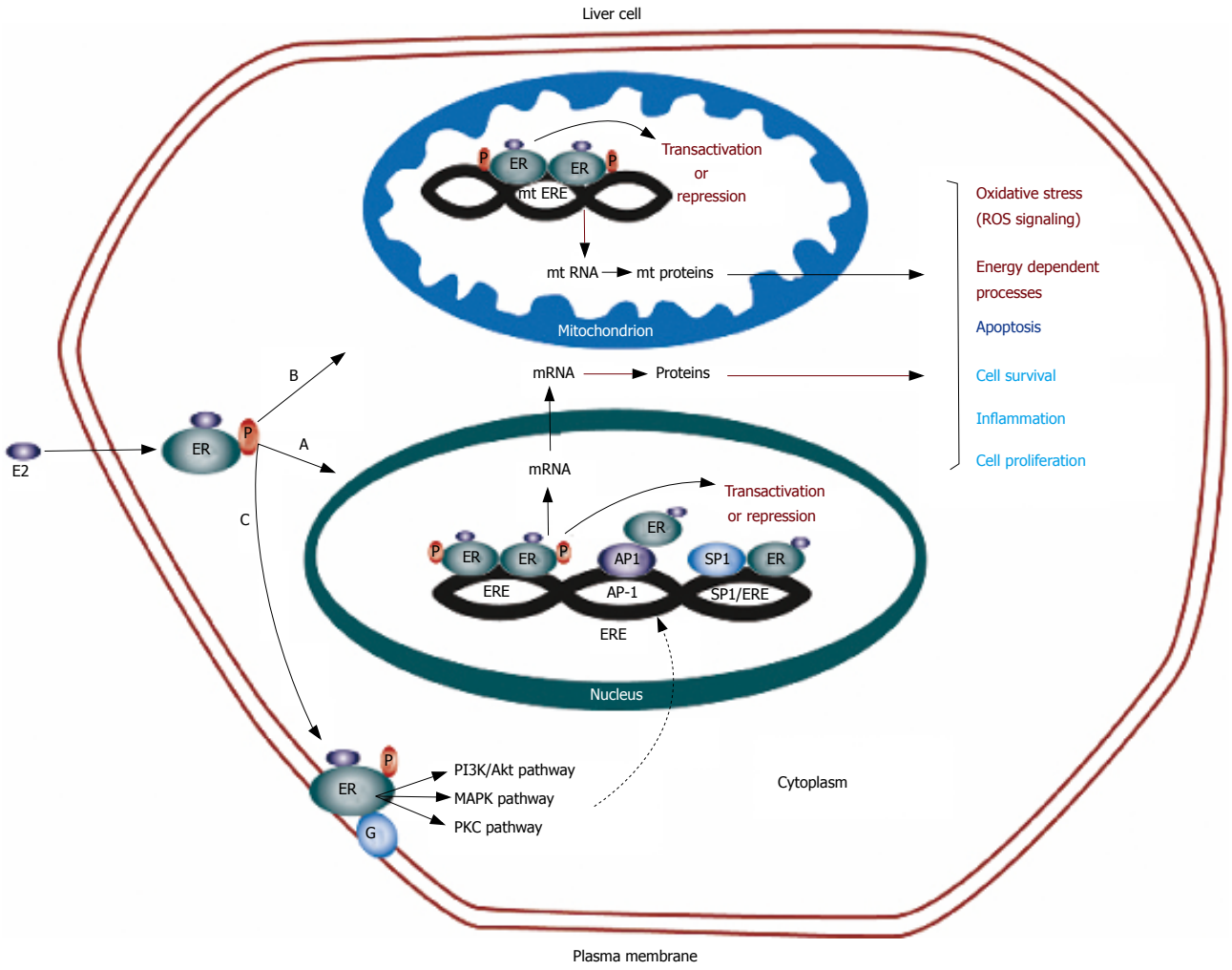


Figure 1 Genomic and non-genomic functions of estrogen mediated by estrogen receptors (ERs) localized in different sub-cellular organelles of a liver cell. A: Genomic actions include translocation of ligand-activated ERs to the nuclei for regulating gene transcription. This transcriptional control is carried out by binding to DNA at a sequence containing either full estrogen response element (ERE) site or an ERE half site adjacent to the binding site for another transcription factor like Sp1. An additional mechanism involves gene regulation at alternate response element through protein-protein interactions with other transcription factors (AP-1, CREB, NF-κB); B: Activated ERs control mitochondrial gene transcription by binding to ERE like sequences (mt ERE) leading to modulation of mitochondrial functions including metabolism, oxidative stress and apoptosis; C: Membrane localized ERs are G-protein coupled receptors that activate various protein kinase pathways. These signal transduction cascades in turn regulate functions of many transcription factors resulting in modulation of expression of a number of genes involved in cell proliferation, survival, apoptosis and inflammation. Genomic and non-genomic actions of estrogen converge to exhibit a fine degree of control for the regulation of transcription by ERs in a liver cell. Modified from: Chen *et al*[110] 2005.

for non-genomic actions of estrogens. This possible convergence of genomic and non-genomic actions at multiple response elements provides an extremely fine degree of control for the regulation of transcription for ERs (Figure 1)[105,110]. In the following section, we discuss the findings of recent *in vitro* studies highlighting the significance of ER in mediating genomic and non-genomic actions of estrogen in liver cells to modulate the expression of a number of genes involved in cellular processes central to carcinogenesis.

Telomerase activation has been implicated in hepatocarcinogenesis and the expression of human telomerase reverse transcriptase (hTERT) that encodes for the catalytic subunit of the multicomponent enzyme telomerase hTERT is the prerequisite for telomerase activation[111,112]. Several studies indicated that estrogen regulates transactivation of the hTERT gene by direct interaction of activated ER with an imperfect ERE sequence in the hTERT promoter[113]. Estrogen treatment has been shown to up-regulate the expression of hTERT mRNA and protein in three normal human hepatic cell lines (hc-cells, hNheps and WRL-68) expressing ERα to varying degrees[90]. Furthermore, estrogen exposure prevents shortening of telomeres and decreases the number of cells undergoing senescence, indicating that estradiol acts as a positive modulator of the hTERT gene in the liver[90]. However, the mechanism of ER-mediated transactivation of hTERT in the liver is not well understood. In contrast, in HepG2 cells, estrogen modulation of telomerase activity has been found to be regulated post-transcriptionally *via* the IP3/PKC pathway[114,115]. IP3 production has been shown to be up-regulated by estrogens in HepG2 cells[114]. Furthermore, estradiol-induced IP3/PKC-alpha production is dependent on either ERα or ERβ expression in both HepG2 and Hela cells[114]. It is

hypothesized that membrane ER-mediated IP3/PKC-alpha pathway represents an alternative signaling pathway utilized by cells when low ER levels are unable to activate classic ER-mediated genomic mechanisms as in HepG2 cells[114].

A similar regulatory mechanism has been observed in case of estrogen modulation of expression of cyclin D1 gene in hepatoma cells. Cyclin D1, important for progression of cells through G1 phase of cell cycle, is a well defined target for estrogen action in mammary carcinoma[116,117], although no detectable estrogen responsive element like sequence in the cyclin D1 gene promoter has been reported in these cells[118]. The cyclin D1 mechanism identified in mammary carcinoma cells involves direct interaction of ERα and Sp1 or ERα and Ap-1[119]. Interestingly, Marino *et al*[23] demonstrated that in HepG2 cells, estrogen-induced activation of cyclin D1 transcription can occur independently of the transcriptional activity of ER. They further showed that the effect of 17-beta estradiol on HepG2 cells is mediated by activation of the MAPK/ERK pathway by membrane-localized ER that increases the expression of cyclin D1 gene through activation of AP-1 transcription factor[23], suggesting that non-genomic signaling pathways play an the pivotal role in estrogen-mediated regulation of gene expression at multiple response elements.

Besides, modulating the molecules involved in cell cycle control and cell proliferation, estrogen has also been shown to regulate the expression of genes crucial for apoptosis of hepatocytes and dysregulation of apoptosis in hepatic cells is reported to be a significant factor in accelerating hepatocarcinogenesis or tumor progression in HCC[120]. The Bcl-2 family of proteins regulates one of the key steps in the conserved apoptotic pathway. Among the members of this family, Bcl-2 and Bcl-xL act as inhibitors of apoptosis where as Bax and Bak promote apoptosis[121,122]. Ethinyl estradiol is known to increase the levels of Bcl-2 protein in cultured female rat hepatocytes[123]. Estradiol and idoxifene, two selective estrogen receptor modulators, are known to induce the expression of Bcl-2 protein in male rat liver tissues[124]. Omoya *et al*[9] and Inoue *et al*[81] also demonstrated that estradiol is able to stimulate the expression of Bcl-2 and Bcl-xL and to suppress Bad expression in oxidative stress-induced early apoptotic rat hepatocytes. Similar findings have been recently documented in response to estradiol treatment of Huh-7 human hepatoma cells describing a dose dependent increase in expression of Bcl-2 and Bcl-xL and a reduction in Bad levels[22]. No change was observed in expression of pro-apoptotic protein Bax. The regulation of Bcl-2 gene expression by estrogen in mammary carcinoma cells has been shown to be mediated indirectly through activation of Sp-1 transcription factor[125]. However, the precise mechanism of Bcl-2 transactivation in hepatocytes has not been clearly understood.

One of the most interesting mechanisms of transcriptional regulation at alternate response elements by estrogen is through inhibition of transcription factor NF-κB. Studies demonstrating a mutually antagonistic cross-talk between these families of transcription factors have been recently reviewed[126]. The ER has been shown to mediate opposition of NF-κB functions at various levels by inhibiting the activation of signaling pathways, preventing nuclear translocation, blocking DNA binding or inhibiting recruitment of co-activators for transcription[126]. Estrogen has been shown to bring about its anti-inflammatory and anti-oxidant effects on liver cells by suppressing the NF-κB activity as evident from the following studies. It was reported that 17 beta-estradiol-bound ERα interferes with cytokine-induced activation of a NF-κB reporter in HepG2 cells, suggesting that estrogen exerts its anti-inflammatory and protective effects on human liver cells[127]. Moreover, in an *in vivo* model, estrogen treatment has been shown to block the induction of hepatic expression of inflammatory vascular cell adhesion molecule-1 (VCAM-1), tumor necrosis factor-α (TNF-α), and regulate normal T-cell expression and secretion upon activation[128]. In a mouse model of DEN-induced HCC, ERα was suggested to suppress IL-6 production, a pro-inflammatory molecule, through the involvement of the NF-κB pathway[83]. Estrogen has also been reported to suppress oxidative stress-induced reactive oxygen species (ROS) generation, lipid peroxidation, activation of AP-1 and NF-κB as well as loss of Cu-Zn SOD activity in cultured rat hepatocytes[2,9].

In addition to genomic and non-genomic actions of estrogen mediated by nuclear and membrane ER, mitochondria have also recently been identified as important targets of estrogen and ERs[110]. Early binding studies on sub-cellular fractions indicated that ER is present in rat liver mitochondria[129]. Both ERα and ERβ have been reported to be present in the mitochondria of human HepG2 cells[130-132]. The mitochondrial genome has been shown to contain sequences that have partial homology to the estrogen responsive elements[132-134]. Both ERα and ERβ bind to mitochondrial DNA and the binding can be increased by estradiol using mobility shift assays and surface plasmon resonance[135]. These results suggest that estradiol is directly involved in the regulation of mitochondrial DNA transcription (Figure 1). Regulation of apoptosis and oxidative metabolism by estrogens in mitochondria may be important in the normal liver and in the development of HCC.

Ethinyl estradiol treatment has been shown to elevate the expression levels of mitochondrial DNA-encoded cytochrome C oxidase subunit Ⅲ (CO Ⅲ) and ATP synthase 6 *in vivo* as well as in HepG2 cells[136]. This increased expression of mitochondrial transcripts is accompanied by increased mitochondrial superoxide production and respiratory chain activity that require cytochrome P450-mediated biotransformation of ethinyl estradiol and 17-beta estradiol to catechol metabolites[136,137]. In addition to CO Ⅲ, the levels of CO Ⅰ and CO Ⅱ encoded by mitochondrial DNA have also been found to be elevated in ethinyl estradiol treated female rat hepatocytes. This effect is accompanied

by increased mitochondrial superoxide production, high ATP levels and increased Bcl2 production, and is suggested to play a role in ethinyl estradiol-mediated inhibition of apoptosis[123]. In contrast, 17-beta estradiol and 17-beta estradiol like compounds, diethylstilbestrol (DES), tamoxifen and genistein, have been found to induce apoptotic effects in human hepatoma Hep3B cell line[138]. These compounds cause the leaking of cytochrome C from mitochondria and activation of caspase-3 in an ER dependent manner. In another study, the two isoforms, ERα and ERβ, showed their opposing actions on apoptosis in a poorly differentiated HCC cell line HA22T[139]. Over-expressed ERβ but not ERα induces the expression of caspase-8 and TNF-α in HA22T cells in response to estradiol treatment, indicating that the death receptor-mediated apoptotic pathway is activated[139].

Differential roles of ERα and ERβ have also been observed in non-genomic actions of estrogen in the liver[21,23]. There is indirect evidence that membrane ER may exist in human liver as the binding of gold tagged estrogen-BSA conjugate on the surface of clathrin-coated pits in HepG2 cells has been demonstrated by electron microscopic visualization[140]. The non-genomic mechanism of action of sex steroids on the plasma membrane involves the activation of protein kinase cascades (Figure 1). Two major cascades, protein kinase C, and mitogen-activated protein (MAP) kinase are active and important in carcinogenic liver cells. Protein kinase C cascade and its second messenger IP3 are important in cell proliferation and have been discussed in this review in context of transcriptional regulation of hTERT expression by estrogen. The mitogen-activated protein (MAP) kinase cascade is another pathway that is regulated by the action of sex steroids at the plasma membrane. This complex signaling cascade involves three major pathways: ERK, p38, and JNK[141]. In HepG2 cells, estradiol has been found to rapidly increase the phosphorylation of ERK[21,23]. Naringenin, an anti-estrogenic flavonone, induces the activation of p38 in ERα containing HepG2 cells or in ERβ containing human colon adenocarcinoma DLD-1 cells[142], suggesting that naringenin has an antiestrogenic effect only on the ERα expressing cells, whereas it mimicks the estradiol effects on ERβ expressing cells. The role of ERα and ERβ in the regulation of MAP kinase cascade has been further studied in cell lines expressing either ERα or ERβ[21]. It was found that estrogen-bound ERα can rapidly activate the ERK and AKT signal transduction pathways leading to cell cycle progression and inhibition of apoptosis, whereas estrogen-complexed ERβ can induce rapid phosphorylation of p38 leading the cells to the apoptotic cycle and cell death. These studies further support the functional antagonism between ERα and ERβ with respect to estrogen-induced cell proliferation and emphasize the need to study the independent and interactive role of both isoforms in hepatocarcinogenesis.

Androgen receptor

In comparison with ER, there is limited information about genomic and non-genomic functions of AR in the liver. Like ER, AR has also been shown to regulate gene transcription by binding to androgen responsive sequences (ARE)[143,144]. Yoon *et al*[145] demonstrated that androgen can directly regulate the expression of transformation growth factor-beta 1 (TGF-β1) through binding of AR to ARE in TGF-β1 promoter, suggesting that such activation might regulate the progression of HCC in both human and animal models[145]. Furthermore, AR has been shown to interact with a newly identified transcription factor, paternally expressed gene 10 (PEG 10) in hepatoma cell line[146]. PEG 10 has growth promoting properties and is implicated in hepatocarcinogenesis[147,148]. Dihydrotestosterone (DHT) promotes hepatoma formation in nude mice through PEG 10 activation. In addition, DHT treatment is shown to up-regulate hTERT expression in hepatoma cell lines in a PEG-10 dependent manner[146]. These studies indicate that PEG-10-mediated transactivation of target genes by AR has an essential role in hepatocarcinogenesis.

To the best of our knowledge, AR has not been detected in the liver mitochondria. The information about the membrane localization of AR in human liver cells is also lacking. However, AR has been reported to occur in the plasma membranes of male rat liver[149]. Androgens are also involved in the regulation of the MAP kinase signaling pathways as orchiectomy of H-ras 12V transgenic mice decreases phospho-MEK and phospho-ERK in liver tissues. In addition, orchiectomy reduces hepatotumorigenesis in male mice while ovariectomy increases phospho-MEK and phospho-ERK in liver tissue from female mice, but ovariectomy does not affect the incidence of tumorigenesis[150]. Detailed investigations are urgently needed to confirm the existence of non-genomic signaling actions of androgens in liver and the role of AR in mediating these functions.

INTERACTION OF ER AND AR WITH VIRAL PROTEINS IN HBV AND HCV PATHOGENESIS

Chronic infection with HBV and HCV is the major cause of increasing incidence of HCC worldwide. Several reports support the role of HBV and HCV proteins in disturbing cellular homeostasis and causing malignant transformation of hepatocytes[151,152]. Recent studies in hepatoma cell lines suggest the interactive role of ERs and AR with HBV and HCV proteins in viral pathogenesis.

Estrogen receptor and viral proteins

Han *et al*[24] recently reported that HBV protein (HBx) interacts with ERα. HBx is a multifunctional protein involved in neoplastic transformation in cultured cells and can induce HCC in transgenic mice. HBx associates with both ERα and delta 5 deletion variant of ERα (vERα) and inhibits ERα transcriptional activity by recruiting histone deacetylase enzyme, HDAC-1[24].

HDAC-1 belongs to the family of enzymes involved in deacetylation of hyperacetylated histone tails, leading to compaction of chromatin and transcritptional repression[153]. Both HBx and vERα have additive effects on suppression of ERα transactivation[24].

ERα has also been shown to interact with nonstructural (NS) 5B protein of HCV[25]. NS5B is a RNA-dependent RNA polymerase, which plays a central role in viral genome replication[154]. HCV replication takes place in a replication complex consisting of viral RNA and non structural proteins including NS5B[155]. The replication complex forms on the surface of intracellular membranes including endoplasmic reticulum membrane and is associated with lipid rafts rich in caveolin 2 (CAV 2) on these membranes[156-158]. Using chemical biology approach, Watashi *et al*[25] demonstrated that ERα facilitates the interaction of NS5B with CAV 2 in lipid rafts and hence promotes the participation of NS5B in HCV replication complex. However, they did not find that ERβ affects HCV replication in the same study[25]. An important observation of the study is that tamoxifen inhibits ERα actions and suppresses HCV genome replication, further supporting the potential for anti-ER drugs in developing new anti-HCV strategies.

Androgen receptor and viral proteins

Like ER, HBx protein has also been shown to interact with AR. HBx functions as a positive transcriptional co-regulator to increase AR-mediated transcriptional activity. This transcription enhancement is increased in the presence of androgen in a concentration-responsive manner. However, HBx does not physically associate with ligand-bound AR in the nuclei, suggesting that HBx augments AR activity by increasing the phosphorylation of AR through HBx-mediated activation of the c-Src kinase signaling pathway[159]. In contrast, Zheng *et al*[160] demonstrated that HBx can physically bind to AR in the liver and alter the subcellular localization of AR both in the presence and absence of dihydrotestosterone (DHT). Further studies indicated that HBx can enhance the gene transactivation activity of AR by enhancing its DNA binding activity in a DHT-dependent manner.

CONCLUSION

Taken together, studies on hepatoma cell lines, HCC tissues and animal models of hepatocarcinogenesis, highlight the importance of sex hormones and their receptors in HCC pathogenesis. Further investigations are urgently needed to elucidate the precise mechanism of action of estrogens, androgens and their receptors in regulating normal liver physiology and pathophysiology of chronic liver diseases resulting in HCC.

A thorough re-examination of studies conducted so far to detect the expression of ER and AR in liver tissues is needed using newer specific, sensitive and quantitative methods. With the emerging significance of ERβ and the availability of isoform specific antibodies, the relative levels of both ERα and ERβ can be determined. The studies on mRNA expression in liver tissues have demonstrated the presence of deletion mutants (variant forms) that need to be further validated at protein levels for establishing their significance in diagnosis and prognosis of HCC. Considering the male predominance of HCC and the wide gap in the information available on AR in liver, detailed mechanistic studies need to be conducted to reveal the mechanism of androgen function in normal liver and HCC. In addition, evaluation of ER and AR status at premalignant stages of chronic liver disease due to different etiological factors is required for critical understanding of their role in HCC pathogenesis.

Recent *in vitro* studies focusing on molecular interaction of hormonal receptors with viral proteins need to be further confirmed in *in vivo* animal models. Currently available HBV and HCV transgenic mouse models as well as human hepatocyte xenograft models can serve as a valuable preclinical tools to validate the importance of sex hormone receptors in chronic liver disease development and progression to HCC. Thus, with the availability of state of the art technologies, the time is ripe to embark on to move this important field forward. Well designed, systematic studies employing adequate tools to study ERs and ARs in chronic liver disease and HCC may contribute to the development of novel therapeutics or prognostic markers. These studies may also be further helpful in resolving controversies about the use of hormonal therapy for HCC.

ACKNOWLEDGMENTS

The authors thank Ms. Peggy Billups for help in graphics and design of liver cell illustration.

REFERENCES

1 **El-Serag HB**, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 2007; **132**: 2557-2576
2 **Shimizu I**. Impact of oestrogens on the progression of liver disease. *Liver Int* 2003; **23**: 63-69
3 **De Maria N**, Manno M, Villa E. Sex hormones and liver cancer. *Mol Cell Endocrinol* 2002; **193**: 59-63
4 **Granata OM**, Carruba G, Montalto G, Miele M, Bellavia V, Modica G, Blomquist CH, Castagnetta LA. Altered androgen metabolism eventually leads hepatocellular carcinoma to an impaired hormone responsiveness. *Mol Cell Endocrinol* 2002; **193**: 51-58
5 **Farinati F**, Cardin R, Bortolami M, Grottola A, Manno M, Colantoni A, Villa E. Estrogens receptors and oxidative damage in the liver. *Mol Cell Endocrinol* 2002; **193**: 85-88
6 **Farinati F**, De Maria N, Marafin C, Fagiuoli S, Della Libera G, Naccarato R. Hepatocellular carcinoma in alcoholic cirrhosis: is sex hormone imbalance a pathogenetic factor? *Eur J Gastroenterol Hepatol* 1995; **7**: 145-150
7 **Shimizu I**, Ito S. Protection of estrogens against the progression of chronic liver disease. *Hepatol Res* 2007; **37**: 239-247
8 **Shimizu I**, Yasuda M, Mizobuchi Y, Ma YR, Liu F, Shiba M, Horie T, Ito S. Suppressive effect of oestradiol on chemical hepatocarcinogenesis in rats. *Gut* 1998; **42**: 112-119
9 **Omoya T**, Shimizu I, Zhou Y, Okamura Y, Inoue H, Lu G, Itonaga M, Honda H, Nomura M, Ito S. Effects of idoxifene and estradiol on NF-kappaB activation in cultured rat hepatocytes undergoing oxidative stress. *Liver* 2001; **21**:

183-191
10 **Gavrilova-Jordan LP**, Price TM. Actions of steroids in mitochondria. *Semin Reprod Med* 2007; **25**: 154-164
11 **Boonyaratanakornkit V**, Edwards DP. Receptor mechanisms mediating non-genomic actions of sex steroids. *Semin Reprod Med* 2007; **25**: 139-153
12 **Pettersson K**, Gustafsson JA. Role of estrogen receptor beta in estrogen action. *Annu Rev Physiol* 2001; **63**: 165-192
13 **Nagasue N**, Ito A, Yukaya H, Ogawa Y. Estrogen receptors in hepatocellular carcinoma. *Cancer* 1986; **57**: 87-91
14 **Nagasue N**, Ito A, Yukaya H, Ogawa Y. Androgen receptors in hepatocellular carcinoma and surrounding parenchyma. *Gastroenterology* 1985; **89**: 643-647
15 **Nagasue N**, Kohno H, Chang YC, Hayashi T, Utsumi Y, Nakamura T, Yukaya H. Androgen and estrogen receptors in hepatocellular carcinoma and the surrounding liver in women. *Cancer* 1989; **63**: 112-116
16 **Negro F**, Papotti M, Pacchioni D, Galimi F, Bonino F, Bussolati G. Detection of human androgen receptor mRNA in hepatocellular carcinoma by in situ hybridisation. *Liver* 1994; **14**: 213-219
17 **Ohnishi S**, Murakami T, Moriyama T, Mitamura K, Imawari M. Androgen and estrogen receptors in hepatocellular carcinoma and in the surrounding noncancerous liver tissue. *Hepatology* 1986; **6**: 440-443
18 **Villa E**, Dugani A, Moles A, Camellini L, Grottola A, Buttafoco P, Merighi A, Ferretti I, Esposito P, Miglioli L, Bagni A, Troisi R, De Hemptinne B, Praet M, Callea F, Manenti F. Variant liver estrogen receptor transcripts already occur at an early stage of chronic liver disease. *Hepatology* 1998; **27**: 983-988
19 **Iavarone M**, Lampertico P, Seletti C, Francesca Donato M, Ronchi G, del Ninno E, Colombo M. The clinical and pathogenetic significance of estrogen receptor-beta expression in chronic liver diseases and liver carcinoma. *Cancer* 2003; **98**: 529-534
20 **Acconcia F**, Kumar R. Signaling regulation of genomic and nongenomic functions of estrogen receptors. *Cancer Lett* 2006; **238**: 1-14
21 **Acconcia F**, Totta P, Ogawa S, Cardillo I, Inoue S, Leone S, Trentalance A, Muramatsu M, Marino M. Survival versus apoptotic 17beta-estradiol effect: role of ER alpha and ER beta activated non-genomic signaling. *J Cell Physiol* 2005; **203**: 193-201
22 **Cheng X**, Shimizu I, Yuan Y, Wei M, Shen M, Huang H, Urata M, Sannomiya K, Fukuno H, Hashimoto-Tamaoki T, Ito S. Effects of estradiol and progesterone on tumor necrosis factor alpha-induced apoptosis in human hepatoma HuH-7 cells. *Life Sci* 2006; **79**: 1988-1994
23 **Marino M**, Acconcia F, Bresciani F, Weisz A, Trentalance A. Distinct nongenomic signal transduction pathways controlled by 17beta-estradiol regulate DNA synthesis and cyclin D(1) gene transcription in HepG2 cells. *Mol Biol Cell* 2002; **13**: 3720-3729
24 **Han J**, Ding L, Yuan B, Yang X, Wang X, Li J, Lu Q, Huang C, Ye Q. Hepatitis B virus X protein and the estrogen receptor variant lacking exon 5 inhibit estrogen receptor signaling in hepatoma cells. *Nucleic Acids Res* 2006; **34**: 3095-3106
25 **Watashi K**, Inoue D, Hijikata M, Goto K, Aly HH, Shimotohno K. Anti-hepatitis C virus activity of tamoxifen reveals the functional association of estrogen receptor with viral RNA polymerase NS5B. *J Biol Chem* 2007; **282**: 32765-32772
26 **Fechner RE**. Benign hepatic lesions and orally administered contraceptives. A report of seven cases and a critical analysis of the literature. *Hum Pathol* 1977; **8**: 255-268
27 **Vana J**, Murphy GP, Aronoff BL, Baker HW. Primary liver tumors and oral contraceptives. Results of a survey. *JAMA* 1977; **238**: 2154-2158
28 **Duffy MJ**, Duffy GJ. Estradiol receptors in human liver. *J Steroid Biochem* 1978; **9**: 233-235
29 **Molteni A**, Bahu RM, Battifora HA, Fors EM, Reddy JK, Rao MS, Scarpelli DG. Estradiol receptor assays in normal and neoplastic tissues. A possible diagnostic acid for tumor differentiation. *Ann Clin Lab Sci* 1979; **9**: 103-108
30 **Friedman MA**, Demanes DJ, Hoffman PG Jr. Hepatomas: hormone receptors and therapy. *Am J Med* 1982; **73**: 362-366
31 **Iqbal MJ**, Wilkinson ML, Johnson PJ, Williams R. Sex steroid receptor proteins in foetal, adult and malignant human liver tissue. *Br J Cancer* 1983; **48**: 791-796
32 **Kohigashi K**, Fukuda Y, Imura H. Estrogen receptors in hepatocellular carcinoma: is endocrine therapy for hepatocellular carcinoma likely to be effective? *Gastroenterol Jpn* 1987; **22**: 322-330
33 **Eagon PK**, Francavilla A, DiLeo A, Elm MS, Gennari L, Mazzaferro V, Colella G, Van Thiel DH, Strazl TE. Quantitation of estrogen and androgen receptors in hepatocellular carcinoma and adjacent normal human liver. *Dig Dis Sci* 1991; **36**: 1303-1308
34 **Nagasue N**, Kohno H, Chang YC, Yamanoi A, Nakamura T, Yukaya H, Hayashi T. Clinicopathologic comparisons between estrogen receptor-positive and -negative hepatocellular carcinomas. *Ann Surg* 1990; **212**: 150-154
35 **Nagasue N**, Kohno H, Chang Y, Hayashi T, Nakamura T. Specificity of androgen receptors of hepatocellular carcinoma and liver in humans. *Hepatogastroenterology* 1990; **37**: 474-479
36 **Nagasue N**, Kohno H, Yamanoi A, Kimoto T, Chang YC, Nakamura T. Progesterone receptor in hepatocellular carcinoma. Correlation with androgen and estrogen receptors. *Cancer* 1991; **67**: 2501-2505
37 **Francavilla A**, Panella C, Amoruso A, Giangaspero A, Gennari L, Mazzaferro V, Colella G, Van Thiel DH, Starzl TE. Role of estrogens and epidermal growth factor in hepatocellular carcinoma (HCC). *Dig Dis Sci* 1991; **36**: 1299-1302
38 **Eagon PK**, Elm MS, Stafford EA, Porter LE. Androgen receptor in human liver: characterization and quantitation in normal and diseased liver. *Hepatology* 1994; **19**: 92-100
39 **Wong LY**, Chan SH, Oon CJ, Rauff A. Immunocytochemical localization of testosterone in human hepatocellular carcinoma. *Histochem J* 1984; **16**: 687-692
40 **Pacchioni D**, Papotti M, Andorno E, Bonino F, Mondardini A, Oliveri F, Brunetto M, Bussolati G, Negro F. Expression of estrogen receptor mRNA in tumorous and non-tumorous liver tissue as detected by in situ hybridization. *J Surg Oncol Suppl* 1993; **3**: 14-17
41 **Boix L**, Bruix J, Castells A, Fuster J, Bru C, Visa J, Rivera F, Rodes J. Sex hormone receptors in hepatocellular carcinoma. Is there a rationale for hormonal treatment? *J Hepatol* 1993; **17**: 187-191
42 **Jonas S**, Bechstein WO, Heinze T, Kling N, Lobeck H, Tullius SG, Steinmueller T, Neuhaus P. Female sex hormone receptor status in advanced hepatocellular carcinoma and outcome after surgical resection. *Surgery* 1997; **121**: 456-461
43 **Ng IO**, Ng M, Fan ST. Better survival in women with resected hepatocellular carcinoma is not related to tumor proliferation or expression of hormone receptors. *Am J Gastroenterol* 1997; **92**: 1355-1358
44 **Moon WS**, Chang K, Tarnawski AS. Overexpression of metastatic tumor antigen 1 in hepatocellular carcinoma: Relationship to vascular invasion and estrogen receptor-alpha. *Hum Pathol* 2004; **35**: 424-429
45 **Xing BC**, Wang JH, Wang Y, Hao CY, Huang XF, Wang Y. [Expression of wild type and variant estrogen receptors in human hepatocellular carcinoma.] *Beijingdaxue Xuebao* 2004; **36**: 620-622
46 **Meza-Junco J**, Montano-Loza AJ, Gamboa-Dominguez A, Green-Renner D. Expression of oestrogen and growth factor receptors in hepatocellular carcinoma. *Clin Oncol* (R Coll Radiol) 2007; **19**: 801-802
47 **Vizoso FJ**, Rodriguez M, Altadill A, Gonzalez-Dieguez ML, Linares A, Gonzalez LO, Junquera S, Fresno-Forcelledo F, Corte MD, Rodrigo L. Liver expression of steroid

hormones and Apolipoprotein D receptors in hepatocellular carcinoma. *World J Gastroenterol* 2007; **13**: 3221-3227
48 **Kuiper GG**, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* 1996; **93**: 5925-5930
49 **Walter P**, Green S, Greene G, Krust A, Bornert JM, Jeltsch JM, Staub A, Jensen E, Scrace G, Waterfield M. Cloning of the human estrogen receptor cDNA. *Proc Natl Acad Sci USA* 1985; **82**: 7889-7893
50 **Green S**, Walter P, Greene G, Krust A, Goffin C, Jensen E, Scrace G, Waterfield M, Chambon P. Cloning of the human oestrogen receptor cDNA. *J Steroid Biochem* 1986; **24**: 77-83
51 **Villa E**, Camellini L, Dugani A, Zucchi F, Grottola A, Merighi A, Buttafoco P, Losi L, Manenti F. Variant estrogen receptor messenger RNA species detected in human primary hepatocellular carcinoma. *Cancer Res* 1995; **55**: 498-500
52 **Wang AG**, Lee KY, Kim SY, Choi JY, Lee KH, Kim WH, Wang HJ, Kim JM, Park MG, Yeom YI, Kim NS, Yu DY, Lee DS. The expression of estrogen receptors in hepatocellular carcinoma in Korean patients. *Yonsei Med J* 2006; **47**: 811-816
53 **Murphy LC**, Dotzlaw H, Leygue E, Coutts A, Watson P. The pathophysiological role of estrogen receptor variants in human breast cancer. *J Steroid Biochem Mol Biol* 1998; **65**: 175-180
54 **Villa E**, Dugani A, Fantoni E, Camellini L, Buttafoco P, Grottola A, Pompei G, De Santis M, Ferrari A, Manenti F. Type of estrogen receptor determines response to antiestrogen therapy. *Cancer Res* 1996; **56**: 3883-3885
55 **Villa E**, Moles A, Ferretti I, Buttafoco P, Grottola A, Del Buono M, De Santis M, Manenti F. Natural history of inoperable hepatocellular carcinoma: estrogen receptors' status in the tumor is the strongest prognostic factor for survival. *Hepatology* 2000; **32**: 233-238
56 **Villa E**, Colantoni A, Camma C, Grottola A, Buttafoco P, Gelmini R, Ferretti I, Manenti F. Estrogen receptor classification for hepatocellular carcinoma: comparison with clinical staging systems. *J Clin Oncol* 2003; **21**: 441-446
57 **Villa E**, Colantoni A, Grottola A, Ferretti I, Buttafoco P, Bertani H, De Maria N, Manenti F. Variant estrogen receptors and their role in liver disease. *Mol Cell Endocrinol* 2002; **193**: 65-69
58 **Leygue E**, Dotzlaw H, Watson PH, Murphy LC. Altered estrogen receptor alpha and beta messenger RNA expression during human breast tumorigenesis. *Cancer Res* 1998; **58**: 3197-3201
59 **Macdonald JS**, Lippman ME, Woolley PV, Petrucci PP, Schein PS. Hepatic estrogen and progesterone receptors in an estrogen-associated hepatic neoplasm. *Cancer Chemother Pharmacol* 1978; **1**: 135-138
60 **Bojar H**, Schutte J, Staib W, Broelsch C. Does human liver contain estrogen receptors? A comparative study of estrogen binding in human and rodent liver. *Klin Wochenschr* 1982; **60**: 417-425
61 **Porter LE**, Elm MS, Van Thiel DH, Dugas MC, Eagon PK. Characterization and quantitation of human hepatic estrogen receptor. *Gastroenterology* 1983; **84**: 704-712
62 **Porter LE**, Elm MS, Van Thiel DH, Eagon PK. Hepatic estrogen receptor in human liver disease. *Gastroenterology* 1987; **92**: 735-745
63 **Ohnami S**, Nakata H, Nagafuchi Y, Zeze F, Eto S. [Estrogen receptors in human gastric, hepatocellular, and gallbladder carcinomas and normal liver tissues] *Gan To Kagaku Ryoho* 1988; **15**: 2923-2928
64 **Hamazaki K**, Miura H, Sakai H, Sato S, Yunoki M, Miichi N, Noda T, Mori M, Orita K. [Estrogen and androgen receptors in hepatocellular carcinoma and in noncancerous liver tissue] *Gan No Rinsho* 1989; **35**: 1109-1113
65 **Wilkinson ML**, Iqbal MJ, Williams R. Characterisation of high affinity binding sites of androgens in primary hepatocellular carcinoma. *Clin Chim Acta* 1985; **152**: 105-113
66 **Bannister P**, Meystre CM, Losowsky MS. Androgen receptor concentrations in needle biopsy specimens of human liver. *Liver* 1988; **8**: 28-31
67 **Nagasue N**, Chang YC, Hayashi T, Galizia G, Kohno H, Nakamura T, Yukaya H. Androgen receptor in hepatocellular carcinoma as a prognostic factor after hepatic resection. *Ann Surg* 1989; **209**: 424-427
68 **Nagasue N**, Yamanoi A, Kohno H, Kimoto T, Chang Y, Taniura H, Uchida M, Nakamura T. Androgen receptor in cirrhotic liver, adenomatous hyperplastic nodule and hepatocellular carcinoma in the human. *Hepatogastroenterology* 1992; **39**: 455-460
69 **Boix L**, Castells A, Bruix J, Sole M, Bru C, Fuster J, Rivera F, Rodes J. Androgen receptors in hepatocellular carcinoma and surrounding liver: relationship with tumor size and recurrence rate after surgical resection. *J Hepatol* 1995; **22**: 616-622
70 **Zhang X**, He L, Lu Y, Liu M, Huang X. Androgen receptor in primary hepatocellular carcinoma and its clinical significance. *Chin Med J* (Engl) 1998; **111**: 1083-1086
71 **Tavian D**, De Petro G, Pitozzi A, Portolani N, Giulini SM, Barlati S. Androgen receptor mRNA under-expression in poorly differentiated human hepatocellular carcinoma. *Histol Histopathol* 2002; **17**: 1113-1119
72 **Nagasue N**, Yu L, Yukaya H, Kohno H, Nakamura T. Androgen and oestrogen receptors in hepatocellular carcinoma and surrounding liver parenchyma: impact on intrahepatic recurrence after hepatic resection. *Br J Surg* 1995; **82**: 542-547
73 **Engstrom PF**, Levin B, Moertel CG, Schutt A. A phase II trial of tamoxifen in hepatocellular carcinoma. *Cancer* 1990; **65**: 2641-2643
74 **Castells A**, Bruix J, Bru C, Ayuso C, Roca M, Boix L, Vilana R, Rodes J. Treatment of hepatocellular carcinoma with tamoxifen: a double-blind placebo-controlled trial in 120 patients. *Gastroenterology* 1995; **109**: 917-922
75 **Liu CL**, Fan ST, Ng IO, Lo CM, Poon RT, Wong J. Treatment of advanced hepatocellular carcinoma with tamoxifen and the correlation with expression of hormone receptors: a prospective randomized study. *Am J Gastroenterol* 2000; **95**: 218-222
76 **Villa E**, Ferretti I, Grottola A, Buttafoco P, Buono MG, Giannini F, Manno M, Bertani H, Dugani A, Manenti F. Hormonal therapy with megestrol in inoperable hepatocellular carcinoma characterized by variant oestrogen receptors. *Br J Cancer* 2001; **84**: 881-885
77 **Chow PK**, Tai BC, Tan CK, Machin D, Win KM, Johnson PJ, Soo KC. High-dose tamoxifen in the treatment of inoperable hepatocellular carcinoma: A multicenter randomized controlled trial. *Hepatology* 2002; **36**: 1221-1226
78 **Di Maio M**, De Maio E, Morabito A, D'Aniello R, De Feo G, Gallo C, Perrone F. Hormonal treatment of human hepatocellular carcinoma. *Ann N Y Acad Sci* 2006; **1089**: 252-261
79 **Di Maio M**, Daniele B, Pignata S, Gallo C, De Maio E, Morabito A, Piccirillo MC, Perrone F. Is human hepatocellular carcinoma a hormone-responsive tumor? *World J Gastroenterol* 2008; **14**: 1682-1689
80 **Alvaro D**, Alpini G, Onori P, Perego L, Svegliata Baroni G, Franchitto A, Baiocchi L, Glaser SS, Le Sage G, Folli F, Gaudio E. Estrogens stimulate proliferation of intrahepatic biliary epithelium in rats. *Gastroenterology* 2000; **119**: 1681-1691
81 **Inoue H**, Shimizu I, Lu G, Itonaga M, Cui X, Okamura Y, Shono M, Honda H, Inoue S, Muramatsu M, Ito S. Idoxifene and estradiol enhance antiapoptotic activity through estrogen receptor-beta in cultured rat hepatocytes. *Dig Dis Sci* 2003; **48**: 570-580
82 **Zhou Y**, Shimizu I, Lu G, Itonaga M, Okamura Y, Shono M, Honda H, Inoue S, Muramatsu M, Ito S. Hepatic stellate cells contain the functional estrogen receptor beta but not the estrogen receptor alpha in male and female rats. *Biochem*

Biophys Res Commun 2001; **286**: 1059-1065
83 **Naugler WE**, Sakurai T, Kim S, Maeda S, Kim K, Elsharkawy AM, Karin M. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. *Science* 2007; **317**: 121-124
84 **Lee JS**, Chu IS, Mikaelyan A, Calvisi DF, Heo J, Reddy JK, Thorgeirsson SS. Application of comparative functional genomics to identify best-fit mouse models to study human cancer. *Nat Genet* 2004; **36**: 1306-1311
85 **Mishkin SY**, Farber E, Ho RK, Mulay S, Mishkin S. Evidence for the hormone dependency of hepatic hyperplastic nodules: inhibition of malignant transformation after exogenous 17 beta-estradiol and tamoxifen. *Hepatology* 1983; **3**: 308-316
86 **Eagon PK**, Chandar N, Epley MJ, Elm MS, Brady EP, Rao KN. Di(2-ethylhexyl)phthalate-induced changes in liver estrogen metabolism and hyperplasia. *Int J Cancer* 1994; **58**: 736-743
87 **Eagon PK**, Elm MS, Epley MJ, Shinozuka H, Rao KN. Sex steroid metabolism and receptor status in hepatic hyperplasia and cancer in rats. *Gastroenterology* 1996; **110**: 1199-1207
88 **Waalkes MP**, Liu J, Chen H, Xie Y, Achanzar WE, Zhou YS, Cheng ML, Diwan BA. Estrogen signaling in livers of male mice with hepatocellular carcinoma induced by exposure to arsenic in utero. *J Natl Cancer Inst* 2004; **96**: 466-474
89 **Waalkes MP**, Liu J, Ward JM, Diwan BA. Enhanced urinary bladder and liver carcinogenesis in male CD1 mice exposed to transplacental inorganic arsenic and postnatal diethylstilbestrol or tamoxifen. *Toxicol Appl Pharmacol* 2006; **215**: 295-305
90 **Sato R**, Maesawa C, Fujisawa K, Wada K, Oikawa K, Takikawa Y, Suzuki K, Oikawa H, Ishikawa K, Masuda T. Prevention of critical telomere shortening by oestradiol in human normal hepatic cultured cells and carbon tetrachloride induced rat liver fibrosis. *Gut* 2004; **53**: 1001-1009
91 **Shimomura M**, Higashi S, Mizumoto R. 32P-postlabeling analysis of DNA adducts in rats during estrogen-induced hepatocarcinogenesis and effect of tamoxifen on DNA adduct level. *Jpn J Cancer Res* 1992; **83**: 438-444
92 **Campen D**, Maronpot R, Lucier G. Dose-response relationships in promotion of rat hepatocarcinogenesis by 17 alpha-ethinylestradiol. *J Toxicol Environ Health* 1990; **29**: 257-268
93 **Yamagiwa K**, Higashi S, Mizumoto R. Effect of alcohol ingestion on carcinogenesis by synthetic estrogen and progestin in the rat liver. *Jpn J Cancer Res* 1991; **82**: 771-778
94 **Tsuchiya Y**, Nakajima M, Yokoi T. Cytochrome P450-mediated metabolism of estrogens and its regulation in human. *Cancer Lett* 2005; **227**: 115-124
95 **Nemoto Y**, Toda K, Ono M, Fujikawa-Adachi K, Saibara T, Onishi S, Enzan H, Okada T, Shizuta Y. Altered expression of fatty acid-metabolizing enzymes in aromatase-deficient mice. *J Clin Invest* 2000; **105**: 1819-1825
96 **Yasuda M**, Shimizu I, Shiba M, Ito S. Suppressive effects of estradiol on dimethylnitrosamine-induced fibrosis of the liver in rats. *Hepatology* 1999; **29**: 719-727
97 **Shimizu I**, Mizobuchi Y, Yasuda M, Shiba M, Ma YR, Horie T, Liu F, Ito S. Inhibitory effect of oestradiol on activation of rat hepatic stellate cells in vivo and in vitro. *Gut* 1999; **44**: 127-136
98 **Ostrowski JL**, Ingleton PM, Underwood JC, Parsons MA. Increased hepatic androgen receptor expression in female rats during diethylnitrosamine liver carcinogenesis. A possible correlation with liver tumor development. *Gastroenterology* 1988; **94**: 1193-1200
99 **Tejura S**, Rodgers GR, Dunion MH, Parsons MA, Underwood JC, Ingleton PM. Sex-steroid receptors in the diethylnitrosamine model of hepatocarcinogenesis: modifications by gonadal ablation and steroid replacement therapy. *J Mol Endocrinol* 1989; **3**: 229-237
100 **Liang L**, Lu M, Huang J. [Antiandrogen treatment for nude mice model with ectopic transplanted human HCC] *Zhonghua Yixue Zazhi* 1998; **78**: 299-300
101 **Matsuura B**, Taniguchi Y, Ohta Y. Effect of antiandrogen treatment on chemical hepatocarcinogenesis in rats. *J Hepatol* 1994; **21**: 187-193
102 **Nagasue N**, Yu L, Yamaguchi M, Kohno H, Tachibana M, Kubota H. Inhibition of growth and induction of TGF-beta 1 in human hepatocellular carcinoma with androgen receptor by cyproterone acetate in male nude mice. *J Hepatol* 1996; **25**: 554-562
103 **Maruyama S**, Nagasue N, Dhar DK, Yamanoi A, El-Assal ON, Satoh K, Okita K. Preventive effect of FK143, a 5alpha-reductase inhibitor, on chemical hepatocarcinogenesis in rats. *Clin Cancer Res* 2001; **7**: 2096-2104
104 **Liang L**, Zhou Z, Lu M, Peng B, Lu H, Yin X, He Q, Huang J. [Dynamic change of androgen-receptor in tumor and surrounding liver tissue of nude mice model with transplanted human HCC] *Zhonghua Waike Zazhi* 1998; **36**: 525-527
105 **Björnström L**, Sjöberg M. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. *Mol Endocrinol* 2005; **19**: 833-842
106 **Nilsson S**, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, Gustafsson JA. Mechanisms of estrogen action. *Physiol Rev* 2001; **81**: 1535-1565
107 **O'Lone R**, Frith MC, Karlsson EK, Hansen U. Genomic targets of nuclear estrogen receptors. *Mol Endocrinol* 2004; **18**: 1859-1875
108 **Göttlicher M**, Heck S, Herrlich P. Transcriptional cross-talk, the second mode of steroid hormone receptor action. *J Mol Med* 1998; **76**: 480-489
109 **Lösel R**, Wehling M. Nongenomic actions of steroid hormones. *Nat Rev Mol Cell Biol* 2003; **4**: 46-56
110 **Chen JQ**, Yager JD, Russo J. Regulation of mitochondrial respiratory chain structure and function by estrogens/estrogen receptors and potential physiological/pathophysiological implications. *Biochim Biophys Acta* 2005; **1746**: 1-17
111 **Hytiroglou P**, Theise ND. Telomerase activation in human hepatocarcinogenesis. *Am J Gastroenterol* 2006; **101**: 839-841
112 **Rudolph KL**, Chang S, Millard M, Schreiber-Agus N, DePinho RA. Inhibition of experimental liver cirrhosis in mice by telomerase gene delivery. *Science* 2000; **287**: 1253-1258
113 **Kyo S**, Takakura M, Kanaya T, Zhuo W, Fujimoto K, Nishio Y, Orimo A, Inoue M. Estrogen activates telomerase. *Cancer Res* 1999; **59**: 5917-5921
114 **Marino M**, Distefano E, Trentalance A, Smith CL. Estradiol-induced IP(3) mediates the estrogen receptor activity expressed in human cells. *Mol Cell Endocrinol* 2001; **182**: 19-26
115 **Brandt S**, Heller H, Schuster KD, Grote J. The tamoxifen-induced suppression of telomerase activity in the human hepatoblastoma cell line HepG2: a result of post-translational regulation. *J Cancer Res Clin Oncol* 2005; **131**: 120-128
116 **Altucci L**, Addeo R, Cicatiello L, Dauvois S, Parker MG, Truss M, Beato M, Sica V, Bresciani F, Weisz A. 17beta-Estradiol induces cyclin D1 gene transcription, p36D1-p34cdk4 complex activation and p105Rb phosphorylation during mitogenic stimulation of G(1)-arrested human breast cancer cells. *Oncogene* 1996; **12**: 2315-2324
117 **Prall OW**, Sarcevic B, Musgrove EA, Watts CK, Sutherland RL. Estrogen-induced activation of Cdk4 and Cdk2 during G1-S phase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E-Cdk2. *J Biol Chem* 1997; **272**: 10882-10894
118 **Herber B**, Truss M, Beato M, Muller R. Inducible regulatory elements in the human cyclin D1 promoter. *Oncogene* 1994; **9**:

1295-1304
119 **Foster JS**, Henley DC, Ahamed S, Wimalasena J. Estrogens and cell-cycle regulation in breast cancer. *Trends Endocrinol Metab* 2001; **12**: 320-327
120 **Park YN**, Chae KJ, Kim YB, Park C, Theise N. Apoptosis and proliferation in hepatocarcinogenesis related to cirrhosis. *Cancer* 2001; **92**: 2733-2738
121 **Finucane DM**, Bossy-Wetzel E, Waterhouse NJ, Cotter TG, Green DR. Bax-induced caspase activation and apoptosis via cytochrome c release from mitochondria is inhibitable by Bcl-xL. *J Biol Chem* 1999; **274**: 2225-2233
122 **Oltvai ZN**, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 1993; **74**: 609-619
123 **Chen J**, Delannoy M, Odwin S, He P, Trush MA, Yager JD. Enhanced mitochondrial gene transcript, ATP, bcl-2 protein levels, and altered glutathione distribution in ethinyl estradiol-treated cultured female rat hepatocytes. *Toxicol Sci* 2003; **75**: 271-278
124 **Lu G**, Shimizu I, Cui X, Itonaga M, Tamaki K, Fukuno H, Inoue H, Honda H, Ito S. Antioxidant and antiapoptotic activities of idoxifene and estradiol in hepatic fibrosis in rats. *Life Sci* 2004; **74**: 897-907
125 **Dong L**, Wang W, Wang F, Stoner M, Reed JC, Harigai M, Samudio I, Kladde MP, Vyhlidal C, Safe S. Mechanisms of transcriptional activation of bcl-2 gene expression by 17beta-estradiol in breast cancer cells. *J Biol Chem* 1999; **274**: 32099-32107
126 **Kalaitzidis D**, Gilmore TD. Transcription factor cross-talk: the estrogen receptor and NF-kappaB. *Trends Endocrinol Metab* 2005; **16**: 46-52
127 **Harnish DC**, Scicchitano MS, Adelman SJ, Lyttle CR, Karathanasis SK. The role of CBP in estrogen receptor cross-talk with nuclear factor-kappaB in HepG2 cells. *Endocrinology* 2000; **141**: 3403-3411
128 **Evans MJ**, Eckert A, Lai K, Adelman SJ, Harnish DC. Reciprocal antagonism between estrogen receptor and NF-kappaB activity in vivo. *Circ Res* 2001; **89**: 823-830
129 **Pietras RJ**, Szego CM. Partial purification and characterization of oestrogen receptors in subfractions of hepatocyte plasma membranes. *Biochem J* 1980; **191**: 743-760
130 **Chen JQ**, Yager JD. Estrogen's effects on mitochondrial gene expression: mechanisms and potential contributions to estrogen carcinogenesis. *Ann N Y Acad Sci* 2004; **1028**: 258-272
131 **Solakidi S**, Psarra AM, Sekeris CE. Differential subcellular distribution of estrogen receptor isoforms: localization of ERalpha in the nucleoli and ERbeta in the mitochondria of human osteosarcoma SaOS-2 and hepatocarcinoma HepG2 cell lines. *Biochim Biophys Acta* 2005; **1745**: 382-392
132 **Psarra AM**, Solakidi S, Sekeris CE. The mitochondrion as a primary site of action of steroid and thyroid hormones: presence and action of steroid and thyroid hormone receptors in mitochondria of animal cells. *Mol Cell Endocrinol* 2006; **246**: 21-33
133 **Sekeris CE**. The mitochondrial genome: a possible primary site of action of steroid hormones. *In Vivo* 1990; **4**: 317-320
134 **Demonacos CV**, Karayanni N, Hatzoglou E, Tsiriyiotis C, Spandidos DA, Sekeris CE. Mitochondrial genes as sites of primary action of steroid hormones. *Steroids* 1996; **61**: 226-232
135 **Chen JQ**, Eshete M, Alworth WL, Yager JD. Binding of MCF-7 cell mitochondrial proteins and recombinant human estrogen receptors alpha and beta to human mitochondrial DNA estrogen response elements. *J Cell Biochem* 2004; **93**: 358-373
136 **Chen J**, Gokhale M, Li Y, Trush MA, Yager JD. Enhanced levels of several mitochondrial mRNA transcripts and mitochondrial superoxide production during ethinyl estradiol-induced hepatocarcinogenesis and after estrogen treatment of HepG2 cells. *Carcinogenesis* 1998; **19**: 2187-2193
137 **Chen J**, Li Y, Lavigne JA, Trush MA, Yager JD. Increased mitochondrial superoxide production in rat liver mitochondria, rat hepatocytes, and HepG2 cells following ethinyl estradiol treatment. *Toxicol Sci* 1999; **51**: 224-235
138 **Huang EJ**, Wu CC, Huang HP, Liu JY, Lin CS, Chang YZ, Lin JA, Lin JG, Chen LM, Lee SD, Kuo WW, Huang CY. Apoptotic and anti-proliferative effects of 17beta-estradiol and 17beta-estradiol-like compounds in the Hep3B cell line. *Mol Cell Biochem* 2006; **290**: 1-7
139 **Huang EJ**, Wu CC, Lee SD, Chen JH, Liu JY, Ko JL, Lin JA, Lu MC, Chen LM, Huang CY, Kuo WW. Opposing action of estrogen receptors alpha and beta on tumor necrosis factor-alpha gene expression and caspase-8-mediated apoptotic effects in HA22T cells. *Mol Cell Biochem* 2006; **287**: 137-145
140 **Moats RK 2nd**, Ramirez VD. Electron microscopic visualization of membrane-mediated uptake and translocation of estrogen-BSA:colloidal gold by hep G2 cells. *J Endocrinol* 2000; **166**: 631-647
141 **Shimada K**, Nakamura M, Ishida E, Konishi N. Molecular roles of MAP kinases and FADD phosphorylation in prostate cancer. *Histol Histopathol* 2006; **21**: 415-422
142 **Totta P**, Acconcia F, Leone S, Cardillo I, Marino M. Mechanisms of naringenin-induced apoptotic cascade in cancer cells: involvement of estrogen receptor alpha and beta signalling. *IUBMB Life* 2004; **56**: 491-499
143 **Claessens F**, Verrijdt G, Haelens A, Callewaert L, Moehren U, d'Alesio A, Tanner T, Schauwaers K, Denayer S, Van Tilborgh N. Molecular biology of the androgen responses. *Andrologia* 2005; **37**: 209-210
144 **Gelmann EP**. Molecular biology of the androgen receptor. *J Clin Oncol* 2002; **20**: 3001-3015
145 **Yoon G,** Kim JY, Choi YK, Won YS, Lim IK. Direct activation of TGF-beta1 transcription by androgen and androgen receptor complex in Huh7 human hepatoma cells and its tumor in nude mice. *J Cell Biochem* 2006; **97**: 393-411
146 **Jie X**, Lang C, Jian Q, Chaoqun L, Dehua Y, Yi S, Yanping J, Luokun X, Qiuping Z, Hui W, Feili G, Boquan J, Youxin J, Jinquan T. Androgen activates PEG10 to promote carcinogenesis in hepatic cancer cells. *Oncogene* 2007; **26**: 5741-5751
147 **Okabe H**, Satoh S, Furukawa Y, Kato T, Hasegawa S, Nakajima Y, Yamaoka Y, Nakamura Y. Involvement of PEG10 in human hepatocellular carcinogenesis through interaction with SIAH1. *Cancer Res* 2003; **63**: 3043-3048
148 **Tsou AP**, Chuang YC, Su JY, Yang CW, Liao YL, Liu WK, Chiu JH, Chou CK. Overexpression of a novel imprinted gene, PEG10, in human hepatocellular carcinoma and in regenerating mouse livers. *J Biomed Sci* 2003; **10**: 625-635
149 **Konoplya EF**, Popoff EH. Identification of the classical androgen receptor in male rat liver and prostate cell plasma membranes. *Int J Biochem* 1992; **24**: 1979-1983
150 **Wang AG**, Moon HB, Chun SY, Lee TH, Yu DY, Lee DS. Orchiectomy reduces hepatotumorigenesis of H-ras12V transgenic mice via the MAPK pathway. *Life Sci* 2006; **79**: 1974-1980
151 **Murakami S**. Hepatitis B virus X protein: structure, function and biology. *Intervirology* 1999; **42**: 81-99
152 **Levrero M**. Viral hepatitis and liver cancer: the case of hepatitis C. *Oncogene* 2006; **25**: 3834-3847
153 **Xue Y**, Wong J, Moreno GT, Young MK, Cote J, Wang W. NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. *Mol Cell* 1998; **2**: 851-861
154 **Bartenschlager R**, Lohmann V. Novel cell culture systems for the hepatitis C virus. *Antiviral Re*s 2001; **52**: 1-17
155 **Lohmann V**, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 1999; **285**: 110-113
156 **Aizaki H**, Lee KJ, Sung VM, Ishiko H, Lai MM. Characterization of the hepatitis C virus RNA replication

complex associated with lipid rafts. *Virology* 2004; **324**: 450-461
157 **Miyanari Y**, Hijikata M, Yamaji M, Hosaka M, Takahashi H, Shimotohno K. Hepatitis C virus non-structural proteins in the probable membranous compartment function in viral genome replication. *J Biol Chem* 2003; **278**: 50301-50308
158 **Shi ST**, Lee KJ, Aizaki H, Hwang SB, Lai MM. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. *J Virol* 2003; **77**: 4160-4168
159 **Chiu CM**, Yeh SH, Chen PJ, Kuo TJ, Chang CJ, Chen PJ, Yang WJ, Chen DS. Hepatitis B virus X protein enhances androgen receptor-responsive gene expression depending on androgen level. *Proc Natl Acad Sci USA* 2007; **104**: 2571-2578
160 **Zheng Y**, Chen WL, Ma WL, Chang C, Ou JH. Enhancement of gene transactivation activity of androgen receptor by hepatitis B virus X protein. *Virology* 2007; **363**: 454-461

S- Editor Zhong XY **L- Editor** Wang XL **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5962

World J Gastroenterol 2008 October 21; 14(39): 5962-5968
World Journal of Gastroenterology ISSN 1007-9327

REVIEW

MYC and gastric adenocarcinoma carcinogenesis

Danielle Queiroz Calcagno, Mariana Ferreira Leal, Paulo Pimentel Assumpção, Marília de Arruda Cardoso Smith, Rommel Rodríguez Burbano

Danielle Queiroz Calcagno, Rommel Rodríguez Burbano, Human Cytogenetics Laboratory, Biology Science Institute, Federal University of Pará, Belém PA 66075-900, Brazil
Mariana Ferreira Leal, Marília de Arruda Cardoso Smith, Genetics Division, Department of Morphology and Genetics, Federal University of São Paulo, São Paulo SP 04023-900, Brazil
Paulo Pimentel Assumpção, João de Barros Barreto University Hospital, Federal University of Pará, Belém PA 66073-000, Brazil
Author contributions: Calcagno DQ, Leal MF, Assumpção PP, Smith MAC and Burbano RR contributed equally to this work; the authors wrote the paper based on results of their own experience and recent literature sources (PubMed, ISI Web of Science) on gastric cancer.
Supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, DQC, MACS and RRB) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, MFL)
Correspondence to: Rommel Rodríguez Burbano, Laboratório de Citogenética Humana, Departamento de Biologia, Instituto de Ciências Biológicas, Campus Universitário do Guamá/ Universidade Federal do Pará, Av. Augusto Correa 01, Belém, PA 66075-900, Brazil. rommel@ufpa.br
Telephone: +55-91-32429254 Fax: +55-91-32111601
Received: April 10, 2008 Revised: June 7, 2008
Accepted: June 14, 2008
Published online: October 21, 2008

Abstract

MYC is an oncogene involved in cell cycle regulation, cell growth arrest, cell adhesion, metabolism, ribosome biogenesis, protein synthesis, and mitochondrial function. It has been described as a key element of several carcinogenesis processes in humans. Many studies have shown an association between *MYC* deregulation and gastric cancer. *MYC* deregulation is also seen in gastric preneoplastic lesions and thus it may have a role in early gastric carcinogenesis. Several studies have suggested that amplification is the main mechanism of *MYC* deregulation in gastric cancer. In the present review, we focus on the deregulation of the *MYC* oncogene in gastric adenocarcinoma carcinogenesis, including its association with *Helicobacter pylori* (*H pylori*) and clinical applications.

Key words: *MYC*; Gastric adenocarcinoma; Gastric preneoplastic lesions; Gastric carcinogenesis; *Helicobacter pylori*

Peer reviewer: Anna Linda Zignego, Professor of Medicine, MASVE Center, Department of Internal Medicine, University of Florence, School of Medicine, Viale Morgagni 85, 50134, Florence, Italy

Calcagno DQ, Leal MF, Assumpção PP, Smith MAC, Burbano RR. *MYC* and gastric adenocarcinoma carcinogenesis. *World J Gastroenterol* 2008; 14(39): 5962-5968 Available from: URL: http://www.wjgnet.com/1007-9327/14/5962.asp DOI: http://dx.doi.org/10.3748/wjg.14.5962

INTRODUCTION

A temporal decline in gastric cancer (GC) incidence has been seen in several countries, including Brazil[1,2]. However, this cancer causes nearly one million deaths a year worldwide and is still a serious public health cancer[3], especially in the Pará State, Northern Brazil, where mortality rates are higher than the national average rate[2]. GC is usually diagnosed at advanced stages and the single curative therapy available requires surgical resection[4].

Over 95% of gastric malignancies are adenocarcinomas[5]. They are subdivided into two main histological types: well-differentiated or intestinal-type, and undifferentiated or diffuse-type[6]. Intestinal-type gastric tumors predominate in high-risk geographic areas whereas diffuse-type tumors are more common in low-risk areas[7].

The identification of peculiar genetic characteristics of gastric tumors may help predict prognosis of GC patients and allow more accurate therapeutic approaches. Genetic analyses of GC suggest that there occur structural and functional alterations of several oncogenes and tumor suppressor genes, as well as genetic instability[8]. Additionally, GC has been an interesting carcinogenesis model. Evidence suggests that intestinal- and diffuse-type gastric carcinomas develop through distinct genetic pathways due to different genetic alterations identified in these histological types[9,10].

MYC (*C-MYC*) oncogene has been described as a key element of several carcinogenesis processes in humans[11]. In the present review, we focus on the deregulation of the *MYC* oncogene in gastric carcinogenesis.

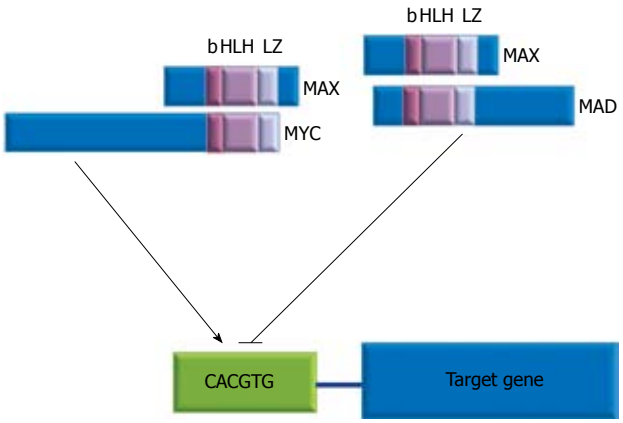


Figure 1 Activation of MYC target genes by the interaction between MYC:MAX or their repression by MAX:MAD. Domains that are common to each protein and are involved in heterodimerization are shown; b: Basic region; HLH: Helix-loop-helix; LZ: Leucine zipper. E-box sequence is shown in green.

MYC AND CANCER

MYC gene was found to be the cellular homolog of retroviral v-myc oncogene about 30 years ago[12-14]. It is located on chromosomal region 8q24.1, has 3 exons[15,16] and encodes a nuclear phosphoprotein[17].

MYC has to heteromerize with MAX, a protein expressed constitutively, to acquire DNA-binding activity. MYC/MAX dimmers are made viable by a basic region helix-loop-helix leucine-zipper motif (bHLH-Zip), conserved sequences in the carboxyl terminus of both proteins. MYC/MAX dimmers bind to E-box sequence CACGTG in the promoters of specific target genes and stimulate their transcription[18].

MYC has an effect on up to about 15% of genes in genomes of many organisms, from flies to humans[19]. Groups of genes involved in cell cycle regulation, metabolism, ribosome biogenesis, protein synthesis, and mitochondrial function are over-represented in the Myc target gene network.

MYC also consistently represses genes involved in cell growth arrest and cell adhesion[20]. Dominguez-Sola *et al*[21] recently showed that Myc interacts with the pre-replicative complex and localizes to early sites of DNA synthesis. Thus, it also has a direct role in the control of DNA replication.

MYC regulates transcription from its targets through several mechanisms, including recruitment of histone acetylases, chromatin modulating proteins, basal transcription factors and DNA methyltransferase[22-26].

Protein products of *MYC* target genes go on to mediate the downstream effects of *MYC* on cell biology. MYC is then rapidly degraded, and the pathway switches to a transcriptionally repressive state when MAX dimerizes with a group of related bHLH-Zip proteins, the MAD family, that act as MYC antagonists[27] (Figure 1).

MYC expression might be regulated transcriptionally (initiation and elongation), post-transcriptionally (mRNA stability and translation) or post-translationally (protein stability)[28].

MYC is generally recognized as an important regulator of proliferation, growth, differentiation and apoptosis[29,30]. Therefore, it is also accepted that the deregulation of *MYC* expression is a major event in cancer pathogenesis or progression. Deregulated expression of a wild-type MYC protein is sufficient to lead to cellular transformation *in vitro* and tumorigenesis *in vivo*[31].

Recent studies have also found that MYC oncoprotein, in addition to its directly transforming role, can mediate genomic instability *via* the induction of reactive oxygen species and by promoting whole chromosome instability leading to tetraploidy and aneuploidy. MYC's ability to promote chromosomal instability is closely linked to its function as a transcriptional regulator[32]. Our research group reported higher frequency of tetraploid clones in GC cell line[33] and aneuploid cells in primary gastric tumor[34,35].

Oncogenic alterations of *MYC* are commonly induced by events such as point mutations, gene amplification, chromosomal translocation, viral insertion at the *MYC* locus, and resistance of MYC protein to ubiquitin-mediated proteolysis and enhanced transcription or translation by other oncogenic signaling pathways[30].

MYC AND GASTRIC CARCINOGENESIS

MYC overexpression has been described in over 40% of GC[36]. We found that MYC protein was expressed in all cases of both intestinal- and diffuse-type gastric adenocarcinoma samples of individuals from Northern Brazil[37]. Table 1[38-59] shows the proportion of cases with *MYC* aberration in several GC studies.

Several studies have shown the association between MYC expression and histopathologic characteristics. Xu *et al*[51] and Yang *et al*[54] described a significantly higher expression of MYC in intestinal-type than in diffuse-type GC.

Kozma *et al*[50] and Yang *et al*[54] reported that higher MYC expression was associated with the presence of metastasis. Onoda *et al*[41] also found *MYC* mRNA levels were higher in metastatic than in primary lesions. Han *et al*[45] described that patients with high levels of MYC expression had poor disease-free survival. Therefore, MYC expression may represent an aggressive phenotype of GC.

MYC overexpression has also been seen in early GC when tumor invasion is confined to the mucosa or submucosa regardless of the presence of lymph node metastasis[40,41,45,46,52,54,59]. Yang *et al*[54] found a significantly higher expression of MYC in advanced GC than in early stage GC. However, Onoda *et al*[41] reported that MYC expression was found to be more frequent and stronger in early than in advanced lesions. Other studies have not found this same difference.

Several studies demonstrated an increased MYC expression in pre-cancerous gastric lesions and its increased expression also has been associated with *Helicobacter pylori* (*H pylori*) infection. *H pylori* is defined as a carcinogen factor to gastric carcinoma infection by the International Agency for Research on Cancer (IARC)[60].

Table 1 Several MYC studies in gastric cancer

Reference	Cause of MYC deregulation	Increased MYC	Number of cases	Rate (%) of cases with MYC deregulation
[38]	Overexpression	Protein	88	55
[39]	Overexpression	Protein	213	23.5
[40]	Amplification/Overexpression	DNA/Protein	31/51	12.9/41.2
[41]	Overexpression	RNA	51	68.6
[42]	Amplification	DNA	23	26
[43]	Amplification	DNA	21	48
[44]	Amplification	Protein	154	15.5
[45]	Overexpression	Protein	48 advanced/28 early	50/42
[46]	Overexpression	Protein	98 advanced/45 early	28/34
[47]	Amplification	DNA	51	24
[48]	Amplification	DNA	10	30
[49]	Overexpression	Protein	42 advanced/77 early	40.5/15.6
[50]	Amplification/Overexpression	DNA/Protein	23	26
[51]	Overexpression	Protein	30 advanced/ 6 early	63.3/50
[52]	Overexpression	Protein	35 advanced/74 early	34/16
[53]	Overexpression	Protein	84	88.1
[54]	Overexpression	Protein	63	52.4
[55]	Overexpression	Protein	65	61.5
[56]	Amplification	DNA/Protein	11	100
[37]	Amplification/Overexpression	DNA/Protein	7	100
[57]	Overexpression	Protein	204	43
[58]	Overexpression	Protein	71	42.3
[59]	Amplification/Overexpression	DNA/Protein	5 early	100

Chronic gastritis caused by *H pylori* infection may progress to intestinal metaplasia and even to GC[61,62].

Tatsuta *et al*[63] evaluated *MYC* mRNA expression by in situ hybridization in 31 elevated gastric lesions. Patients who had borderline lesions with and without *MYC* overexpression were followed up with repeated endoscopic examinations and gastric biopsies. The authors reported that well-differentiated elevated-type adenocarcinomas were detected in 46% of patients with elevated lesions that presented *MYC* overexpression during a follow-up period of about 15 mo (range, 2-32 mo) and that no cancers were found in patients with elevated lesions without *MYC* overexpression. These sample groups were significantly different. Therefore, *MYC* overexpression may provide a valuable tool for distinguishing between adenomas and well-differentiated elevated-type adenocarcinomas.

Xu *et al*[51] noticed that MYC protein expression increased progressively as follows: chronic active gastritis, gastric ulcer, mild nonclassic proliferation, severe non-classic proliferation, early GC, and progressive GC.

Lan *et al*[53] found that MYC expression was higher in GC than in chronic gastritis, intestinal metaplasia and dysplasia. MYC expression was higher in type Ⅲ intestinal metaplasia with *H pylori* compared to the same metaplasia without infection and the positive rate in dysplasia with *H pylori* was higher than that without infection. Zhang *et al*[55] also reported that MYC expression was higher in chronic atrophic gastritis with severe intestinal metaplasia than that with mild intestinal metaplasia. In chronic atrophic gastritis with severe intestinal metaplasia, MYC expression was higher in cases with *H pylori* infection than in those without infection. Higher MYC expression was also found in GC with *H pylori* infection than in that without infection. Thus, MYC expression was coordinately up-regulated in *H pylori* infected GC and chronic atrophic gastritis with severe intestinal metaplasia. Authors have suggested that *H pylori* infection may affect MYC expression in gastric diseases, especially in chronic atrophic gastritis.

Several studies have shown that patients with preneoplastic and neoplastic gastric epithelial lesions are more likely to be infected by cagA positive strains. *H pylori* cagA is one of the most virulent strains of *H pylori*. Increased cancer risk is described in individuals infected by cagA-positive *H pylori* strains compared with those infected by cagA-negative *H pylori* strains and, in general, in those living in areas with a high rate of cagA-positive *H pylori* strains[64]. Yang *et al*[54] compared MYC expression in gastric tissues (intestinal metaplasia, dysplasia and GC) with and without *H pylori* cagA. These authors found that MYC expression was significantly higher in those lesions of type Ⅲ intestinal metaplasia and dysplasia Ⅱ-Ⅲ with cagA than in those without cagA. Nardone *et al*[64] also suggested that the increased prevalence of MYC expression was in agreement with the high prevalence of cagA positivity seen in the population studied.

Kim *et al*[65] investigated the expression of MYC protein and mRNA in 22 patients with chronic gastritis who had been successfully treated for *H pylori*. Two endoscopic antral biopsies were taken before and 2 mo after *H pylori* eradication. The proportion of gastric antral epithelial cells expressing MYC protein was significantly lower after *H pylori* eradication. *MYC* mRNA expression was not changed by *H pylori* eradication. *H pylori* may affect cell cycle progression and carcinogenesis through post-translational effects on specific gene expression. Nardone *et al*[64] also found that MYC expression disappeared after *H pylori* eradication.

In vitro studies have also confirmed that *H pylori* can

affect *MYC* expression. Yang *et al*[66] described that *H pylori* induces apoptosis in human gastric adenocarcinoma cells mediated by an increased expression of *MYC* mRNA.

Epstein-Barr virus (EBV) is another infectious agent thought to contribute to cancerous transformation of human host cells. EBV infection is seen in about 10% of gastric adenocarcinoma cases[49,58,67]. Ishii *et al*[49] found MYC expression in early stages of EBV-positive GC was higher than that of EBV-negative GC, while MYC expression in advanced stages of EBV-positive GC was lower than that of EBV-negative tumors. It was inferred that EBV might cause the host cell to induce MYC expression in early cancer development, but then negatively affect MYC expression in advanced stages of cancers, making them less likely to have a natural regression *via* apoptosis. Lima *et al*[58] also reported MYC low expression in EBV-positive GC samples. However, Luo *et al*[67] have not found any correlation between EBV and MYC expression in GC, suggesting that EBV does not inhibit MYC expression in advanced stages of EBV-positive gastric cancer.

MECHANISMS OF MYC DEREGULATION IN GASTRIC CANCER

Copy number gains are frequently detected along chromosome 8 in gastric tumors[43,48,56,68-73]. Suzuki *et al*[43] described that chromosome 8 copy number was significantly higher in differentiated than undifferentiated types of GC. Our research group found 8q24.1 gain, where *MYC* is located, exclusively in intestinal subtype with metastasis by comparative genome hybridization (CGH)[72]. However, Koo *et al*[48] reported that amplifications in 8q region were more common in diffuse-type cancer.

Some studies have showed an association between MYC amplification and GC[42-44,48]. We have also previously seen *MYC* amplification in intestinal adenocarcinoma by dual-color fluorescence in situ hybridization (FISH), such as homogeneously staining chromosomal regions and double minutes, supporting our CGH results[56]. Our findings support that these two histological GC types follow different genetic pathways.

Our research group also found that all five early GC cases with MYC overexpression also had three signal to MYC gene by FISH assay, varying between 13% and 26% of cells/case[59]. Suzuki *et al*[43] found *MYC* amplification in all 6 early GC cases studied, varying between 19% and 89% of cells/case, and this rate was not significantly difference from that found in advanced GC samples. These findings suggest that *MYC* amplification can be a critical event to gastric carcinogenesis.

MYC translocation is frequently described in Burkitt's lymphoma. Few studies have also found translocation of the *MYC* locus associated with gastric carcinogenesis. Yamashita *et al*[74] identified chromosomal translocations involved in 8q24 breakpoint by spectral karyotyping (SKY) analysis of established GC cell lines and cancerous ascitic fluids. In a previous study, our findings suggested that translocations can be related to diffuse-type GC using FISH assay[37,56].

Epigenetic events play a significant role in cancer development and progression. DNA methylation is the most studied epigenetic alteration. Some studies also have demonstrated that *MYC* hypomethylation, which leads to its activation, is significantly more common in GC samples than non-cancerous tissues[75,76]. Fang *et al*[77] and Weng *et al*[78] suggest that folate level reduction is associated with upregulation of *MYC* expression and its promoter hypomethylation in GC.

FUTURE PERSPECTIVES

Proto-oncogenes have a major role not only in cancer development, but also in cancer therapies[79]. *MYC* alteration is seen in the early gastric carcinogenesis progress. The detection of *MYC* locus amplification may be used as an auxiliary tool to GC diagnosis and as a predictor of GC aggressiveness.

MYC also could be used as a therapeutical target. Several experimental studies showed that *MYC* inactivation suppresses tumors in animal models, suggesting *MYC* as a molecular target in cancer treatment[80-83].

Chen *et al*[84] evaluated the effect of MYC expression inhibition by recombinant antisense *MYC* adenovirus (Ad-ASc-myc) infected SGC7901 human gastric carcinoma cells, which have *MYC* gene amplification, in the proliferation, apoptosis and growth processes of human gastric tumors in nude mice. It was found that MYC expression inhibition may strongly inhibit cell growth and induce apoptosis in SGC7901 cells. Proliferation of Ad-ASc-myc-infected SGC7901 cells was reduced by 44.1%. Studies involving tumorigenicity in nude mice and experimental therapy in nude mice model using Ad-ASc-myc also support these findings. These studies also suggest that Ad-ASc-myc overexpression may result in the elimination of tumor cells *via* apoptosis and proliferation inhibition, and therefore reduce tumor burden.

Inhibiting MYC expression can be a potential tool for GC treatment in tumors with MYC overexpression. MYC's therapy target may help identifying more specific and less toxic therapeutic agents[30].

REFERENCES

1 **Terry MB**, Gaudet MM, Gammon MD. The epidemiology of gastric cancer. *Semin Radiat Oncol* 2002; **12**: 111-127
2 **Resende AL**, Mattos IE, Koifman S. [Gastric cancer mortality in the State of Para, Brazil, 1980-1997] *Arq Gastroenterol* 2006; **43**: 247-251
3 **World Health Organization (WHO)**. Available from: URL: http://www.who.int/en. Accessed January 29, 2008
4 **Nardone G**. Review article: molecular basis of gastric carcinogenesis. *Aliment Pharmacol Ther* 2003; **17** Suppl 2: 75-81
5 **Smith MG**, Hold GL, Tahara E, El-Omar EM. Cellular and molecular aspects of gastric cancer. *World J Gastroenterol* 2006; **12**: 2979-2990
6 **Crew KD**, Neugut AI. Epidemiology of gastric cancer. *World J Gastroenterol* 2006; **12**: 354-362

7 **Hamilton SR**, Aaltonen LA. Pathology and Genetics of Tumours of the Digestive System. In: WHO Classification of Tumors. Lyon: IARC Press, 2000: 204
8 **Assumpção PP**, Burbano RR. Genética do Câncer Gástrico. 1st ed. In: Linhares E, Laércio L, Takeshi S, editors. Atualização em câncer gástrico. São Paulo: Tecmed Editora Ltda, 2005: 95-121
9 **Chan AO**, Luk JM, Hui WM, Lam SK. Molecular biology of gastric carcinoma: from laboratory to bedside. *J Gastroenterol Hepatol* 1999; **14**: 1150-1160
10 **Baffa R**, Santoro R, Bullrich F, Mandes B, Ishii H, Croce CM. Definition and refinement of chromosome 8p regions of loss of heterozygosity in gastric cancer. *Clin Cancer Res* 2000; **6**: 1372-1377
11 **Oster SK**, Ho CS, Soucie EL, Penn LZ. The myc oncogene: Marvelousl Y Complex. *Adv Cancer Res* 2002; **84**: 81-154
12 **Sheiness D**, Fanshier L, Bishop JM. Identification of nucleotide sequences which may encode the oncogenic capacity of avian retrovirus MC29. *J Virol* 1978; **28**: 600-610
13 **Bishop JM**. Retroviruses and cancer genes. *Adv Cancer Res* 1982; **37**: 1-32
14 **Vennstrom B**, Sheiness D, Zabielski J, Bishop JM. Isolation and characterization of c-myc, a cellular homolog of the oncogene (v-myc) of avian myelocytomatosis virus strain 29. *J Virol* 1982; **42**: 773-779
15 **Dalla-Favera R**, Bregni M, Erikson J, Patterson D, Gallo RC, Croce CM. Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. *Proc Natl Acad Sci USA* 1982; **79**: 7824-7827
16 **Battey J**, Moulding C, Taub R, Murphy W, Stewart T, Potter H, Lenoir G, Leder P. The human c-myc oncogene: structural consequences of translocation into the IgH locus in Burkitt lymphoma. *Cell* 1983; **34**: 779-787
17 **Persson H**, Leder P. Nuclear localization and DNA binding properties of a protein expressed by human c-myc oncogene. *Science* 1984; **225**: 718-721
18 **Cowling VH**, Cole MD. Mechanism of transcriptional activation by the Myc oncoproteins. *Semin Cancer Biol* 2006; **16**: 242-252
19 **Fernandez PC**, Frank SR, Wang L, Schroeder M, Liu S, Greene J, Cocito A, Amati B. Genomic targets of the human c-Myc protein. *Genes Dev* 2003; **17**: 1115-1129
20 **Dang CV**, O'Donnell KA, Zeller KI, Nguyen T, Osthus RC, Li F. The c-Myc target gene network. *Semin Cancer Biol* 2006; **16**: 253-264
21 **Dominguez-Sola D**, Ying CY, Grandori C, Ruggiero L, Chen B, Li M, Galloway DA, Gu W, Gautier J, Dalla-Favera R. Non-transcriptional control of DNA replication by c-Myc. *Nature* 2007; **448**: 445-451
22 **McMahon SB**, Van Buskirk HA, Dugan KA, Copeland TD, Cole MD. The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins. *Cell* 1998; **94**: 363-374
23 **Eberhardy SR**, Farnham PJ. Myc recruits P-TEFb to mediate the final step in the transcriptional activation of the cad promoter. *J Biol Chem* 2002; **277**: 40156-40162
24 **O'Connell BC**, Cheung AF, Simkevich CP, Tam W, Ren X, Mateyak MK, Sedivy JM. A large scale genetic analysis of c-Myc-regulated gene expression patterns. *J Biol Chem* 2003; **278**: 12563-12573
25 **Kanazawa S**, Soucek L, Evan G, Okamoto T, Peterlin BM. c-Myc recruits P-TEFb for transcription, cellular proliferation and apoptosis. *Oncogene* 2003; **22**: 5707-5711
26 **Brenner C**, Deplus R, Didelot C, Loriot A, Vire E, De Smet C, Gutierrez A, Danovi D, Bernard D, Boon T, Pelicci PG, Amati B, Kouzarides T, de Launoit Y, Di Croce L, Fuks F. Myc represses transcription through recruitment of DNA methyltransferase corepressor. *EMBO J* 2005; **24**: 336-346
27 **Ayer DE**, Kretzner L, Eisenman RN. Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity. *Cell* 1993; **72**: 211-722
28 **Sears RC**. The life cycle of C-myc: from synthesis to degradation. *Cell Cycle* 2004; **3**: 1133-1137
29 **Meyer N**, Kim SS, Penn LZ. The Oscar-worthy role of Myc in apoptosis. *Semin Cancer Biol* 2006; **16**: 275-287
30 **Vita M**, Henriksson M. The Myc oncoprotein as a therapeutic target for human cancer. *Semin Cancer Biol* 2006; **16**: 318-330
31 **Chung HJ**, Levens D. c-myc expression: keep the noise down! *Mol Cells* 2005; **20**: 157-166
32 **Prochownik EV**, Li Y. The ever expanding role for c-Myc in promoting genomic instability. *Cell Cycle* 2007; **6**: 1024-1029
33 **Lima EM**, Rissino JD, Harada ML, Assumpcao PP, Demachki S, Guimaraes AC, Casartelli C, Smith MA, Burbano RR. Conventional cytogenetic characterization of a new cell line, ACP01, established from a primary human gastric tumor. *Braz J Med Biol Res* 2004; **37**: 1831-1838
34 **Costa Guimarães A**, Gonçalves Quintana L, Ferreira Leal M, Satomi Takeno S, Pimentel Assumpção P, Moura Lima E, Salim Khayat A, Suchi Chen E, de Arruda Cardoso Smith M, Rodríguez Burbano R. Aneuploidy of chromosome 8 detected by fluorescence in situ hybridisation in ACP01 cell line gastric adenocarcinoma. *Clin Exp Med* 2006; **6**: 129-133
35 **Assumpcao PP**, Seabra AD, Leal MF, Guimaraes AC, Calcagno DQ, Khayat AS, Smith MC, Burbano RR. Chromosome instability in carcinomas. *Int J Morphol* 2006; **24**: 335-338
36 **Milne AN**, Sitarz R, Carvalho R, Carneiro F, Offerhaus GJ. Early onset gastric cancer: on the road to unraveling gastric carcinogenesis. *Curr Mol Med* 2007; **7**: 15-28
37 **Calcagno DQ**, Leal MF, Seabra AD, Khayat AS, Chen ES, Demachki S, Assumpcao PP, Faria MH, Rabenhorst SH, Ferreira MV, de Arruda Cardoso Smith M, Burbano RR. Interrelationship between chromosome 8 aneuploidy, C-MYC amplification and increased expression in individuals from northern Brazil with gastric adenocarcinoma. *World J Gastroenterol* 2006; **12**: 6207-6211
38 **Spandidos DA**, Karayiannis M, Yiagnisis M, Papadimitriou K, Field JK. Immunohistochemical analysis of the expression of the c-myc oncoprotein in human stomach cancers. *Digestion* 1991; **50**: 127-134
39 **Ninomiya I**, Yonemura Y, Matsumoto H, Sugiyama K, Kamata T, Miwa K, Miyazaki I, Shiku H. Expression of c-myc gene product in gastric carcinoma. *Oncology* 1991; **48**: 149-153
40 **Nakata B**, Onoda N, Chung YS, Maeda K, Nishimura S, Yashiro M, Nitta A, Kubo T, Kato Y, Sowa M. [Correlation between malignancy of gastric cancer and c-myc DNA amplification or overexpression of c-myc protein] *Gan To Kagaku Ryoho* 1995; **22** Suppl 2: 176-179
41 **Onoda N**, Maeda K, Chung YS, Yano Y, Matsui-Yuasa I, Otani S, Sowa M. Overexpression of c-myc messenger RNA in primary and metastatic lesions of carcinoma of the stomach. *J Am Coll Surg* 1996; **182**: 55-59
42 **Hajdu J**, Kozma L, Kiss I, Szentkereszty Z, Szakall S, Ember I. Is the presence of distant metastasis associated with c-myc amplification in gastric cancer? *Acta Chir Hung* 1997; **36**: 119-121
43 **Suzuki S**, Tenjin T, Watanabe H, Matsushima S, Shibuya T, Tanaka S. Low level c-myc gene amplification in gastric cancer detected by dual color fluorescence in situ hybridization analysis. *J Surg Oncol* 1997; **66**: 173-178
44 **Hara T**, Ooi A, Kobayashi M, Mai M, Yanagihara K, Nakanishi I. Amplification of c-myc, K-sam, and c-met in gastric cancers: detection by fluorescence in situ hybridization. *Lab Invest* 1998; **78**: 1143-1153
45 **Han S**, Kim HY, Park K, Cho HJ, Lee MS, Kim HJ, Kim YD. c-Myc expression is related with cell proliferation and associated with poor clinical outcome in human gastric cancer. *J Korean Med Sci* 1999; **14**: 526-530
46 **Sanz-Ortega J**, Steinberg SM, Moro E, Saez M, Lopez JA, Sierra E, Sanz-Esponera J, Merino MJ. Comparative study of tumor angiogenesis and immunohistochemistry for p53, c-ErbB2, c-myc and EGFr as prognostic factors in gastric

cancer. *Histol Histopathol* 2000; **15**: 455-462
47 **Kitayama Y**, Igarashi H, Sugimura H. Different vulnerability among chromosomes to numerical instability in gastric carcinogenesis: stage-dependent analysis by FISH with the use of microwave irradiation. *Clin Cancer Res* 2000; **6**: 3139-3146
48 **Koo SH**, Kwon KC, Shin SY, Jeon YM, Park JW, Kim SH, Noh SM. Genetic alterations of gastric cancer: comparative genomic hybridization and fluorescence In situ hybridization studies. *Cancer Genet Cytogenet* 2000; **117**: 97-103
49 **Ishii H**, Gobe G, Kawakubo Y, Sato Y, Ebihara Y. Interrelationship between Epstein-Barr virus infection in gastric carcinomas and the expression of apoptosis-associated proteins. *Histopathology* 2001; **38**: 111-119
50 **Kozma L**, Kiss I, Hajdu J, Szentkereszty Z, Szakall S, Ember I. C-myc amplification and cluster analysis in human gastric carcinoma. *Anticancer Res* 2001; **21**: 707-710
51 **Xu AG**, Li SG, Liu JH, Gan AH. Function of apoptosis and expression of the proteins Bcl-2, p53 and C-myc in the development of gastric cancer. *World J Gastroenterol* 2001; **7**: 403-406
52 **Ishii HH**, Gobe GC, Pan W, Yoneyama J, Ebihara Y. Apoptosis and cell proliferation in the development of gastric carcinomas: associations with c-myc and p53 protein expression. *J Gastroenterol Hepatol* 2002; **17**: 966-972
53 **Lan J**, Xiong YY, Lin YX, Wang BC, Gong LL, Xu HS, Guo GS. Helicobacter pylori infection generated gastric cancer through p53-Rb tumor-suppressor system mutation and telomerase reactivation. *World J Gastroenterol* 2003; **9**: 54-58
54 **Yang GF**, Deng CS, Xiong YY, Gong LL, Wang BC, Luo J. Expression of nuclear factor-kappa B and target genes in gastric precancerous lesions and adenocarcinoma: association with Helicobactor pylori cagA (+) infection. *World J Gastroenterol* 2004; **10**: 491-496
55 **Zhang GX**, Gu YH, Zhao ZQ, Xu SF, Zhang HJ, Wang HD, Hao B. Coordinate increase of telomerase activity and c-Myc expression in Helicobacter pylori-associated gastric diseases. *World J Gastroenterol* 2004; **10**: 1759-1762
56 **Calcagno DQ**, Leal MF, Taken SS, Assumpcao PP, Demachki S, Smith Mde A, Burbano RR. Aneuploidy of chromosome 8 and C-MYC amplification in individuals from northern Brazil with gastric adenocarcinoma. *Anticancer Res* 2005; **25**: 4069-4074
57 **Milne AN**, Carvalho R, Morsink FM, Musler AR, de Leng WW, Ristimaki A, Offerhaus GJ. Early-onset gastric cancers have a different molecular expression profile than conventional gastric cancers. *Mod Pathol* 2006; **19**: 564-572
58 **Lima VP**, de Lima MA, Andre AR, Ferreira MV, Barros MA, Rabenhorst SH. H pylori (CagA) and Epstein-Barr virus infection in gastric carcinomas: correlation with p53 mutation and c-Myc, Bcl-2 and Bax expression. *World J Gastroenterol* 2008; **14**: 884-891
59 **Costa Raiol LC**, Figueira Silva EC, Mendes da Fonseca D, Leal MF, Guimaraes AC, Calcagno DQ, Khayat AS, Assumpcao PP, de Arruda Cardoso Smith M, Burbano RR. Interrelationship between MYC gene numerical aberrations and protein expression in individuals from northern Brazil with early gastric adenocarcinoma. *Cancer Genet Cytogenet* 2008; **181**: 31-35
60 **Cancer Databases and Other Resourcess**. International Agency for Research on Cancer (IARC) page. Available from: URL: http://www.iarc.fr
61 **Wu MS**, Chen SY, Shun CT, Lee WJ, Wang HP, Wang TH, Chen CJ, Lin JT. Increased prevalence of Helicobacter pylori infection among patients affected with intestinal-type gastric cancer at non-cardiac locations. *J Gastroenterol Hepatol* 1997; **12**: 425-428
62 **Wu MS**, Shun CT, Wu CC, Hsu TY, Lin MT, Chang MC, Wang HP, Lin JT. Epstein-Barr virus-associated gastric carcinomas: relation to H. pylori infection and genetic alterations. *Gastroenterology* 2000; **118**: 1031-1038
63 **Tatsuta M**, Iishi H, Baba M, Nakaizumi A, Uehara H, Taniguchi H. Expression of c-myc mRNA as an aid in histologic differentiation of adenoma from well differentiated adenocarcinoma in the stomach. *Cancer* 1994; **73**: 1795-1799
64 **Nardone G**, Staibano S, Rocco A, Mezza E, D'armiento FP, Insabato L, Coppola A, Salvatore G, Lucariello A, Figura N, De Rosa G, Budillon G. Effect of Helicobacter pylori infection and its eradication on cell proliferation, DNA status, and oncogene expression in patients with chronic gastritis. *Gut* 1999; **44**: 789-799
65 **Kim SS**, Meitner P, Konkin TA, Cho YS, Resnick MB, Moss SF. Altered expression of Skp2, c-Myc and p27 proteins but not mRNA after H. pylori eradication in chronic gastritis. *Mod Pathol* 2006; **19**: 49-58
66 **Yang Y**, Deng CS, Peng JZ, Wong BC, Lam SK, Xia HH. Effect of Helicobacter pylori on apoptosis and apoptosis related genes in gastric cancer cells. *Mol Pathol* 2003; **56**: 19-24
67 **Luo B**, Wang Y, Wang XF, Gao Y, Huang BH, Zhao P. Correlation of Epstein-Barr virus and its encoded proteins with Helicobacter pylori and expression of c-met and c-myc in gastric carcinoma. *World J Gastroenterol* 2006; **12**: 1842-1848
68 **Sakakura C**, Mori T, Sakabe T, Ariyama Y, Shinomiya T, Date K, Hagiwara A, Yamaguchi T, Takahashi T, Nakamura Y, Abe T, Inazawa J. Gains, losses, and amplifications of genomic materials in primary gastric cancers analyzed by comparative genomic hybridization. *Genes Chromosomes Cancer* 1999; **24**: 299-305
69 **Panani AD**, Ferti AD, Avgerinos A, Raptis SA. Numerical aberrations of chromosome 8 in gastric cancer detected by fluorescence in situ hybridization. *Anticancer Res* 2004; **24**: 155-159
70 **Kitayama Y**, Sugimura H. Nonrandom chromosomal numerical abnormality as a new molecular cytogenetic tumor marker--a retrospective study of 60 gastric cancer cases. *Rinsho Byori* 2005; **53**: 881-886
71 **Assumpcao PP**, Ishak G, Chen ES, Takeno SS, Leal MF, Guimaraes AC, Calcagno DQ, Khayat AS, Demachki S, Smith Mde A, Burbano RR. Numerical aberrations of chromosome 8 detected by conventional cytogenetics and fluorescence in situ hybridization in individuals from northern Brazil with gastric adenocarcinoma. *Cancer Genet Cytogenet* 2006; **169**: 45-49
72 **Burbano RR**, Assumpcao PP, Leal MF, Calcagno DQ, Guimaraes AC, Khayat AS, Takeno SS, Chen ES, De Arruda Cardoso Smith M. C-MYC locus amplification as metastasis predictor in intestinal-type gastric adenocarcinomas: CGH study in Brazil. *Anticancer Res* 2006; **26**: 2909-2914
73 **Yang S**, Jeung HC, Jeong HJ, Choi YH, Kim JE, Jung JJ, Rha SY, Yang WI, Chung HC. Identification of genes with correlated patterns of variations in DNA copy number and gene expression level in gastric cancer. *Genomics* 2007; **89**: 451-459
74 **Yamashita Y**, Nishida K, Okuda T, Nomura K, Matsumoto Y, Mitsufuji S, Horiike S, Hata H, Sakakura C, Hagiwara A, Yamagishi H, Taniwaki M. Recurrent chromosomal rearrangements at bands 8q24 and 11q13 in gastric cancer as detected by multicolor spectral karyotyping. *World J Gastroenterol* 2005; **11**: 5129-5135
75 **Fang JY**, Zhu SS, Xiao SD, Jiang SJ, Shi Y, Chen XY, Zhou XM, Qian LF. Studies on the hypomethylation of c-myc, c-Ha-ras oncogenes and histopathological changes in human gastric carcinoma. *J Gastroenterol Hepatol* 1996; **11**: 1079-1082
76 **Fang JY**, Cheng ZH, Chen YX, Lu R, Yang L, Zhu HY, Lu LG. Expression of Dnmt1, demethylase, MeCP2 and methylation of tumor-related genes in human gastric cancer. *World J Gastroenterol* 2004; **10**: 3394-3398
77 **Fang JY**, Xiao SD, Zhu SS, Yuan JM, Qiu DK, Jiang SJ. Relationship of plasma folic acid and status of DNA methylation in human gastric cancer. *J Gastroenterol* 1997; **32**: 171-175
78 **Weng YR**, Sun DF, Fang JY, Gu WQ, Zhu HY. Folate

levels in mucosal tissue but not methylenetetrahydrofol ate reductase polymorphisms are associated with gastric carcinogenesis. *World J Gastroenterol* 2006; **12**: 7591-7597

79 **Gschwind A**, Fischer OM, Ullrich A. The discovery of receptor tyrosine kinases: targets for cancer therapy. *Nat Rev Cancer* 2004; **4**: 361-370

80 **Boxer RB**, Jang JW, Sintasath L, Chodosh LA. Lack of sustained regression of c-MYC-induced mammary adenocarcinomas following brief or prolonged MYC inactivation. *Cancer Cell* 2004; **6**: 577-586

81 **Shachaf CM**, Kopelman AM, Arvanitis C, Karlsson A, Beer S, Mandl S, Bachmann MH, Borowsky AD, Ruebner B, Cardiff RD, Yang Q, Bishop JM, Contag CH, Felsher DW. MYC inactivation uncovers pluripotent differentiation and tumour dormancy in hepatocellular cancer. *Nature* 2004; **431**: 1112-1117

82 **Shachaf CM**, Felsher DW. Tumor dormancy and MYC inactivation: pushing cancer to the brink of normalcy. *Cancer Res* 2005; **65**: 4471-4474

83 **Yu D**, Dews M, Park A, Tobias JW, Thomas-Tikhonenko A. Inactivation of Myc in murine two-hit B lymphomas causes dormancy with elevated levels of interleukin 10 receptor and CD20: implications for adjuvant therapies. *Cancer Res* 2005; **65**: 5454-5461

84 **Chen JP**, Lin C, Xu CP, Zhang XY, Fu M, Deng YP, Wei Y, Wu M. Molecular therapy with recombinant antisense c-myc adenovirus for human gastric carcinoma cells in vitro and in vivo. *J Gastroenterol Hepatol* 2001; **16**: 22-28

S- Editor Zhong XY **L- Editor** Li M **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5969

World J Gastroenterol 2008 October 21; 14(39): 5969-5979
World Journal of Gastroenterology ISSN 1007-9327

REVIEW

Insight into congenital absence of the portal vein: Is it rare?

Guo-Hua Hu, Lai-Gen Shen, Jin Yang, Jin-Hua Mei, Yue-Feng Zhu

Guo-Hua Hu, Lai-Gen Shen, Jin Yang, Jin-Hua Mei, Yue-Feng Zhu, Department of General Surgery, Sir Run Run Shaw Hospital, Medical College of Zhejiang University, Hangzhou 310016, Zhejiang Province, China
Author contributions: Shen LG designed this work; Hu GH wrote the paper; Shen LG, Yang J, Mei JH and Zhu YF contributed equally to the revision process.
Correspondence to: Lai-Gen Shen, Department of General Surgery, Sir Run Run Shaw Hospital, Medical College of Zhejiang University, Hangzhou 310016, Zhejiang Province, China. huguo2873@163.com
Telephone: +86-571-86090073 Fax: +86-571-86044817
Received: January 29, 2008 Revised: May 27, 2008
Accepted: June 3, 2008
Published online: October 21, 2008

Abstract

Congenital absence of portal vein (CAPV) was a rare event in the past. However, the number of detected CAPV cases has increased in recent years because of advances in imaging techniques. Patients with CAPV present with portal hypertension (PH) or porto-systemic encephalopathy (PSE), but these conditions rarely occur until the patients grow up or become old. The patients usually visit doctors for the complications of venous shunts, hepatic or cardiac abnormalities detected by ultrasonography (US), computed tomography (CT) and magnetic resonance imaging (MRI). The etiology of this disease is not clear, but most investigators consider that it is associated with abnormal embryologic development of the portal vein. Usually, surgical intervention can relieve the symptoms and prevent occurrence of complications in CAPV patients. Moreover, its management should be stressed on a case-by-case basis, depending on the type or anatomy of the disease, as well as the symptoms and clinical conditions of the patient.

Key words: Congenital absence of portal vein; Abernethy malformation; Focal nodular hyperplasia

Peer reviewer: Mitsuo Shimada, Professor, Department of Digestive and Pediatric Surgery, Tokushima University, Kuramoto 3-18-15, Tokushima 770-8503, Japan

Hu GH, Shen LG, Yang J, Mei JH, Zhu YF. Insight into congenital absence of the portal vein: Is it rare? *World J Gastroenterol* 2008; 14(39): 5969-5979 Available from: URL: http://www.wjgnet.com/1007-9327/14/5969.asp DOI: http://dx.doi.org/10.3748/wjg.14.5969

INTRODUCTION

The first account of congenital absence of the portal vein (CAPV) was given by John Abernethy in 1793[1], based on a postmortem examination of a 10-mo-old female, which revealed the termination of portal vein in the inferior vena cava at the insertion level of the renal veins and multiple congenital abnormalities other than CAPV. It was reported that complete portosystemic shunts not perfusing the liver *via* portal vein are defined as type Ⅰ, whereas partial shunts with a remaining degree of portal perfusion to the liver are defined as type Ⅱ[2]. Furthermore, type Ⅰ is sub-classified into types Ⅰa and Ⅰb depending on the anatomy of portal vein. In type Ⅰa, the splenic vein (SV) and superior mesenteric vein (SMV) drain separately in type Ⅰa, while both drain together in type Ⅰb after uniting to form a common trunk[3]. Howard and Davenport[4] suggested that the congenital diversion of portal blood away from the liver, by either an end-to-side or a side-to-side shunt, is known as the Abernethy malformation. Thus, CAPV associated with extra hepatic portocaval shunts can be referred to as an Abernethy type Ⅰ malformation (Figure 1).

ANATOMY AND EMBRYOLOGY

The portal vein returns blood from the intraperitoneal section of the gastrointestinal tract and the spleen, pancreas, and biliary apparatus, while SV and SMV return blood form the portal vein. At the porta hepatis, the portal vein is subdivided into right and left branches (besides providing the quadrate lobe with an additional branch). These branches ramify to form small vessels that drain into the sinusoids[5]. CAPV with an extrahepatic porto-caval shunt means that the mesenteric vasculature where splanchnic blood bypasses the liver through a congenital shunt vessel, completely drains into the systemic circulations, such as inferior vena cava, left renal vein, right atrium, iliac vein, left hepatic vein (HV), and azygos vein[6-8]. Strictly speaking, CAPV is also characterized by complete absence of venules within the portal areas, which has been confirmed by liver biopsy[9,10]. The case seems to be a complete portal vein agenesis[10]. Similarly, complete absence of the portal

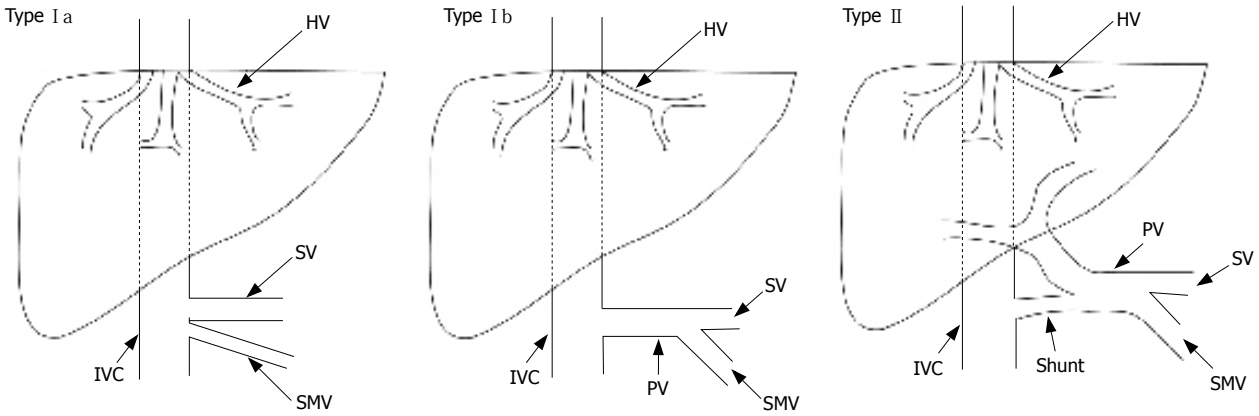


Figure 1 Schematic demonstration of different types of Abernethy abnormality[2]. SMV: Superior mesenteric vein; HV: Hepatic vein; SV: Splenic vein; PV: Portal vein; IVC: Inferior vena cava.

vein with intrahepatic portal venules can be named portal vein atresia.

The portal vein develops embryologically between the 4th and 10th weeks[10,11]. In the 4th week embryo, three paired venous systems are present: umbilical veins of chorionic origin, vitelline veins from the yolk sac and cardinal veins from the body of the embryo. Towards the end of the 4th week, three cross links are formed between the right and left vitelline veins[12]. The intrahepatic portal veins develop from the superior link, while the extrahepatic portal vein forms during the selective involution of the caudal part of the right and left vitelline veins. Selective involution of these communications generates the fully developed portal vein[11]. Primary failure to form this critical anastomosis will lead to complete or partial absence of the portal system. As a result, mesenteric and splenic venous flow cannot but drain into renal veins, HVs, or directly into the inferior vena cava (IVC)[6-8,13], with relatively poor perfusion of the liver[14].

CLINICAL MANIFESTATIONS

CAPV can cause a broad spectrum of clinical manifestations, which can be divided into two groups: concomitant congenital abnormalities and sequent syndromes. The former can be referred to as a congenital hepatopathy and a congenital cardiopathy, which can be explained by its close relation to the development of cardiovascular system.

Systemic shunting of the visceral venous return can lead to abnormal hepatic development, function and regeneration secondary to the absence of portal hepatotrophic factor, resulting in the development of focal nodular hyperplasia (FNH) and hepatic tumors[15-17]. Other than CAPV, many cases are presented with liver abnormalities, including FNH[7,15,18-30] or nodular regenerative hyperplasia (NRH)[8,31-35]. Several patients have been found to have combined hepatocellular adenoma[31,36,37]. These are generally considered benign parenchymatous lesions, and also have the potential to deteriorate into hepatoblastoma[26,28] and hepatocellular carcinoma (HCC)[12,20,38,39]. CAPV, though rare, could be found along with chronic hepatitis[20] and liver cirrhosis[17]. Gocmen *et al*[40] reported a 7-year-old boy with congenital hepatic fibrosis (CHF). Intrahepatic calcification was detected during prenatal diagnosis in a fetus. The liver volume is often larger than normal because of the regenerative or hyperplasia nodular change and sometime hepatomegaly might be observed[15]. Moreover, the mass can cause a series of mechanical pressure symptomes, such as mild intermittent jaundice and pruritus[35]. Nevertheless, the liver size may be small due to insufficient portal vein supply or lobular hypoplasia[41,42]. Abnormal hepatic development, with aberrant lobation, absence of ligamentum teres and falciform ligament, has been noted in Hellwegg's patients[43]. Liver dysfunction (LD), frequently found in liver tumors, also occurs in patients without liver mass.

CAPV may result from a embryologic insult which causes defect of the cardiovascular system and complicated cardiogenesis could be affected by the insult or the systemic diversion of portal venous flow[11]. Congenital cardiac diseases including atrial septal defect (ASD)[2, 21,26,28,37,44,45] or patent foramen ovale[43], ventricular septal defect (VSD)[16,17,21,26,31,40], and patent ductus arterious (PDA)[12,23,43,44] are frequently observed along with CAPV. Bellah *et al*[45] have also detected a fatal congenital hypertrophic cardiomyopathy with ultrasound in an infant. Dextrocardia[1] and Mesocardia[44] that reported congenital cardiac abnormalities are rarely found in CAPV cases. Congenital stenosis of aortic valve[11] and pulmonary artery valve[45], found in a few CAPV cases, can cause tricuspid regurgitation[44]. Even stenosis[11] or coarctation[12] has been observed in aorta. Most frequently encountered Cardiovascular lesions, most frequently encountered in CAPV patients with Goldenhar syndrome, are tetralogy of Fallot and VSD[46]. Cardiomegaly has been noted in CAPV patients[41,47], which may result from congenital insult or increased blood flow into the heart with a certain degree of congestive heart failure. A large number of non-heart abnormalities are found in all the cases reviewed[3,9,14,27,34-36,39,48,49].

Besides CAPV, visceral or cutaneous vascular

malformations have been reported, such as double SV[1], double inferior vena cava[40], left sided IVC[2,17,26], hepatic artery originating from superior mesenteric artery[8,50], intrapulmonary shunting[7], azygos and hemiazygos continuation[1,17,43,48], and skin hemangioma[14,43]. Obvious compensatory changes may be found in blood vessels, especially in veins returning visceral blood or ramus anastomoticus, such as the SV[51], inferior mesenteric vein[33,52], left renal vein[44,53], azygos system[30,51], IVC[14,44,53], and right atrium[14]. Hepatic artery is enlarged and hypertrophied[1,19] due to the same reason.

Polysplenia[1,2,4,17,26,54], megalosplenia[14,24,51] and hypersplenism[51] are the frequently encountered splanchnic abnormalities other than hepatic and cardiac abnormalities, which may be due to the embryonic impairment or portal hypertension (PH). The latter is a fault because CAPV accompanying PH has merely been observed in several cases[9,16,55], which is inconsistent with our general deduction. One reasonable explanation is out of the congenital adaptation.

It was reported that CAPV patients could also have congenital biliary atresia (CBA)[2,4,17,21], congenital choledochal cyst[29], and intrahepatic gallbladder[43]. The urinogenital system including cystic dysplasia of the kidneys[22,24], bilateral ureteropelvic obstruction of the kidneys[45], vesicoureteral reflux[33], crossed fuses renal ectopia[33,40], and hypospadias[56], is also involved. Nonfunctioning pancreatic tumor[25], ulcerative colitis(UC)[33], juvenile polyposis[21], inguinal hernia[23,37], and even situs inversus viscerum[2,4], are sporadically observed in CAPV patients.

The skeletal systematic abnormalities, such as radial hypoplasia[33] and congenital absence of the first metacarpophalangeal complex of the right hand[33], are noted in Grazioli's patients. Vertebral abnormalities or hemivertebra scoliolosis[15,40,57], and oculoauriculovertebral dysplasia or Goldenhar's syndrome[16,26,28,58] exhibit thoracic hemivertebrae, right maxillary hypoplasia, mild micrognathia, and short fifth fingers, are not uncommon in CAPV patients.

Sequent syndromes, which can result in poor prognosis[27], are mainly related to LD or liver abnormalities besides CAPV malformation. Although most cases do not possess any liver abnormalities[1,3,9,27,43-45,47-49,54,57], CAPV patients suffer from different levels of LD[2-4,9,15,16,20,23,24,26-29,34,37,38,44,47,55,56] possibly due to the lack of portal flow. Hepatic encephalopathy, hepatopulmonary syndrome (HPS), and hepatorenal syndrome are closely related to metabolic disorder because of liver lesions, including hyperammonemia[3,27,41,59] and galactosemia[20,27]. Toxic compounds produced in the digestion process only bypass the liver into the systemic circulation in CAPV patients, and are prone to cause hepatic encephalopathy[23,26,27,34,40,49,56]. Mild CAPV patients present with cognitive retardation[33] or mental retardation[27], and their symptoms are merely drowsiness or delirium[56]. Wakamoto *et al*[23] reported the first case of sub-clinical porto-systemic encephalopathy (PSE) with CAPV. When it becomes worse, tremor or orthostatic disturbance[56], abscess in the brain[60], even epilepsy[38] and cerebellar meningioma[55] may occur. If the porto-systematic shunt ratio is high enough, HPS[27,30,60-63] may occur, including cyanosis[30] of the hands, feet, and lips, digital clubbing and pectus excavatum[60], bronchial asthma[8,23] or hypoxemia-induced bronchial stenosis[56], pulmonary hypertension[56,64]. When the shunt ratio is over 90%, CAPV patients would have hematuria and proteinuria, namely hydropigenous nephritis[65]. CAPV patients also could have chronic renal failure[33,34]. Gonadal hormonal disorder[33,47,66] can result in hypergalactocemia[66-68], primary amenorrhoea and signs of virilization[33]. Satoh's patients present with hyperandrogenism, insulin resistant hyperinsulinaemia, and hyperglycaemia[47]. A few cases of PH[9,16,55], rectal bleeding[9,21,33,42], anaemia[31,33] and peripheral edema[21] have also been reported. All the metabolic disorders described above can lead to growth retardation[21,40,56], small head or microcephaly[14,38].

Routine clinical examination can find the above or other diseases in CAPV patients, suggesting that there are a large number of asymptomatic CAPV patients.

ETIOLOGY

Cardiovascular system

Embryologically, paired vitelline veins enter the embryo with yolk stalk, anastomose with each other around the developing duodenum forming a loop, and pass through the septum transversum to the sinus venosus. Portal venous system development occurs depending on selective apoptosis of the bilaterial vitelline veins and their median links before entering the septum transversum[33]. The whole process is complex and coinciding, any insult may affect the development resulting in a preduodenal portal vein, CAPV and duplications, as well as communications between the portal and pulmonary veins[10,12,16,28]. The pathogenesis of CAPV may be attributed to excessive involution of the peri-intestinal vitelline venous loop[10,13,22,69], or to total failure of the vitelline veins to establish the critical anastomosis with hepatic sinusoids[4]. Behind the abnormalities, the initiative event may be referred to genetic mutation or chromatosome variation as CAPV has been sometimes reported in conjunction with chromosomal disorders[70], such as translocation (2,10)[21] and turner syndrome (45, XO)[6,71]. The associated extrahepatic portosystemic shunts may occur due to the persistent subcardinohepatic anastomosis with the vitelline veins. The subcardinohepatic anastomosis connects the vitelline vein that develops into the portal system and the right subcardinal vein that develops into the renal segment of the IVC, as well as forms the hepatic segment of the IVC, thus accounting for the high incidence of draining points at the suprarenal IVC[3].

Cardiac malformations are frequently observed in patients with CAPV[11,72], the close relationship between the development of vitelline veins and the heart in embryonic life may be responsible for the association

between cardiovascular malformations and CAPV[12,40,45]. The cardiac abnormalities may result from a some embryogenic insults and compensate for the congestive effect of portal vein absence and shunting[37], indicating that prenatal insult occurs during the concurrent development of the heart and gastrointestinal tract. However, it was also supposed that systemic shunt of the portal venous flow could adversely affect the hepatic and cardiac development and function[11]. It was reported that concomitant atrial and ventricular septal defects related to CAPV may be attributed to a congenital adaptive change occurring during the development from the embryonic stage, which tends to compensate for the congestive effects of portal venous aplasia[10]. CAPV can result in cardiomegaly or even congestive heart failure due to the shunts of blood flow[10].

Liver

The importance of intact portal vein flow following liver resection or transplantation has been recognized both in experiments and in clinical practice[46,73]. The lack of portal flow can affect the development, function and regenerative pability of the liver. The importance of certain substances, such as insulin and glucagon, is underscored because of CAPV[73], and these substances are no longer supplied to the liver through the mesenteric blood flow and lead to hepatic hypoplasia because they help maintain the hepatic structure and function[74-77]. The hepatic volume is extremely small compared with the standard one as detected by computed tomography (CT) volumetry[56]. However, as far as the regenerative ability of the liver is concerned, such an assumption is questionable. In fact, in cases of CAPV, the regenerative ability appears to be normal after liver lobectomy and trisegmentectomy[16,37].

CAPV is frequently observed with hepatic tumors and tumor-like conditions, such as FNH and NRH[40], suggesting that intrahepatic changes due to hemodynamic imbalance participate in the development of liver tumors[8]. In CAPV patients, liver is supplied only by the hepatic artery in the absence of the portal vein[18]. Many CAPV patients have an enlarged and hypertrophied hepatic artery with a high flow[1,19,50,54] as well as the absence of portal vein, or the presence of hypervascular liver tumor[28]. Such conditions affect the development, function and regenerative capacity of liver, thus predisposing to the development of nodular dysplasia, hepatocarcinoma, or other benign and malignant hepatic tumors[38]. It was reported that abnormal hepatic circulation is one of the etiological factors for hepatocellular hyperplastic nodular lesions[78-80]. Both CAPV and other situations, such as Budd-Chiari syndrome, cause abnormal hepatic flow or peripheral portal venous thrombosis[81,82]. HCC can occur in patients with chronic Budd-Chiari syndrome[82]. Cells in the hyperplastic nodules contain fat deposits[8,83,84], which can be differentiated from other masses.

Marois *et al*[16] demonstrated that abnormal thin-walled vessels filled in a retrograde fashion from hepatic arteries, may be too weak to burden the changed flow. FNH is an uncommon benign tumor-like lesion of well-circumscribed hyperplastic liver parenchyma, often with central stellate scars. These lesions are hypervascular and can be supplied exclusively with arterial blood[79,85]. FNH, derived from acquired thrombosis, has been reported as well[85]. NRH is due to obstruction or narrowing of portal branches caused by thrombosis or atrophy of areas with severely impaired blood flow, and hypertrophy of areas with a relatively mild impairment of blood flow, leading to nodular formation[80].

Yoshidome *et al*[86] observed morphological alterations in the liver parenchyma of patients with congenital portocaval shunts, and proposed that morphological changes in the liver of patients with cirrhosis and acquired portocaval shunts as well as HCC may be explained by a common mechanism, namely reduced portal flow. However, circulatory disturbance alone cannot explain the pathogenesis and the underlying unknown mechanism[25]. Simple occlusion of the portal vein and a compensatory increase in arterial blood flow have been proved insufficient for nodule formation[82]. Although rare, fibrosis may develop due to hemodynamic imbalance[20], because flow disturbance only affects hyperplastic hepatic cells but not mesenchymal cells. CAPV occasionally involves HCC, and 40% of HCC patients have no cirrhosis or chronic liver disease[87,88], suggesting that HCC is related to genetic alterations[38]. Indeed, early genetic alterations or the common genetic pathways of hepatic tumors and CAPV would allow accurate comprehension of the commensalisms.

Intrahepatic bile ductules develop from the primitive ductal plate. It was reported that the portal vein plays a crucial role in the formation and remodelling of the ductal plate[89]. Lack of remodeling of the ductal plates results in persistence of an excess of embryonic bile duct structures. This is why biliary atresia and choledochal cysts concur in CAPV patients.

PSE

When the portal vein is absent, toxic metabolites such as ammonia and bile acids collected from the gastrointestinal tract have to bypass the liver directly drainage into the systemic circulation, thus may initiate hepatic encephalopathy. Interestingly, PSE is rarely observed in CAPV patients with mild hyperammonemia and CAPV patients show no clinical manifestations of hepatic encephalopathy until they become obvious[41]. Only a small number of CAPV cases present with subclinical PSE[23,90-92]. Although PSE is not usually observed in CAPV patients, the serum ammonia level in such patients is not always highly elevated. In fact, a significantly low blood ammonia level in SMV is discovered in patients with CAPV[36,37], suggesting that this low level might indicate the presence of a homeostatic control mechanism[37]. The presence of compensatory alterations in intestinal bacterial flora has been suggested as an explanation[9,15,16]. Kamiya[36] analyzed intestinal flora in faeces of CAPV patients and healthy persons before operation, and did not isolate any microorganisms with a strong urease activity. However,

intestinal microorganisms isolated from the faeces of patients after operation produced as much urease *in vitro* as from healthy volunteers[36], indicating that some inhibitory factors for urease-positive microorganisms may exist in the intestinal tract. It was reported that *lactulose* is effective on hyperammonemia of hepatic encephalopathy by inducing a remarkable growth of *Lactobacillus* to produce lactic acids which interfere with urease-producing microorganisms[93,94]. It is also possible that proteolysis might be inhibited in the intestine of patients, causing decreased production of ammonia[36]. Kavukcu *et al*[95] hold the opposite opinion as among the varieties of bacteria displaying urease activity, only three species have been detected: *Klebsiella pneumonia*, *Enterococcus avium*, and *Peptostreptococcus productus*. Moreover, the number of these bacteria is extremely small with no significant differences observed in the flora in fecal specimens obtained before, during, and after surgery[95]. Another likely explanation is that PSE might be due to the increased sensitivity of an aging brain to ammonia and other toxic materials[23] or that homeostatic control may gradually become disordered with increasing age[52]. The brain sensitivity to ammonia or other toxic metabolites may increase with aging[96]. Healthy brain may tolerate to high ammonia levels, while aging brain may not cope with high ammonia and other metabolites and develop symptoms[96-98]. Such mechanisms may contribute to the delayed presentation with hyperammonemia-related encephalopathy[99]. Moreover, another plausible one is that the thin anastomoses at birth slowly become large as the patient ages. The shunt ratio may play a certain role in the occurrence of symptoms[100]. Certain special unknown mechanisms underlying CAPV lead to the delayed PSE.

Others

Bile acids are synthesized in the liver, secreted into bile ducts, and expelled into the intestinal lumen where they are reabsorbed into the systemic circulation *via* the lymphatic system followed by hepatic uptake from the portal vein, and then metabolized in the liver. However, when the portal vein is absent, these fatty acids, after absorption by the intestinal tract, assume the form of chylomicrons that are transported into the vena cava. Before arriving at the liver, blood from the vena cava reaches the capillaries of peripheral tissues, including adipose and muscular tissue where fat is accumulated, and this presumably constitutes the cause of obesity in such cases[37]. Gitzelmann *et al*[67] detected patients with hypergalactosaemia along with congenital portosystemic shunts[66-68], and proposed that high blood galactose found in newborns is useful for detecting this abnormality. Hypergalactosaemia might also result from insulin resistance (IR) which is correlated to LD[47]. Imbalance between vasodilator and vasoconstrictor substances has been reported in CAPV patients with HPS[27,30,60-63], and decrease in metabolism or synthesis of these substances in the liver is responsible for the imbalance[60]. Mehrotra *et al*[101] observed extrahepatic portal vein obstruction in children with high serum levels of growth hormone and somatostatin (IGF- I), and showed that there is some resistance to the action of growth hormones[101]. Chronic anemia (secondary to loss of blood caused by bleeding and/or hypersplenism), and intestinal venous congestion with secondary malabsorption may interfere with the growth rate[31]. Liver dysfunction causing hepatotrophic hormone deprivation also results in growth retardation[40,59,102]. Takeichi[34] reported a CAPV case of patient with chronic renal failure in a vicious circle of toxic materials, leading to severe encephalopathy with coma, waiting for liver transplantation (LT).

However, since CAPV cases have individual series of presentations, more or less, subtle or obvious, the pathology of these manifestations seems to be complicated and covered. Further study is needed to confirm its etiology by analyzing more entities.

IMAGEOLOGY

Imaging abnormality is often coincidentally discovered in children with portal vein disorder. Routine liver examination can reveal the extraordinary mass in majority of these children, triggering further imaging exploration. Portosystemic shunt diagnosis is usually based on clinicopathologic and portographic findings[103]. Portography, ultrasonography, scintigraphy, CT and magnetic imagining are used in diagnosis of this portal vein disorder.

Doppler ultrasonography has been extensively used in evaluating vessels of the abdomen. It is most useful for determining flow direction and pattern besides liver tumor. However, the most important advantage of this technique is its noninvasiveness and no requirement for anesthesia. It yields much useful information about the detailed vascular anatomy as well as hemodynamics[104]. Prenatal screening and intraoperative ultrasonography is especially superior to other techniques[104]. Although color Doppler sonography first depicted the image of the absent portal vein in most cases, ultrasonography (US) may fail to accurately detect the associated extrahepatic shunts because of its subtle US features[31]. Experience and good ultrasound system, knowledge of the examination protocol, and familiarity about the ultrasound anatomy of abdominal vessels and portal vein abnormalities, contribute to the accurate diagnosis of CAPV.

Accurate depiction of intra and extrahepatic vascular anatomy will undoubtedly guide management decisions and surgical or angiographic approaches[21]. Cross-sectional imaging (CT and MR) is very helpful in depicting the course of portosystemic shunt and in identifying absent vessels and type of malformations[42,50]. Currently, 3D-computed tomography angiography (3D-CTA) and magnetic resonance angiography (MRA) can confirm CAPV and visualize the portosystemic shunt[50]. It was reported that multi-slice CTA displays even small vascular branches and has superior spatial resolution to MRA[105]. The posterior or short gastric veins cannot be visualized

in patients with portal vein disorders by conventional angiographic portography, but can be clearly revealed by 3D-CT portography[106]. However, a breath-holding technique has been recommended to prevent motion artifacts during scanning by Tsuji *et al*[8] who assumed that if a patient is able to hold the breath, 3D-CTA can easily capture the entire abnormal vasculature during one breath holding. Unfortunately, CAPV predominately occurs in children difficult to hold breath that urges us to search the substitute. MRA is also a reliable and noninvasive diagnostic modality for the portal venous system[49]. MR imaging can be used both in diagnosis of CAPV and in evaluation of focal hepatic lesions[41]. In addition, previous reports indicate that the presence of a portosystemic shunt may cause lesions of middle cerebellar peduncles, which are responsible for cerebellar symptoms[91]. MR imaging can be used to find cerebral lesions. It was recently reported that conventional high-resolution MR angiography seems unnecessary[41], as the spatial resolution time of MR angiography is almost equivalent. Since 3D-MRA obtained from quality multiplanar reformatted images and volume-rendered images has the advantage of high temporal resolution, contamination from overlapping vascular structures can be avoided, flow dynamics can be assessed, and quality feature is obtainable even in young children with free breaths because the technique is relatively insensitive to motion artefacts[41]. Perhaps, it will be widespread in the diagnosis of CAPV several years later.

Although cross-sectional imaging in most cases can approximately suggest the diagnosis of portal vein disorder, the definitive diagnosis can be made only with catheter angiography[15] and by additional histological analysis of the hepatic parenchyma that demonstrates the absence of hepatic portal venules within the portal triad[35]. Mesenteric portovenography, a usually indirect technique depicting the portal system anatomy, can clarify CAPV abnormality and extrahepatic shunts. In recent years, CO_2-wedged venography is considered a good and safe technique for demonstrating the portal circulation[107]. It has such advantages over indirect portography obtained during visceral arteriography[108] as only a venous puncture is required, free and wedged pressure measurements can be obtained with no iodinated contrast medium injected, and transvenous liver biopsy can be made at the same time. However, opacification of portal vein branches could not be obtained[35].

Portovenography can facilitate measurement of the pressure gradient indicating vein blood flow and selected embolism shunts as a therapy. In dogs, transvenous retrograde portography[109] is less invasive than operative mesenteric portography and allows measurement of portal pressures before and after temporary shunt ligation. It also helps differentiate rich-vessel tumor and confirm parenchymal magnetic resonance imaging (MRI) findings. Conventional angiography is not good for children, although it is fairly safe[49,110].

Rectal portal scintigraphy plays an important part in suspected abnormalities of portal circulation and is precise to quantitate portosystemic shunts and valuable for clinical diagnosis[111]. During performing this kind of examination, shunt indexes (SI) are calculated, relative portal hemodynamics can be observed noninvasively, and portal collateral circulation can be detected as well[112]. This technique is hopeful to be extensively applied in detecting CAPV if not expensive.

DIFFERENTIAL DIAGNOSIS

Definitive diagnosis of CAPV should exclude many seemingly resemble cases. For example, histologically confirmed absence of portal vein in the liver is mandatory in the diagnosis. Abernethy type Ⅱ was previously misclassified as CAPV[21]. Kerlan *et al*[96] reported a case similar to CAPV, but surgery for closing the fistula between portal vein and inferior cava, revealed an intrahepatic portal vein. Absence of stigmata in patients with portal venous hypertension is an important clue to the final diagnosis[40]. Radiologically, absence of the portal vein must be distinguished from portal vein thrombosis[15,28], based on the absence of venous collaterals or other secondary signs of PH, such as splenomegaly or ascites. Compensatory hypertrophy of the hepatic artery may be present[19]. Appel *et al*[51] assumed that it is a secondary phenomenon, most properly due to thrombotic occlusion of the extrahepatic portal vein. However, gradual thrombosis of the portal vein stem may allow the development of collaterals without acute dramatic episodes, similar to CAPV. The term of portal vein "aplasia" or "agenesis" in such cases is inadequate since intrahepatic bile ducts are normal[51,113]. Extrinsic compression of tumors, such as HCC and extrahepatic malignant tumor, especially pancreatic adenocarcinoma[5], is another reason. If the portal flow is ceased, initial thrombus arises asymptomatically, the only sign may be the formation of new vessels, which on Doppler US is known as "portal cavernoma" or "cavernomatous transformation" due to the blood volume at the site[102]. It is not easy to differentiate this condition from Abernethy. It was reported that hepatic nodules with rich artery blood flow may prevent influx of portal blood resulting in increased sinusoidal pressure, the portal vein will not be visualized at portography[82]. Owing to the progressive growth of tumor in the omentum and mesentery, increased portal flow would produce extrahepatic portosystemic venous shunts[114].

During fetal life, ductus venosus is the continuation of umbilical vein, which directly inflows into the inferior vena cava, allowing blood returning through the umbilical vein to bypass the portal venous system[115,116]. Failure to close patent ductus venosus within 2 wk after birth would lead to portosystemic encephalopathy or malformations, including congenital heart disease and minor abnormalities[117]. Hepatic nodular lesions, such as FNH, and PSE, have been reported in patients with patent ductus venosus[117,118]. Although the clinical manifestations of patent ductus venosus and CAPV are similar, the mechanism is different. The treatment of

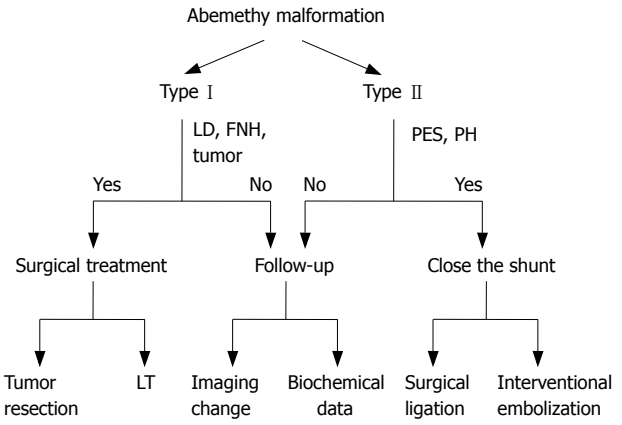


Figure 2 Schematic demonstration of decision-making advices for treatment of Abernethy malformation. LD: Liver dysfunction; FNH: Focal nodular hyperplasia; PSE: Porto-systemic encephalopathy; PH: Portal hypertension; LT: Liver transplantation.

CAPV needs liver transplantation and surgical ligation of the patent ductus venosus[18].

It is difficult to differentiate hepatic benign lesions (FNH, FNH-like lesions, and NRH) from malignant lesions (HCC or hepatoblastoma) by imaging except in cases of such abnormal hepatic circulation while transvenous liver biopsy is a valuable alternative diagnostic tool[35,119]. Intrahepatic portal vasculature abnormalities should be differentiated from congenital diseases, such as idiopathic non-cirrhotic PH, portal venous hypoplasia, or hepatic microvascular dysplasia. Histologic findings of these liver diseases are similar to those seen in congenital liver diseases. Thus, it is not possible to differentiate these diseases from congenital shunts[120]. CAPV with PSE should be differentiated from mental disorder involving hyperammonemia or galactosemia due to metabolic deficiencies[121].

Potential etiologies of extrahepatic shunts include shunt formation in association with PH and mesenteric adhesion due to prior abdominal surgery, abdominal trauma, and congenital shunts[97,98,122]. As a result, congenital extrahepatic portosystemic shunts in CAPV patients must be confirmed by excluding the existence of the three liver diseases.

TREATMENT

Treatment options for extrahepatic portosystemic venous shunts strongly depend on the type of Abernethy abnormalities (Figure 2). Balloon-occluded retrograde transvenous obliteration (BRTO), embolization with metallic coils and surgical correction of the shunts are available[97]. In type Ⅱ patients previously diagnosed with CAPV, occlusion of the shunt is indicated in case of serious symptoms such as hepatic encephalopathy[4] or lateral bleeding. The occlusion techniques include surgical ligation[56,96,123] and interventional embolization[124-126]. Otake *et al*[99] used coils as embolic materials, because they can progressively occlude the shunt, avoiding acute overload of the portal venous system, and confirmed that there is no evidence that the shunt vessels were recanalized after a two-year follow-up period. The treatment for venous shunts in type I patients without severe symptoms other than liver tumors and LDs is inactive, indicating that close clinical, biochemical, and imaging follow-up should be performed.

The treatment for such patients depends on the conditions of liver neoplasm, such as size and histology[20,50]. It involves LT for hepatoblastoma[26] and chemotherapy for hepatoblastoma after resection of the right hepatic lobe[16]. The choice of treatment for liver tumor in CAPV patients is radical resection of the tumor[39], although mostly it is benign, because the mass becomes larger and then progresses to malignancy[8,26]. Morse *et al*[28] reported a patient with CAPV who finally underwent LT for hepatoblastoma initially diagnosed as FNH 2 years ago[26,28], suggesting that long-term follow-up and monitoring for malignancy are mandatory, even for benign tumors.

After tumor resection, liver regeneration is said to be dependent on hepatotropic factors in the portal venous blood[37,73]. However, in the patients resected for liver tumor reported[12,15,16,24,37], the resection was uncomplicated and the postoperative course uneventful, despite the absence of gut-derived hepatotropic factors to stimulate liver regeneration. Stimuli other than those transported with the portal blood stream must thus be sufficient to ensure an adequate postoperative liver regeneration in these patients[39].

Some authors hold that increased blood flow comes mainly from the SV in patients with PH and hypersplenism or megalosplenia, indicating that partial splenic embolization can decrease the blood flow and pressure of the main portal vein, similar to the conjoint effects of splenectomy and devascularization[127]. Surgical decompression is also recommended for selected children in order to promote their growth[128].

CAPV has been thought to be asymptomatic and has no indication for LT, and only a few cases having been reported[2,17,27,56,129]. More and more surgeons hold that LT is necessary when medical therapy cannot relieve CAPV-associated abnormalities, such as CAPV-associated cirrhosis caused by biliary atresia[4,17], diffuse hepatoblastoma involving both lobes of the liver[26], and severe portosystemic encephalopathy[56]. No surgical method is available for reconstructing the portal structures of the native liver[56,59]. Shinkai *et al*[56] reported that LT is an effective surgical treatment for symptomatic CAPV patients when the disease is unresponsive to medical treatment, and believe that prophylactic LT is justified for patients with CAPV before the development of fatal pulmonary complications, such as pulmonary hypertension or HPS, which might complicate or preclude LT[56]. Woodle *et al*[17] successfully transplanted liver for a biliary atresia patient, and assumed that CAPV is not a contraindication for LT. Taoube *et al*[130] performed the first paediatric liver transplant for a patient with portal venous agenesia, using the de piggy-back technique.

Recently, auxiliary partial orthotopic liver

transplantation (APOLT) was developed in order to reverse fulminant hepatic failure (FHF), which is advantageous over the orthotopic liver transplantation (OLT) and avoids eliminating regeneration of the native liver and a life-long immune suppression[131]. It also has been utilized as an aid in small-for-size grafts to larger recipients during living donor liver transplantation (LDLT)[131]. Soejima *et al*[27] performed LT for a male patient using a left lateral segment graft from his mother to preserve his native right lobe. Configuration of the donor PV, hepatic artery, HV and bile duct was normal. The results indicate that APOLT is an ideal procedure for patients with CAPV[27]. However, APOLT has certain drawbacks, such as portal steal phenomenon and potential risk of developing tumors in the remnant native liver.

Both the anatomy of portal vein and the function of liver can be restored, and other liver dysfunctions-associated complications may be relieved after LT, such as disappearance of high-intensity lesions in the brain[59].

In conclusion, the prognosis of CAPV patients depends on congenital heart disease, liver disease, and the site of portosystemic shunts. The outcome of CAPV patients with no other abnormalities is different. A long-term follow-up including laboratory tests and image screening is recommended for CAPV patients[20,35,50,126].

REFERENCES

1 **Abernethy J**. Account of two instances of uncommon formation in the viscera of the human body. *Philos Trans R Soc* 1793; **83**: 59-66
2 **Morgan G**, Superina R. Congenital absence of the portal vein: two cases and a proposed classification system for portasystemic vascular anomalies. *J Pediatr Surg* 1994; **29**: 1239-1241
3 **Kohda E**, Saeki M, Nakano M, Masaki H, Ogawa K, Nirasawa M, Hiramatsu K. Congenital absence of the portal vein in a boy. *Pediatr Radiol* 1999; **29**: 235-237
4 **Howard ER**, Davenport M. Congenital extrahepatic portocaval shunts--the Abernethy malformation. *J Pediatr Surg* 1997; **32**: 494-497
5 **Schettino GC**, Fagundes ED, Roquete ML, Ferreira AR, Penna FJ. Portal vein thrombosis in children and adolescents. *J Pediatr* (Rio J) 2006; **82**: 171-178
6 **Noe JA**, Pittman HC, Burton EM. Congenital absence of the portal vein in a child with Turner syndrome. *Pediatr Radiol* 2006; **36**: 566-568
7 **Turkbey B**, Karcaaltincaba M, Demir H, Akcoren Z, Yuce A, Haliloglu M. Multiple hyperplastic nodules in the liver with congenital absence of portal vein: MRI findings. *Pediatr Radiol* 2006; **36**: 445-448
8 **Tsuji K**, Naoki K, Tachiyama Y, Fukumoto A, Ishida Y, Kuwabara T, Sumida T, Tatsugami M, Nagata S, Ohgoshi H, Uraki S, Hidaka T, Akagi Y, Ishine M, Saeki S, Chayama K. A case of congenital absence of the portal vein. *Hepatol Res* 2005; **31**: 43-47
9 **Komatsu S**, Nagino M, Hayakawa N, Yamamoto H, Nimura Y. Congenital absence of portal venous system associated with a large inferior mesenteric-caval shunt: a case report. *Hepatogastroenterology* 1995; **42**: 286-290
10 **Marks C**. Developmental basis of the portal venous system. *Am J Surg* 1969; **117**: 671-681
11 **Massin M**, Verloes A, Jamblin P. Cardiac anomalies associated with congenital absence of the portal vein. *Cardiol Young* 1999; **9**: 522-525
12 **Joyce AD**, Howard ER. Rare congenital anomaly of the portal vein. *Br J Surg* 1988; **75**: 1038-1039
13 **Fasouliotis SJ**, Achiron R, Kivilevitch Z, Yagel S. The human fetal venous system: normal embryologic, anatomic, and physiologic characteristics and developmental abnormalities. *J Ultrasound Med* 2002; **21**: 1145-1158
14 **Venkat-Raman N**, Murphy KW, Ghaus K, Teoh TG, Higham JM, Carvalho JS. Congenital absence of portal vein in the fetus: a case report. *Ultrasound Obstet Gynecol* 2001; **17**: 71-75
15 **Matsuoka Y**, Ohtomo K, Okubo T, Nishikawa J, Mine T, Ohno S. Congenital absence of the portal vein. *Gastrointest Radiol* 1992; **17**: 31-33
16 **Marois D**, van Heerden JA, Carpenter HA, Sheedy PF 2nd. Congenital absence of the portal vein. *Mayo Clin Proc* 1979; **54**: 55-59
17 **Woodle ES**, Thistlethwaite JR, Emond JC, Whitington PF, Vogelbach P, Yousefzadeh DK, Broelsch CE. Successful hepatic transplantation in congenital absence of recipient portal vein. *Surgery* 1990; **107**: 475-479
18 **Kim T**, Murakami T, Sugihara E, Hori M, Wakasa K, Nakamura H. Hepatic nodular lesions associated with abnormal development of the portal vein. *AJR Am J Roentgenol* 2004; **183**: 1333-1338
19 **De Gaetano AM**, Gui B, Macis G, Manfredi R, Di Stasi C. Congenital absence of the portal vein associated with focal nodular hyperplasia in the liver in an adult woman: imaging and review of the literature. *Abdom Imaging* 2004; **29**: 455-459
20 **Tanaka Y**, Takayanagi M, Shiratori Y, Imai Y, Obi S, Tateishi R, Kanda M, Fujishima T, Akamatsu M, Koike Y, Hamamura K, Teratani T, Ishikawa T, Shiina S, Kojiro M, Omata M. Congenital absence of portal vein with multiple hyperplastic nodular lesions in the liver. *J Gastroenterol* 2003; **38**: 288-294
21 **Murray CP**, Yoo SJ, Babyn PS. Congenital extrahepatic portosystemic shunts. *Pediatr Radiol* 2003; **33**: 614-620
22 **Altavilla G**, Cusatelli P. Ultrastructural analysis of the liver with portal vein agenesis: a case report. *Ultrastruct Pathol* 1998; **22**: 477-483
23 **Wakamoto H**, Manabe K, Kobayashi H, Hayashi M. Subclinical portal-systemic encephalopathy in a child with congenital absence of the portal vein. *Brain Dev* 1999; **21**: 425-428
24 **Guariso G**, Fiorio S, Altavilla G, Gamba PG, Toffolutti T, Chiesura-Corona M, Tedeschi U, Zancan L. Congenital absence of the portal vein associated with focal nodular hyperplasia of the liver and cystic dysplasia of the kidney. *Eur J Pediatr* 1998; **157**: 287-290
25 **Motoori S**, Shinozaki M, Goto N, Kondo F. Case report: congenital absence of the portal vein associated with nodular hyperplasia in the liver. *J Gastroenterol Hepatol* 1997; **12**: 639-643
26 **Barton JW 3rd**, Keller MS. Liver transplantation for hepatoblastoma in a child with congenital absence of the portal vein. *Pediatr Radiol* 1989; **20**: 113-114
27 **Soejima Y**, Taguchi T, Ogita K, Taketomi A, Yoshizumi T, Uchiyama H, Ohno T, Shimada M, Maehara Y. Auxiliary partial orthotopic living donor liver transplantation for a child with congenital absence of the portal vein. *Liver Transpl* 2006; **12**: 845-849
28 **Morse SS**, Taylor KJ, Strauss EB, Ramirez E, Seashore JH. Congenital absence of the portal vein in oculoauriculovertebral dysplasia (Goldenhar syndrome). *Pediatr Radiol* 1986; **16**: 437-439
29 **Kinjo T**, Aoki H, Sunagawa H, Kinjo S, Muto Y. Congenital absence of the portal vein associated with focal nodular hyperplasia of the liver and congenital choledochal cyst: a case report. *J Pediatr Surg* 2001; **36**: 622-625
30 **Cheung KM**, Lee CY, Wong CT, Chan AK. Congenital absence of portal vein presenting as hepatopulmonary syndrome. *J Paediatr Child Health* 2005; **41**: 72-75

31 **Koizumi J**, Yamashita T, Dowaki S, Tobita K, Shimamura K, Hirabayashi K, Imai Y, Imaizumi T. Hepatobiliary and pancreatic: Hepatic adenoma, focal nodular hyperplasia and congenital absence of the portal vein. *J Gastroenterol Hepatol* 2006; **21**: 619

32 **Arana E**, Marti-Bonmati L, Martinez V, Hoyos M, Montes H. Portal vein absence and nodular regenerative hyperplasia of the liver with giant inferior mesenteric vein. *Abdom Imaging* 1997; **22**: 506-508

33 **Grazioli L**, Alberti D, Olivetti L, Rigamonti W, Codazzi F, Matricardi L, Fugazzola C, Chiesa A. Congenital absence of portal vein with nodular regenerative hyperplasia of the liver. *Eur Radiol* 2000; **10**: 820-825

34 **Takeichi T**, Okajima H, Suda H, Hayashida S, Iwasaki H, Ramirez MZ, Ueno M, Asonuma K, Inomata Y. Living domino liver transplantation in an adult with congenital absence of portal vein. *Liver Transpl* 2005; **11**: 1285-1288

35 **Collard B**, Maleux G, Heye S, Cool M, Bielen D, George C, Roskams T, Van Steenbergen W. Value of carbon dioxide wedged venography and transvenous liver biopsy in the definitive diagnosis of Abernethy malformation. *Abdom Imaging* 2006; **31**: 315-319

36 **Kamiya S**, Taniguchi I, Yamamoto T, Sawamura S, Kai M, Ohnishi N, Tsuda M, Yamamura M, Nakasaki H, Yokoyama S. Analysis of intestinal flora of a patient with congenital absence of the portal vein. *FEMS Immunol Med Microbiol* 1993; **7**: 73-80

37 **Nakasaki H**, Tanaka Y, Ohta M, Kanemoto T, Mitomi T, Iwata Y, Ozawa A. Congenital absence of the portal vein. *Ann Surg* 1989; **210**: 190-193

38 **Pichon N**, Maisonnette F, Pichon-Lefievre F, Valleix D, Pillegand B. Hepatocarcinoma with congenital agenesis of the portal vein. *Jpn J Clin Oncol* 2003; **33**: 314-316

39 **Lundstedt C**, Lindell G, Tranberg KG, Svartholm E. Congenital absence of the intrahepatic portion of the portal vein in an adult male resected for hepatocellular carcinoma. *Eur Radiol* 2001; **11**: 2228-2231

40 **Gocmen R**, Akhan O, Talim B. Congenital absence of the portal vein associated with congenital hepatic fibrosis. *Pediatr Radiol* 2007; **37**: 920-924

41 **Goo HW**. Extrahepatic portosystemic shunt in congenital absence of the portal vein depicted by time-resolved contrast-enhanced MR angiography. *Pediatr Radiol* 2007; **37**: 706-709

42 **Gallego C**, Miralles M, Marin C, Muyor P, Gonzalez G, Garcia-Hidalgo E. Congenital hepatic shunts. *Radiographics* 2004; **24**: 755-772

43 **Hellweg G**. Congenital absence of intrahepatic portal venous system simulating Eck fistula; report of a case with necropsy findings. *AMA Arch Pathol* 1954; **57**: 425-430

44 **Laverdiere JT**, Laor T, Benacerraf B. Congenital absence of the portal vein: case report and MR demonstration. *Pediatr Radiol* 1995; **25**: 52-53

45 **Bellah RD**, Hayek J, Teele RL. Anomalous portal venous connection to the suprahepatic vena cava: sonographic demonstration. *Pediatr Radiol* 1989; **20**: 115-117

46 **Williams JW**, Britt LG, Peters TG, Vera SR, Haggitt RC. Portal vein obstruction in patients requiring hepatic resection or transplantation. *Am Surg* 1984; **50**: 465-468

47 **Satoh M**, Yokoya S, Hachiya Y, Hachiya M, Fujisawa T, Hoshino K, Saji T. Two hyperandrogenic adolescent girls with congenital portosystemic shunt. *Eur J Pediatr* 2001; **160**: 307-311

48 **Le Borgne J**, Paineau J, Hamy A, Dupas B, Lerat F, Raoul S, Hamel A, Robert R, Armstrong O, Rogez JM. Interruption of the inferior vena cava with azygos termination associated with congenital absence of portal vein. *Surg Radiol Anat* 2000; **22**: 197-202

49 **Usuki N,** Miyamoto T. A case of congenital absence of the intrahepatic portal vein diagnosed by MR angiography. *J Comput Assist Tomogr* 1998; **22**: 728-729

50 **Kornprat P**, Langner C, Fritz K, Mischinger HJ. Congenital absence of the portal vein in an adult woman: a case report. *Wien Klin Wochenschr* 2005; **117**: 58-62

51 **Appel H**, Loddenkemper C, Schirmacher P, Dienes HP, Sieper J, Rudwaleit M, Golder W, Zeitz M. Congenital absence of the portal vein with splenomegaly and hypersplenism in a young woman. *Digestion* 2003; **67**: 105-110

52 **Nishimoto Y**, Hoshino H, Sato S, Oguri A, Yamada M, Nishimura D, Katada N, Sano H, Kato K. Extrahepatic portosystemic venous shunt without portal hypertension. *Intern Med* 1997; **36**: 886-889

53 **Manning N**, Impey L, Lindsell D, Lakhoo K. Prenatally diagnosed portocaval shunt and postnatal outcome: a case report. *Prenat Diagn* 2004; **24**: 537-540

54 **Kiernan F**. The Anatomy and Physiology of the Liver. *Philos Trans R Soc* 1833; **113**: 711-770

55 **Olling S**, Olsson R. Congenital absence of portal venous system in a 50-year-old woman. *Acta Med Scand* 1974; **196**: 343-345

56 **Shinkai M**, Ohhama Y, Nishi T, Yamamoto H, Fujita S, Take H, Adachi M, Tachibana K, Aida N, Kato K, Tanaka Y, Takemiya S. Congenital absence of the portal vein and role of liver transplantation in children. *J Pediatr Surg* 2001; **36**: 1026-1031

57 **Northrup M**, Mendez-Castillo A, Sethi Y, Churchill R. Congenital absence of the portal vein with an intrahepatic inferior vena cava branch showing hepatopetal flow. *J Ultrasound Med* 2002; **21**: 569-572

58 **Kalifa G**, Brunelle F, Chaumont P. Fistule porto-cave congenitale. *Ann Radiol* (Paris) 1978; **21**: 183-186

59 **Shinkai M**, Ohhama Y, Nishi T, Yamamoto H, Fujita S, Take H, Adachi M, Tachibana K, Aida N, Kato K, Tanaka Y, Takemiya S, Tanaka K. Living related partial liver transplantation for hyperammonemia due to congenital absence of the portal vein. *Transplant Proc* 2000; **32**: 2184

60 **Alvarez AE**, Ribeiro AF, Hessel G, Baracat J, Ribeiro JD. Abernethy malformation: one of the etiologies of hepatopulmonary syndrome. *Pediatr Pulmonol* 2002; **34**: 391-394

61 **Tercier S**, Delarue A, Rouault F, Roman C, Breaud J, Petit P. Congenital portocaval fistula associated with hepatopulmonary syndrome: ligation vs liver transplantation. *J Pediatr Surg* 2006; **41**: e1-e3

62 **Krowka MJ**, Porayko MK, Plevak DJ, Pappas SC, Steers JL, Krom RA, Wiesner RH. Hepatopulmonary syndrome with progressive hypoxemia as an indication for liver transplantation: case reports and literature review. *Mayo Clin Proc* 1997; **72**: 44-53

63 **Egawa H**, Kasahara M, Inomata Y, Uemoto S, Asonuma K, Fujita S, Kiuchi T, Hayashi M, Yonemura T, Yoshibayashi M, Adachi Y, Shapiro JA, Tanaka K. Long-term outcome of living related liver transplantation for patients with intrapulmonary shunting and strategy for complications. *Transplantation* 1999; **67**: 712-717

64 **Imamura H**, Momose T, Kitabayashi H, Takahashi W, Yazaki Y, Takenaka H, Isobe M, Sekiguchi M, Kubo K. Pulmonary hypertension as a result of asymptomatic portosystemic shunt. *Jpn Circ J* 2000; **64**: 471-473

65 **Kuramitsu T**, Komatsu M, Matsudaira N, Naganuma T, Niizawa M, Zeniya A, Yoshida T, Toyoshima I, Chiba M, Masamune O. Portal-systemic encephalopathy from a spontaneous gastrorenal shunt diagnosed by three-dimensional computed tomography and treated effectively by percutaneous vascular embolization. *Liver* 1998; **18**: 208-212

66 **Ono H**, Mawatari H, Mizoguchi N, Eguchi T, Sakura N. Clinical features and outcome of eight infants with intrahepatic porto-venous shunts detected in neonatal screening for galactosaemia. *Acta Paediatr* 1998; **87**: 631-634

67 **Gitzelmann R**, Arbenz UV, Willi UV. Hypergalactosaemia and portosystemic encephalopathy due to persistence of ductus venosus Arantii. *Eur J Pediatr* 1992; **151**: 564-568

68 **Matsumoto T**, Okano R, Sakura N, Kawaguchi Y, Tanaka Y, Ueda K, Ito S, Yamamoto S, Tanaka M, Amano D. Hypergalactosaemia in a patient with portal-hepatic venous and hepatic arterio-venous shunts detected by neonatal screening. *Eur J Pediatr* 1993; **152**: 990-992

69 **Walsh G**, Williams MP. Congenital anomalies of the portal venous system--CT appearances with embryological considerations. *Clin Radiol* 1995; **50**: 174-176

70 **Pipitone S**, Garofalo C, Corsello G, Mongiovì M, Piccione M, Maresi E, Sperandeo V. Abnormalities of the umbilico-portal venous system in Down syndrome: a report of two new patients. *Am J Med Genet A* 2003; **120A**: 528-532

71 **Hofstaetter C**, Plath H, Hansmann M. Prenatal diagnosis of abnormalities of the fetal venous system. *Ultrasound Obstet Gynecol* 2000; **15**: 231-241

72 **Sumida W**, Kaneko K, Ogura Y, Tainaka T, Ono Y, Seo T, Kiuchi T, Ando H. Living donor liver transplantation for congenital absence of the portal vein in a child with cardiac failure. *J Pediatr Surg* 2006; **41**: e9-e12

73 **Starzl TE**, Francavilla A, Halgrimson CG, Francavilla FR, Porter KA, Brown TH, Putnam CW. The origin, hormonal nature, and action of hepatotrophic substances in portal venous blood. *Surg Gynecol Obstet* 1973; **137**: 179-199

74 **Leffert HL**, Koch KS, Moran T, Rubalcava B. Hormonal control of rat liver regeneration. *Gastroenterology* 1979; **76**: 1470-1482

75 **Parker P**, Burr I, Slonim A, Ghishan FK, Greene H. Regression of hepatic adenomas in type Ia glycogen storage disease with dietary therapy. *Gastroenterology* 1981; **81**: 534-536

76 **Starzl TE**, Putnam CW, Porter KA, Halgrimson CG, Corman J, Brown BI, Gotlin RW, Rodgerson DO, Greene HL. Portal diversion for the treatment of glycogen storage disease in humans. *Ann Surg* 1973; **178**: 525-539

77 **Starzl TE**, Francavilla A, Halgrimson CG, Francavilla FR, Porter KA, Brown TH, Putnam CW. The origin, hormonal nature, and action of hepatotrophic substances in portal venous blood. *Surg Gynecol Obstet* 1973; **137**: 179-199

78 **Wanless IR**, Mawdsley C, Adams R. On the pathogenesis of focal nodular hyperplasia of the liver. *Hepatology* 1985; **5**: 1194-1200

79 **Kumagai H**, Masuda T, Oikawa H, Endo K, Endo M, Takano T. Focal nodular hyperplasia of the liver: direct evidence of circulatory disturbances. *J Gastroenterol Hepatol* 2000; **15**: 1344-1347

80 **Wanless IR**, Godwin TA, Allen F, Feder A. Nodular regenerative hyperplasia of the liver in hematologic disorders: a possible response to obliterative portal venopathy. A morphometric study of nine cases with an hypothesis on the pathogenesis. *Medicine* (Baltimore) 1980; **59**: 367-379

81 **Maetani Y**, Itoh K, Egawa H, Haga H, Sakurai T, Nishida N, Ametani F, Shibata T, Kubo T, Tanaka K, Konishi J. Benign hepatic nodules in Budd-Chiari syndrome: radiologic-pathologic correlation with emphasis on the central scar. *AJR Am J Roentgenol* 2002; **178**: 869-875

82 **Kudo M**. Hepatic nodular lesions caused by abnormal hepatic circulation: etiological and clinical aspects. *J Gastroenterol* 2003; **38**: 308-310

83 **Kenmochi K**, Sugihara S, Kojiro M. Relationship of histologic grade of hepatocellular carcinoma (HCC) to tumor size, and demonstration of tumor cells of multiple different grades in single small HCC. *Liver* 1987; **7**: 18-26

84 **Ishii Y**, Inagaki Y, Hirai K, Aoki T. Hepatic encephalopathy caused by congenital extrahepatic portosystemic venous shunt. *J Hepatobiliary Pancreat Surg* 2000; **7**: 524-528

85 **Whelan TJ Jr**, Baugh JH, Chandor S. Focal nodular hyperplasia of the liver. *Ann Surg* 1973; **177**: 150-158

86 **Yoshidome H**, Edwards MJ. An embryological perspective on congenital portacaval shunt: a rare anomaly in a patient with hepatocellular carcinoma. *Am J Gastroenterol* 1999; **94**: 2537-2539

87 **Nzeako UC**, Goodman ZD, Ishak KG. Hepatocellular carcinoma in cirrhotic and noncirrhotic livers. A clinico-histopathologic study of 804 North American patients. *Am J Clin Pathol* 1996; **105**: 65-75

88 **Bralet MP**, Regimbeau JM, Pineau P, Dubois S, Loas G, Degos F, Valla D, Belghiti J, Degott C, Terris B. Hepatocellular carcinoma occurring in nonfibrotic liver: epidemiologic and histopathologic analysis of 80 French cases. *Hepatology* 2000; **32**: 200-204

89 **Desmet VJ**. Congenital diseases of intrahepatic bile ducts: variations on the theme "ductal plate malformation". *Hepatology* 1992; **16**: 1069-1083

90 **Hassall E**, Benson L, Hart M, Krieger DE. Hepatic encephalopathy after portacaval shunt in a noncirrhotic child. *J Pediatr* 1984; **105**: 439-441

91 **Hitoshi S**, Terao Y, Mizuno T, Takeda K, Sakuta M. [A case of portal-systemic encephalopathy presenting characteristic MR images in globus pallidus, hypothalamus, corpus callosum, pontine base, and middle cerebellar peduncle] *Rinsho Shinkeigaku* 1992; **32**: 217-219

92 **Gitlin N**. Subclinical portal-systemic encephalopathy. *Am J Gastroenterol* 1988; **83**: 8-11

93 **Vince A**, Zeegen R, Drinkwater JE, O'Grady F, Dawson AM. The effect of lactulose on the faecal flora of patients with hepatic encephalopathy. *J Med Microbiol* 1974; **7**: 163-168

94 **Atterbury CE**, Maddrey WC, Conn HO. Neomycin-sorbitol and lactulose in the treatment of acute portal-systemic encephalopathy. A controlled, double-blind clinical trial. *Am J Dig Dis* 1978; **23**: 398-406

95 **Kavukcu S**, Turkmen M, Soylu A, Bora S, Yurt P, Unak P. Intestinal urea metabolism: could the bacteria involved in nitrogen cycle lead to reutilization of intestinal urea nitrogen in uremic rabbits? *J Nutr Sci Vitaminol* (Tokyo) 2002; **48**: 24-29

96 **Kerlan RK Jr**, Sollenberger RD, Palubinskas AJ, Raskin NH, Callen PW, Ehrenfeld WK. Portal-systemic encephalopathy due to a congenital portocaval shunt. *AJR Am J Roentgenol* 1982; **139**: 1013-1015

97 **Sudhamshu KC**, Matsutani S, Maruyama H, Fukamachi T, Nomoto H, Akiike T, Ebara M, Saisho H. Portal-systemic encephalopathy in two patients without liver cirrhosis and portal hypertension. *Hepatol Res* 2002; **23**: 122-129

98 **Kiriyama M**, Takashima S, Sahara H, Kurosaka Y, Matsushita M, Akiyama T, Tomita F, Saito H, Kosaka T, Kita I, Kojima Y, Takegawa S. Case report: portal-systemic encephalopathy due to a congenital extrahepatic portosystemic shunt. *J Gastroenterol Hepatol* 1996; **11**: 626-629

99 **Otake M**, Kobayashi Y, Hashimoto D, Igarashi T, Takahashi M, Kumaoka H, Takagi M, Kawamura K, Koide S, Sasada Y, Kageyama F, Kawasaki T, Nakamura H. An inferior mesenteric-caval shunt via the internal iliac vein with portosystemic encephalopathy. *Intern Med* 2001; **40**: 887-890

100 **Uchino T**, Matsuda I, Endo F. The long-term prognosis of congenital portosystemic venous shunt. *J Pediatr* 1999; **135**: 254-256

101 **Mehrotra RN**, Bhatia V, Dabadghao P, Yachha SK. Extrahepatic portal vein obstruction in children: anthropometry, growth hormone, and insulin-like growth factor I. *J Pediatr Gastroenterol Nutr* 1997; **25**: 520-523

102 **Valla DC**, Condat B. Portal vein thrombosis in adults: pathophysiology, pathogenesis and management. *J Hepatol* 2000; **32**: 865-871

103 **Buczinski S**, Duval J, D'Anjou MA, Francoz D, Fecteau G. Portacaval shunt in a calf: clinical, pathologic, and ultrasonographic findings. *Can Vet J* 2007; **48**: 407-410

104 **Konno K**, Ishida H, Uno A, Ohnami Y, Masamune O. Large extrahepatic portosystemic shunt without portal hypertension. *Abdom Imaging* 1997; **22**: 79-81

105 **Prokop M**. Multislice CT angiography. *Eur J Radiol* 2000; **36**: 86-96

106 **Matsumoto A**, Kitamoto M, Imamura M, Nakanishi T, Ono C, Ito K, Kajiyama G. Three-dimensional portography using

multislice helical CT is clinically useful for management of gastric fundic varices. *AJR Am J Roentgenol* 2001; **176**: 899-905
107 **Krajina A**, Lojik M, Chovanec V, Raupach J, Hulek P. Wedged hepatic venography for targeting the portal vein during TIPS: comparison of carbon dioxide and iodinated contrast agents. *Cardiovasc Intervent Radiol* 2002; **25**: 171-175
108 **Martinez-Cuesta A**, Elduayen B, Vivas I, Delgado C, Gonzalez-Crespo I, Bilbao JI. CO(2) wedged hepatic venography: technical considerations and comparison with direct and indirect portography with iodinated contrast. *Abdom Imaging* 2000; **25**: 576-582
109 **Miller MW**, Fossum TW, Bahr AM. Transvenous retrograde portography for identification and characterization of portosystemic shunts in dogs. *J Am Vet Med Assoc* 2002; **221**: 1586-1590, 1574
110 **Waugh JR**, Sacharias N. Arteriographic complications in the DSA era. *Radiology* 1992; **182**: 243-246
111 **Sura PA**, Tobias KM, Morandi F, Daniel GB, Echandi RL. Comparison of 99mTcO4(-) trans-splenic portal scintigraphy with per-rectal portal scintigraphy for diagnosis of portosystemic shunts in dogs. *Vet Surg* 2007; **36**: 654-660
112 **Shiomi S**, Kuroki T, Ueda T, Takeda T, Ikeoka N, Nishiguchi S, Nakajima S, Kobayashi K, Ochi H. Clinical usefulness of evaluation of portal circulation by per rectal portal scintigraphy with technetium-99m pertechnetate. *Am J Gastroenterol* 1995; **90**: 460-465
113 **Crawford JM**. Development of the intrahepatic biliary tree. *Semin Liver Dis* 2002; **22**: 213-226
114 **Yamagami T**, Arai Y, Takeuchi Y, Sueyoshi S, Inaba Y, Maeda T. Intrahepatic portosystemic venous shunt associated with a large abdominal tumour. *Br J Radiol* 1999; **72**: 815-817
115 **Ohnishi K**, Hatano H, Nakayama T, Kohno K, Okuda K. An unusual portal-systemic shunt, most likely through a patent ductus venosus. A case report. *Gastroenterology* 1983; **85**: 962-965
116 **Loberant N**, Barak M, Gaitini D, Herskovits M, Ben-Elisha M, Roguin N. Closure of the ductus venosus in neonates: findings on real-time gray-scale, color-flow Doppler, and duplex Doppler sonography. *AJR Am J Roentgenol* 1992; **159**: 1083-1085
117 **Matsubara T**, Sumazaki R, Saitoh H, Imai H, Nakayama J, Takita H. Patent ductus venosus associated with tumor-like lesions of the liver in a young girl. *J Pediatr Gastroenterol Nutr* 1996; **22**: 107-111
118 **Buukyavuz I**, Ekinci S, Haliloglu M, Senocak ME, Ciftci AO. A case of patent ductus venosus with pulmonary arterio-venous fistula as a rare and unique clinical entity. *Turk J Pediatr* 2004; **46**: 272-274
119 **Kakar S**, Kamath PS, Burgart LJ. Sinusoidal dilatation and congestion in liver biopsy: is it always due to venous outflow impairment? *Arch Pathol Lab Med* 2004; **128**: 901-904
120 **Ferrell EA**, Graham JP, Hanel R, Randell S, Farese JP, Castleman WL. Simultaneous congenital and acquired extrahepatic portosystemic shunts in two dogs. *Vet Radiol Ultrasound* 2003; **44**: 38-42
121 **Kitagawa S**, Gleason WA Jr, Northrup H, Middlebrook MR, Ueberschar E. Symptomatic hyperammonemia caused by a congenital portosystemic shunt. *J Pediatr* 1992; **121**: 917-919
122 **Akahoshi T**, Nishizaki T, Wakasugi K, Mastuzaka T, Kume K, Yamamoto I, Sugimachi K. Portal-systemic encephalopathy due to a congenital extrahepatic portosystemic shunt: three cases and literature review. *Hepatogastroenterology* 2000; **47**: 1113-1116
123 **Ito T**, Ikeda N, Watanabe A, Sue K, Kakio T, Mimura H, Tsuji T. Obliteration of portal systemic shunts as therapy for hepatic encephalopathy in patients with non-cirrhotic portal hypertension. *Gastroenterol Jpn* 1992; **27**: 759-764
124 **Potts JR 3rd**, Henderson JM, Millikan WJ Jr, Sones P, Warren WD. Restoration of portal venous perfusion and reversal of encephalopathy by balloon occlusion of portal systemic shunt. *Gastroenterology* 1984; **87**: 208-212
125 **Uflacker R**, Silva Ade O, d'Albuquerque LA, Piske RL, Mourao GS. Chronic portosystemic encephalopathy: embolization of portosystemic shunts. *Radiology* 1987; **165**: 721-725
126 **Ikeda S**, Sera Y, Ohshiro H, Uchino S, Uchino T, Endo F. Surgical indications for patients with hyperammonemia. *J Pediatr Surg* 1999; **34**: 1012-1015
127 **Numata S**, Akagi K, Sakino I, Ogata H, Kawadoko T, Suzuki N, Nomiyama K, Tsuji H, Fujishima M. [Partial splenic embolization for the treatment of liver cirrhosis with hypersplenism: assessment of clinical response and liver function] *Nippon Shokakibyo Gakkai Zasshi* 1997; **94**: 526-531
128 **Kato T**, Romero R, Koutouby R, Mittal NK, Thompson JF, Schleien CL, Tzakis AG. Portosystemic shunting in children during the era of endoscopic therapy: improved postoperative growth parameters. *J Pediatr Gastroenterol Nutr* 2000; **30**: 419-425
129 **Wojcicki M**, Haagsma EB, Gouw AS, Slooff MJ, Porte RJ. Orthotopic liver transplantation for portosystemic encephalopathy in an adult with congenital absence of the portal vein. *Liver Transpl* 2004; **10**: 1203-1207
130 **Taoube KA**, Alonso Calderon JL, Yandza T, St Vil D, Blanchard H. [Congenital absence of portal vein in a girl with biliary atresia treated with liver transplant] *Cir Pediatr* 1999; **12**: 38-40
131 **Inomata Y**, Kiuchi T, Kim I, Uemoto S, Egawa H, Asonuma K, Fujita S, Hayashi M, Tanaka K. Auxiliary partial orthotopic living donor liver transplantation as an aid for small-for-size grafts in larger recipients. *Transplantation* 1999; **67**: 1314-1319

S- Editor Zhong XY **L- Editor** Wang XL **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5980

World J Gastroenterol 2008 October 21; 14(39): 5980-5989
World Journal of Gastroenterology ISSN 1007-9327

LIVER CANCER

Fibrinogen-like protein 2/fibroleukin prothrombinase contributes to tumor hypercoagulability *via* IL-2 and IFN-γ

Kai Su, Fang Chen, Wei-Ming Yan, Qi-Li Zeng, Li Xu, Dong Xi, Bin Pi, Xiao-Ping Luo, Qin Ning

Kai Su, Fang Chen, Wei-Ming Yan, Qi-Li Zeng, Li Xu, Dong Xi, Bin Pi, Xiao-Ping Luo, Qin Ning, Department of Infectious Diseases and Pediatrics, Institute of Infectious Diseases, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Avenue, Wuhan 430030, Hubei Province, China

Author contributions: Su K, Chen F, Luo XP and Ning Q designed the research; Su K, Chen F, Yan WM, Zeng QL, Xu L, Xi D and Pi B performed the research; Su K and Chen F analyzed data; and Su K, Luo XP and Ning Q wrote the paper.
Supported by The Natural Science Foundation of China, NSFC (30672380, 30571643); National Key Basic Research Program of China (2007CB512900, 2005CB522901, 2005CB522507) and 11th Five-Year Plan Key Project (2006BAI05A07)
Correspondence to: Qin Ning, MD, PhD, Department of Infectious Diseases, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Avenue, Wuhan 430030, Hubei Province, China. qning@tjh.tjmu.edu.cn
Telephone: +86-27-83662391 Fax: +86-27-83662391
Received: April 22, 2008 Revised: September 12, 2008
Accepted: September 17, 2008
Published online: October 21, 2008

Abstract

AIM: To examine the role of Fibrinogen-like protein 2 (fgl2)/fibroleukin in tumor development. Fgl2 has been reported to play a vital role in the pathogenesis in MHV-3 (mouse hepatitis virus) induced fulminant and severe hepatitis, spontaneous abortion, allo- and xeno-graft rejection by mediating "immune coagulation".

METHODS: Tumor tissues from 133 patients with six types of distinct cancers and the animal tumor tissues from human hepatocellular carcinoma (HCC) model on nude mice (established from high metastasis HCC cell line MHCC97LM6) were obtained.

RESULTS: Hfgl2 was detected in tumor tissues from 127 out of 133 patients as well as tumor tissues collected from human HCC nude mice. Hfgl2 was highly expressed both in cancer cells and interstitial inflammatory cells including macrophages, NK cells, and $CD8^+$ T lymphocytes and vascular endothelial cells. Hfgl2 mRNA was localized in cells that expressed hfgl2 protein. Fibrin (nogen) co-localization with hfgl2 expression was determined by dual immunohistochemical staining. *In vitro*, IL-2 and IFN-γ increased hfgl2 mRNA by 10-100 folds and protein expression in both THP-1 and HUVEC cell lines. One-stage clotting assays demonstrated that THP-1 and HUVEC cells expressing hfgl2 had increased procoagulant activity following cytokines stimulation.

CONCLUSION: The hfg12 contributes to the hypercoagulability in cancer and may induce tumor angiogenesis and metastasis *via* cytokine induction.

Key words: Fibrinogen-like protein 2/fibroleukin; Thrombin; Tumor; Coagulation; Cytokine

Peer reviewer: Xin-Xin Zhang, Professor, Department of Infectious Diseases, Rui Jin Hospital, 197, Rui Jin Er Road, Shanghai 200025, China

Su K, Chen F, Yan WM, Zeng QL, Xu L, Xi D, Pi B, Luo XP, Ning Q. Fibrinogen-like protein 2/fibroleukin prothrombinase contributes to tumor hypercoagulability *via* IL-2 and IFN-γ. *World J Gastroenterol* 2008; 14(39): 5980-5989 Available from: URL: http://www.wjgnet.com/1007-9327/14/5980.asp DOI: http://dx.doi.org/10.3748/wjg.14.5980

INTRODUCTION

Fibrinogen-like protein 2 (fgl2)/fibroleukin, also called fg12 prothrombinase, has recently been identified as a new member of fibrinogen-related protein superfamily, with the serine protease activity. Mouse fgl2 (mfgl2) and human fgl2 (hfgl2) are localized in chromosomes 5 and 7, respectively. The biological activity of fgl2 prothrombinase, similar to coagulating factor Xa, can directly catalyze prothrombinase into activated thrombinase, thereby, initiating a cascade coagulating reaction[1]. Several studies indicate that fgl2 is involved in MHV-3 induced fulminant hepatitis and severe or fulminant viral hepatitis in human, spontaneous abortion and xenograft rejection by mediating pathological changes such as immune coagulation, fibrin deposition, and micro-thrombus[2-5]. In addition to its primary role in homeostasis and blood coagulation, thrombin is a potent mitogen that dramatically increases the growth and metastasis potential of tumor cells. Both tissue factor (TF) and thrombin exert their influence on tumor angiogenesis and metastasis through clotting-dependent

and clotting-independent pathways[6,7]. Fgl2 functions as a novel immune coagulant with the ability to generate thrombin directly. Therefore, we propose that fgl2 may contribute to tumor angiogenesis and metastasis through a clotting-dependent pathway.

In the present study, the authors investigated the expression and histological localization of hfgl2, co-localization of fgl2 with fibrin in cancer and the gene regulation of fgl2 upon cytokine induction, in the hope of providing a new point of view on the characteristic hypercoagulability of cancer and a novel anticoagulant target, the fgl2 gene.

MATERIALS AND METHODS

Patients' tumor tissues

Informed consent was obtained from all the participants, and the research protocol was reviewed and approved by the Institutional Review Board of Tongji Hospital, Wuhan, China. Patients were recruited at Tongji Hospital, and 133 tumor samples and their paired adjacent normal tissues were collected. The patients' characteristics are shown in Table 1. The specimens for RNA extraction were frozen in liquid nitrogen until studied. Specimens for immunohistochemical and *in situ* hybridization were fixed in 4% paraform.

Table 1 General data and pathologic diagnosis of hfgl2 positive samples

Diagnosis	Case	Gender		Age (yr)	Subtype	Metastasis
		F	M			
Colon carcinoma	21	12	9	58.27 ± 10.27	AC 21	17
Breast cancer	20	0	20	49.70 ± 11.06	IDC 20	16
Lung cancer	20	17	3	55.17 ± 12.53	SCC 5 SqC 5 AC 7 AdCa 3	19
Gastric cancer	26	13	13	55.8 ± 15.88	AC 26	20
Esophageal carcinoma	18	15	3	56.44 ± 8.63	SqC 15 AC 3	13
Cervix cancer	22	0	22	39.14 ± 6.48	SqC 16 AC 6	22

AC: Adenocarcinoma; IDC: Infiltrating ductal carcinoma; SCC: Small cell carcinoma; SqC: Squamous carcinoma; AdCa: Adenosquamous carcinoma.

Mice

Male BALB/c-nu/nu mice (Shanghai Shilaike Animal Seed Center), 4-6 wk of age, with a body weight of 15.0-18.7 g, were kept in micro-isolated cages housed in Tongji Hospital and fed a standard lab chow diet and water ad libitum. Animals were divided into two groups: tumor-bearing mice (experimental group) and tumor-free mice (control group).

Cell and culture conditions

THP1 and HUVEC cell lines were purchased from Biology Treasure Center of Wuhan University. Human hepatocellular carcinoma (HCC) cell line MHCC97LM6 with high tendency of metastasis were purchased from Liver Cancer Institute, Fudan University, Shanghai. The HUVEC and MHCC97LM6 cell lines were cultured in Dulbecco modified Eagle medium (DMEM), and THP-1 cell lines were maintained in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum (FCS, Gibco Life Technologies), 100 U/mL penicillin, and 100 mg/mL streptomycin and cultured at 37℃, 50 mL/L CO_2, and 95% humidity.

Tumor cell inoculation and quantification of pulmonary metastatic foci

MHCC97LM6 cell lines were cultured *in vitro* by sub-confluent passage in DMEM. Sub-confluent tumor cells were washed with phosphate-buffered saline (PBS), detached by a brief exposure to a 0.125% trypsin and 0.02% EDTA solution, washed in serum-containing media, and then resuspended in cold serum-free medium to get the single cell suspension. The 95% viability of the tumor cells was determined by trypan blue exclusion. The cells were kept in an ice bath until transplanted into mice. A single cell suspension of 9×10^6 cells in 100 μL serum-free media was injected subcutaneously into the dorsal scapular skin of nude mice using a 27-gauge needle. Injection with the same volume of serum-free media served as the negative control. Once a tumor was clearly visible, it was measured daily and the volume estimated by the formula $V = ab^2/2$, where a = longest diameter, b = shortest diameter. After 36 d, the nude mice were sacrificed and the tumors and other organs including brain, heart, lung, liver, kidney, spleen, and small intestine were removed and rinsed in PBS. Aliquot of the tissue specimens were frozen in liquid nitrogen for RNA extraction. Other aliquots were fixed in 4% paraform and prepared for immunohistochemical studies. The lungs were separated into individual lobes and the number of metastatic foci was counted under a microscope with HE stain.

Immunohistochemical staining of fgl2 prothrombinase

Immunohistochemical staining was used to assess fgl2 expression in tumor tissue and HUVEC and THP-1 cell lines. Tissues were fixed with 4% paraform, processed into paraffin, and sectioned. Then they were rehydrated with 0.1 mol/L PBS (pH 7.4) and endogenous peroxidase. Nonspecific binding was blocked by sequential incubation of the sections in 10% hydrogen peroxidase solution for 10 min followed by 10% normal goat serum in PBS at room temperature for 30 min. Thereafter tissue or cultured cell slices were incubated with a polyclonal antibody against fgl2 at a dilution of 1/300 in PBS at 4℃ for 16 h. Subsequently, sections were incubated with immunoperoxidase-conjugated goat IgG fraction to rabbit IgG Fc (Zhongshan Company) at room temperature for 15 min, followed by three washes in PBS. The secondary antibody, an anti-rabbit IgG linked to peroxidase, was incubated with 3,3' -diaminobenzidine chromagen and counterstained with hematoxylin.

Fibrin in human malignant tumor tissues

Fibrin was detected with the use of a rabbit-anti-fibrinogen antibody (Dako Cytomation). This reagent is known to react with fibrinogen and fibrin in mouse and human tissues. The technique used for detection of fibrin was the standard avidin-biotin complex (ABC) method. The biotinylated secondary antibody was an anti-rabbit IgG linked to peroxidase incubated with 3,3'-diaminobenzidine chromagen, followed by counterstaining with hematoxylin.

Dual immunohistochemical staining of hfgl2 and fibrin

Dual staining for hfgl2 and fibrin on the same tissue was performed using a Vectastain ABC kit (Vector Laboratories), with second Abs labeled with AP or HRP, respectively.

Immunohistochemical *staining of macrophages, T lymphocytes, NK cells, and* vascular endothelial cells

Antibodies against CD68,CD57, CD4, CD8 and a monoclonal antibody against von Wille brand factor antigen (NeoMarkers) were individually used at a dilution of 1:50-1:100 in PBS to detect macrophages (Kupffer cells), NK cells, T lymphocytes, and vascular endothelial cells using immunoperoxidase staining *via* similar methodology described above.

Western blot analysis

Cells were solubilized in lysis buffer containing 10 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% TritonX-100, at 4℃ for 30 min. The cell lysates were subjected to centrifugation at 10000 × *g* at 4℃ for 1 min. The supernatants were saved and their protein contents were measured. Thirty mg lysate protein was loaded onto 12% SDS-polyacrylamide gels. After the proteins were separated, they were transferred to a NC membrane. The membrane was blocked and probed with a polyclonal antibody against fgl2 at a dilution of 1:200 in 5% milk in TBS. After washing with TBS and 0.5% Tween-20, the blot was incubated with secondary antibodies conjugated to horseradish-peroxidase. Immunoreactive bands were detected with the enhanced chemiluminescence (ECL) reagent (Pierce).

In situ hybridization

A digoxigenin-11-UTP (Dig-UTP) (Roche)-labeled cDNA probe was cut by EcoRI following subcloning of a 169-bp fragment of mfgl2 cDNA, representing nt 756 (ACTGTGACA …) to 924 (… GAGTAAGGA), into pCR2.1 vector (Invitrogen Life Technologies). The Dig-UTP-labeled probe concentration was determined by immunoenzymatic reaction with chemiluminescent detection, and the probes were stored at -80℃. Tissue sections were deparaffinized in 100% xylene and 100% alcohol, followed by prehybridization in 50% formamide and 2 × SSC at room temperature for 1 h. The hybridization mixture consisted of 50% deionized formamide, 5% dextran sulfate, 250 μg salmon sperm DNA per milliliter, and 2 μg Dig-labeled cDNA probe per milliliter in 2 × SSC. The hybridization mixture with the probe was denatured by heating in an 85℃ water bath for 5 min, chilled on ice for 1 min, and added to tissue sections for hybridization at 42℃ overnight. Post-hybridization washing in a series of dilutions of SSC was followed by application of 3% blocking reagent at room temperature for 30 min. After a brief wash in Tris-HCl buffer (pH 7.5), sections were incubated with polyclonal anti-Dig Fab, conjugated to alkaline phosphatase (AP; Boehringer Manheim), and diluted 1/500 in Tris-HCl buffer. Unbound antibody was removed by two 5-min washes with Tris-HCl buffer. A purple reaction product was developed using AP substrate, 5-bromo-4-chloro-3-indolyl-phosphate, and NBT to sections at room temperature for 120 min. Sections were counterstained with methylene green and mounted with Per mount for viewing.

RNA preparation and quantitative real-time PCR

Total RNA was isolated from tumor specimens and cell lines using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. The concentration and purity of RNA were determined by measuring the absorbance at 260 nm and 280 nm. Subsequently, the cDNAs were synthesized. The nucleotide sequences of the primers for PCR amplification of 169 bp fragment of fgl2 were the following: sense primer, 5'-ACTGTG ACATGGAGACCATG-3', and antisense primer, 5'-T CCTTACTCTTGGTCAGAAG-3'. The amplified 571 bp fragment of GAPDH cDNA was used as an internal control to ensure equal loading and first strand synthesis with forward primer, 5'-ATCACCATCTTCCAGGAG-3' and reverse primer, 5'-TGCTTCACCACCTTCTT G-3'. In the PCR reaction the DNA was amplified over 36 cycles, denatured at 94℃ for 40 s, annealed at 60℃ for 45 s, and extended at 72℃ for 60 s. The real-time PCR reactions were performed using a SYBR green PCR kit (Biotium) in Roche Sequence Detection System. Specificity of the PCR reaction was verified by dissociation-curve analysis and agarose gel electrophoresis. Fgl2 mRNA relative quantification was assigned by reference to standard curve analysis.

Cytokine treatment

THP-1 and HUVEC cell lines were maintained in medium containing 10% FBS in six well plate for 72 h until they reached sub-confluence. Then they were incubated with IL-2 (100 U/mL) or IFN-γ (200 U/mL) in medium for 4 h, 8 h, 12 h and 24 h before they were collected for immunohistochemical staining and real-time PCR studies.

Prothrombinase assays (PCA)

Samples to be assayed for PCA were washed three times with unsupplemented RPMI 1640 and resuspended at a concentration of 10^6/mL. The cells were then subjected to three cycles of freeze-thawing to obtain maximal total cellular procoagulant activity. Milliunits of PCA were determined from a standard curve generated by serial log dilutions of a standard rabbit brain thromboplastin

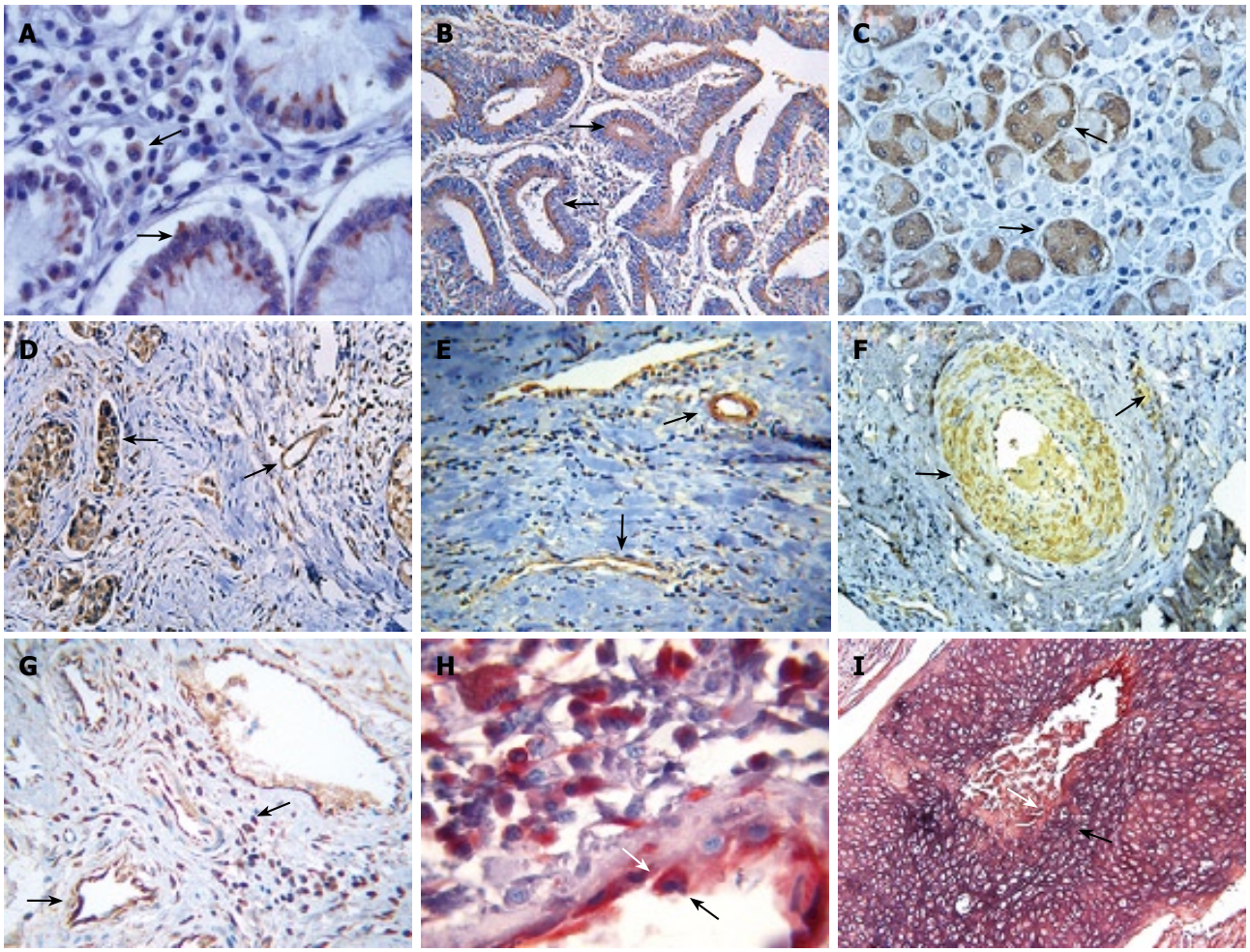


Figure 1 Immunohistochemical analysis of hfgl2 prothrombinase and fibrin in tumor tissues. Fgl2 was verified by immunohistochemistry in colon cancer (**A**, x 400), esophageal cancer (**B**, x 200), gastric cancer (**C**, x 400), breast cancer (**D**, x 200), lung cancer (**E**, × 200) and cervix cancer (**F**, x 100). Fibrin deposition was stained for colon cancer (**G**, x 200). Dual staining of hfgl2 (indigo) and fibrin in colon cancer (**H**, x 1000) and cervix cancer (**I**, x 400) displayed the co-localization of hfgl2 (indigo) and fibrin (scarlet) expression. Cells expressed fgl2 protein and fibrin were detected with antibodies specific for fgl2 (black arrows) and fibrin (white arrow), respectively.

(Sigma) to determine functional shorting of the spontaneous clotting time of normal citrated human platelet-poor plasma. After addition of cellular sample, 0.1 mL of normal plasma and 0.1 mL of 25 mmol/L $CaCl_2$ were added and clotting time was visually determined by the appearance of white precipitate after incubation at 37℃. Human plasmas deficient in specific clotting factors such as factor Ⅱ or factor X (ADI/DELLWIN) were also used as substrate in the clotting assay in place of normal human plasma.

Statistical analysis

Quantitative data were expressed as mean ± SD. Statistical analysis was performed by one-way analysis of variance with $P < 0.05$ considered statistically significant.

RESULTS

Fgl2 expression in tumor tissues from patients

The study population was composed of 133 patients, of whom 107 patients were noted clinically to have metastasis (Table 1). Tumor tissues of the patients were examined for fgl2 expression at both the mRNA and protein levels by *in situ* hybridization and immunohistochemical staining respectively. The normal tissue surrounding the tumor tissue was used as control. Fgl2 was present in cancer cells as well as interstitial infiltrated and vascular endothelium cells of the microvasculature (Figure 1A-F). There was significantly upregulated hfgl2 expression with cancers when compared with those in no magnificent tumor tissues which showed little or no fgl2 expression (data not shown). Dual staining of hfgl2 and fibrin displayed the co-localization of these two molecules, indicating the contribution of highly expressed hfgl2 protein to the hypercoagulability (Figure 1G-I). *In situ* hybridization showed a similar pattern of hfgl2 staining in tumor tissues of the patients (Figure 2).

Cellular source of fgl2 and fibrin deposition in tumor tissues

As shown with staining of serial tumor sections, the majority of CD68+, CD57+, CD8+, and vascular endothelial cells displayed increased expression of fgl2 protein in tumor tissues of the patients (Table 2 and Figure 3).

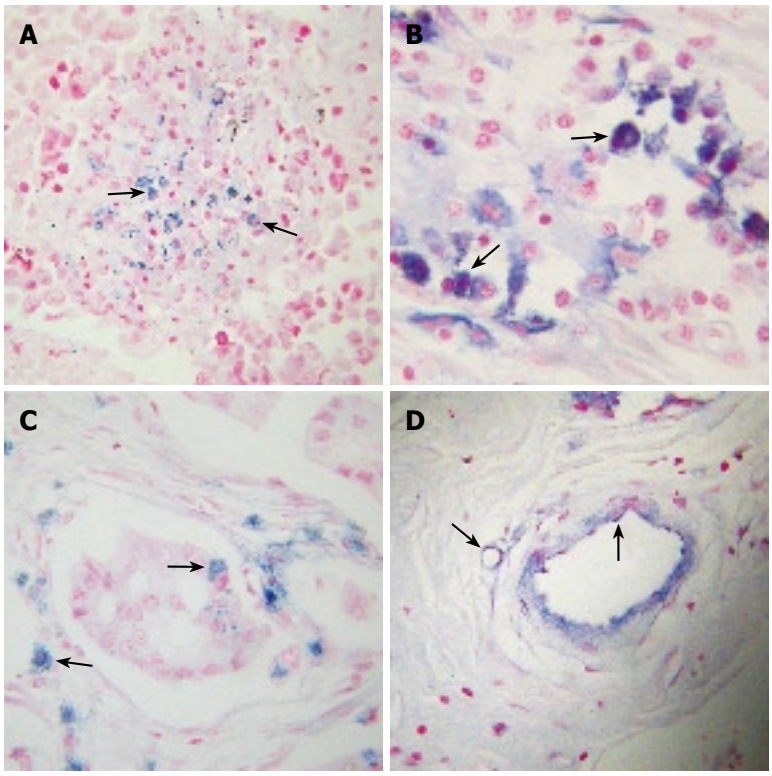


Figure 2 Hfgl2 mRNA detection in human tumor tissues by in situ hybridization. **A**: Hfgl2 mRNA was stained purple in lung small cell carcinoma, hfgl2 mRNA present in the nests of tumor cells, some tumor cells secreted melanin (x 400); **B**: Submucosa of colon descendens tubular adenocarcinoma, hfgl2 mRNA present in the infiltrated cells and single migrating tumor cells (x 400); **C**: Invasive ductal breast carcinoma, hfgl2 mRNA localized to nest tumor cells, exfoliated tumor cells and infiltrated cells around the duct (x 400); **D**: Submucosa of gastric adenocarcinoma, hfgl2 mRNA localized to tumor blood vessel endothelium (x 400). Arrows indicate hfgl2 mRNA locations.

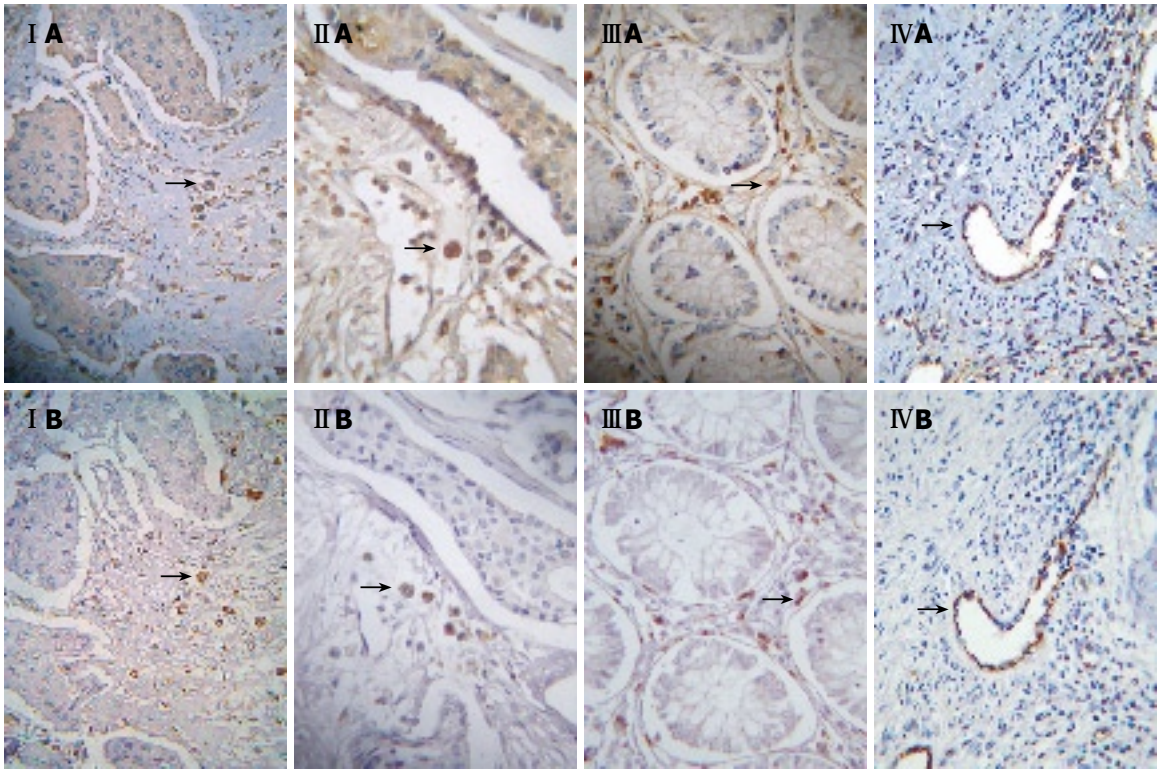


Figure 3 Ⅰ**A** to Ⅳ**A** was stained with fgl2 antibody (black arrows) displaying the fgl2 protein in infiltrative cells. Ⅰ**B** to Ⅳ**B** was a serial section of Ⅰ**A** to Ⅳ**A**, arrows indicate the staining of CD68, CD57, CD8 and vWF respectively.

Hfgl2 expression in tumor tissue from HCC nude mouse model

In the HCC nude mouse model, no evidence of histoincompatibility or tumor rejection was observed based on the rapid development of visible tumors after injection (100% of mice within 3 d). The steady growth of the tumors was found in MHCC97LM6 mice (Figure 4 Ⅰ A and B) whereas tumors were not observed in the control group. Almost all MHCC97LM6 mice developed on site palpable tumors and metastatic foci in lung tissues within 7 d of injection (Figure 4 Ⅰ C). Further studies showed that mfgl2 (mouse fgl2) expression

Table 2 Histological localization and involved cell type in hfgl2 positive samples

Tumor type	Case	Hfgl2 positive	Histological localization and cases				
			Tumor cell	CD57$^+$	CD3$^+$, CD8$^+$	CD68$^+$	VWF
Colon carcinoma	21	21	19	20	18	21	17
Breast cancer	20	20	18	20	20	20	18
Lung cancer	22	20	18	20	19	20	18
Gastric cancer	26	26	25	26	13	26	25
Esophageal carcinoma	19	18	18	16	15	18	14
Cervix cancer	25	22	20	21	21	22	22

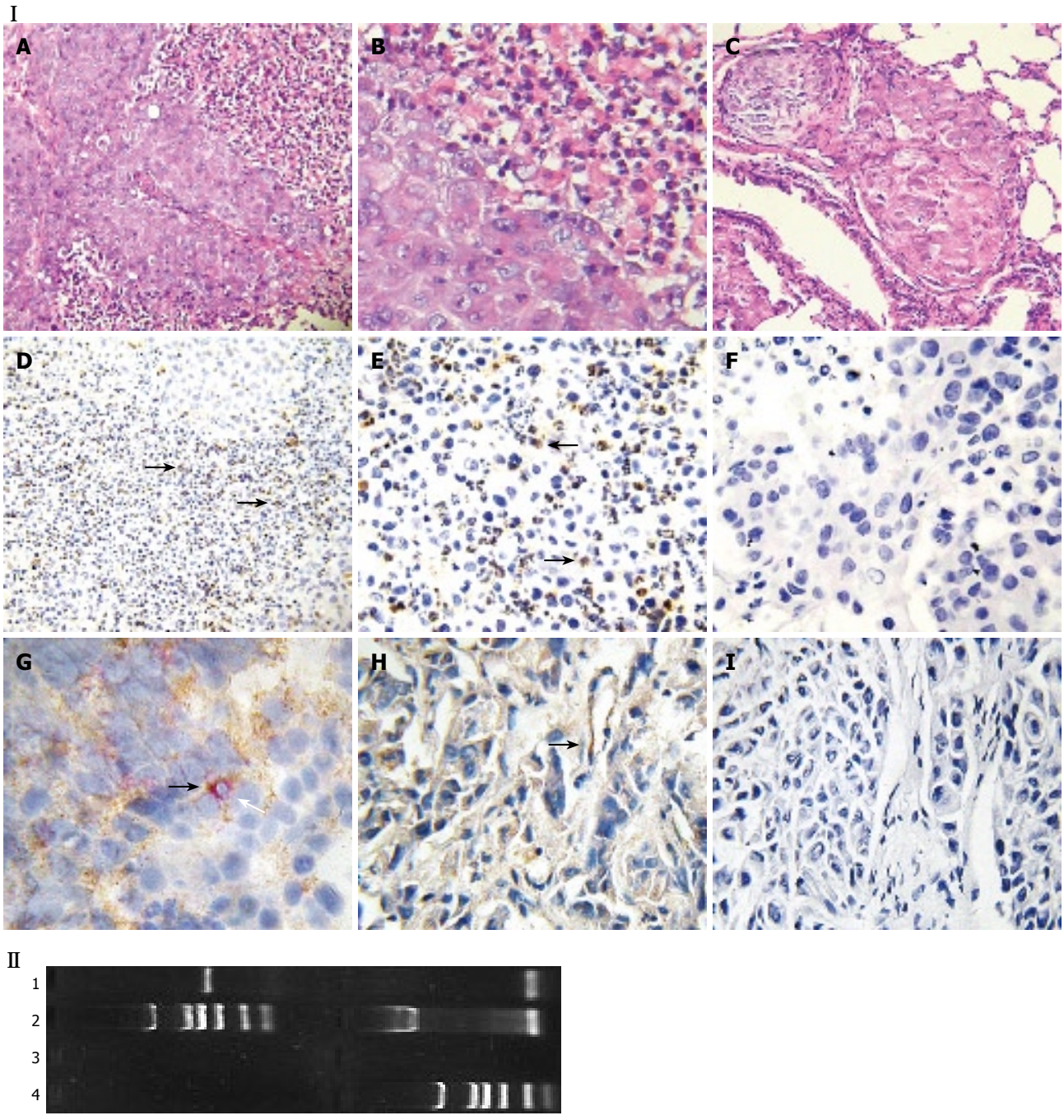


Figure 4 Fgl2 expression evidenced in mouse tumor tissue. Male BALB/c-nu/nu mice were subcutaneously injected MHCC97LM6 cell lines and tumor tissues were harvested 36 d later. Panel Ⅰ: **A**-**C**, HE staining in tumor tissue at injection site (**A**, x 200; **B**, x 400) and lung metastatic tumor tissue (**C**, x 200); **D**-**I**: mfgl2 expression in tumor tissue of human hepatocellular carcinoma (HCC) nude mice model (**D**, SP x 200; **E**, SP x 400; **G**, dual staining of mfgl2 and marker of macrophages; **H**, dual staining of mfgl2 and marker of endothelial cells; **F** and **I**, negative controls). Arrows in **D** and **E** indicate fgl2 positive cells, arrows in **G** and **F** indicate the fgl2 positive macrophage and endothelial cells, respectively. Panel Ⅱ: mfgl2 mRNA expression in tumor tissue (1), PCDNA3.1-fgl2 plasmid as positive control (2), PCDNA3.1 as negative control (3) and DL-2000 marker (4).

in interstitial inflammatory cells and vascular endothelial cells (Figure 4 I D-H). Furthermore the fgl2 was detected at mRNA level in the tumor tissue (Figure 4 Ⅱ).

Increased hfgl2 expression and PCA in IL-2 or IFN-γ stimulated HUVEC and THP-1 cell lines

Endothelium original HUVEC and mononuclear original THP-1 cells were used to investigate the regulatory expression of fgl2 in response to various tumor cytokines involved in tumor development *in vitro*. RT-PCR analysis demonstrated minimal constitutive fgl2 mRNA levels in both cell lines, but increased in response to stimulation with IL-2 or IFN-γ (data not shown). This primary observation was further demonstrated by real-time PCR, which showed a 10-100 fold increase of fgl2 mRNA copies following stimulation of IFN-γ or IL-2 (Figure 5Ⅲ). Immunohistochemical staining and Western-blotting also detected upregulated hfgl2 protein expression upon stimulation of cytokines (Figure 5 I and Ⅱ). The functional measurement of fgl2 protein was carried out by one-stage clotting assay expressed as PCA. Both HUVEC and THP-1 cells displayed basal levels of PCA with a significant increase following IL-2 or IFN-γ stimulation in parallel with fgl2 protein expression (Figure 6A). The induced PCA was independent of factor X, but closely associated with factor Ⅱ, thus demonstrating the PCA was induced by increased expression of fgl2 protein (Figure 6B).

DISCUSSION

The association between thrombosis and cancer was observed by Professor Armand Trousseau in 1865, who noted that patients who present with idiopathic venous thromboembolism (VTE) frequently harbor an occult cancer. We now believe that there are two key mediators of this link: one being the thrombin[6] whose broad substrate specificity supports a variety of cellular effects relevant to tumor growth and metastasis; and the other being the tissue factor (TF)[7], the primary initiator of the coagulation cascade, whose rather ubiquitous presence as a transmembrane receptor on a variety of nucleated cells confers responsibility for the generation of cell-surface thrombin in many pathologic situations *via* both clotting-dependent and clotting-independent mechanisms. TF and thrombin are capable of inducing angiogenesis, the process of generating new blood vessels from preexisting vessels, which is essential for tumor growth and metastasis.

We and many others have described a new procoagulant other than tissue factor and thrombin: fgl2 prothrombinase, a member of the fibrinogen superfamily, which was primarily reported to be produced by activated macrophages, T cells, and endothelial cells. Mouse fgl2 (mfgl2) and human fgl2 (hfgl2), were localized in chromosomes 5 and 7, respectively[1,3,8]. Fgl2 is a 64-70 kDa, type 2 transmembrane protein containing a C-terminal FRED (fibrinogen related extracellular domain). The fgl2 amino acid sequence is 36% homologous to the β and γ fibrinogen chains[9]. There is 78% homology between human and mouse fgl2 with 90% homology in their C-terminal domains containing FRED[3]. Fgl2 functions as a strong prothrombinase which directly cleaves prothrombin to thrombin leading to fibrin deposition in the absence of factor Ⅶ or factor X[10]. The direct prothrombinase activity of fgl2 is implicated in the pathogenesis of several inflammatory disorders including fulminant hepatitis and severe hepatitis, allo- and xeno-graft rejection[4,11,12]. Furthermore, its role is also evidenced in murine and human cytokine induced fetal loss[5,13-15] and neonatal death from contractile dysfunction and rhythm abnormalities during embryonic and postnatal development[16]. The observations that neutralizing Abs to mfgl2 prevent both fibrin deposition and death from MHV-3 infection support its role as a coagulant[17]. Recent studies have shown that inhibition of reticuloendothelial cell mfgl2 expression through the use of gene-targeted fgl2-deficient (fgl2-/-) mice or targeted fgl2 gene with antisense mfgl2 results in the prevention of MHV-3-induced fibrin deposition, liver injury, and death[2,18].

Our study shows that fgl2 prothrombinase was expressed in malignant tumor tissues including colon, breast, lung, gastric, esophageal, and cervical tissues from patients and in HCC nude mouse models. Up-regulation of fgl2 gene expression is evident not only in cancer cells, but also in interstitial infiltrated cells including macrophages, NK cells, $CD8^+$ T lymphocytes, and vascular endothelial cells. Dual staining shows that fibrin (nogen) uniformly co-localized with fgl2 protein. In breast cancer, fgl2 is present predominantly in the same cellular types in which TF was expressed[19]. Other studies have further shown fibrin (nogen) co-localization with TF expression. Cross-linked fibrin (XLF) was found within the endothelium of angiogenic vessels of invasive breast cancer specimens, but not within the vessels of benign breast tumors in histological specimens from the patients[20]. The similar expression patterns of TF and fgl2 have led us to hypothesize that both fgl2 and TF may be responsible for the coagulation cascade in cancer. Fgl2 and TF cleavage of prothrombin to thrombin results in fibrin deposition in the tumor microenvironment (TME). Thrombin-catalyzed, XLF formation is a characteristic histopathological finding in many human and experimental tumors[21].

Fgl2 induces angiogenesis by generating thrombin. Thrombin dramatically increases the growth and metastatic potential of tumor cells *via* clotting dependent and independent mechanisms. The fibrin matrix that develops around tumors provides a provisional proangiogenic scaffold that supports vessel formation and stimulates endothelial cell proliferation and migration through clotting dependent mechanisms. Clotting independent mechanisms are thought to be mediated *via* proteolytic cleavage of the PARs and subsequent activation of G-protein-coupled signal transduction cascades, leading to the upregulation of many angiogenesis-related genes, including VEGF,

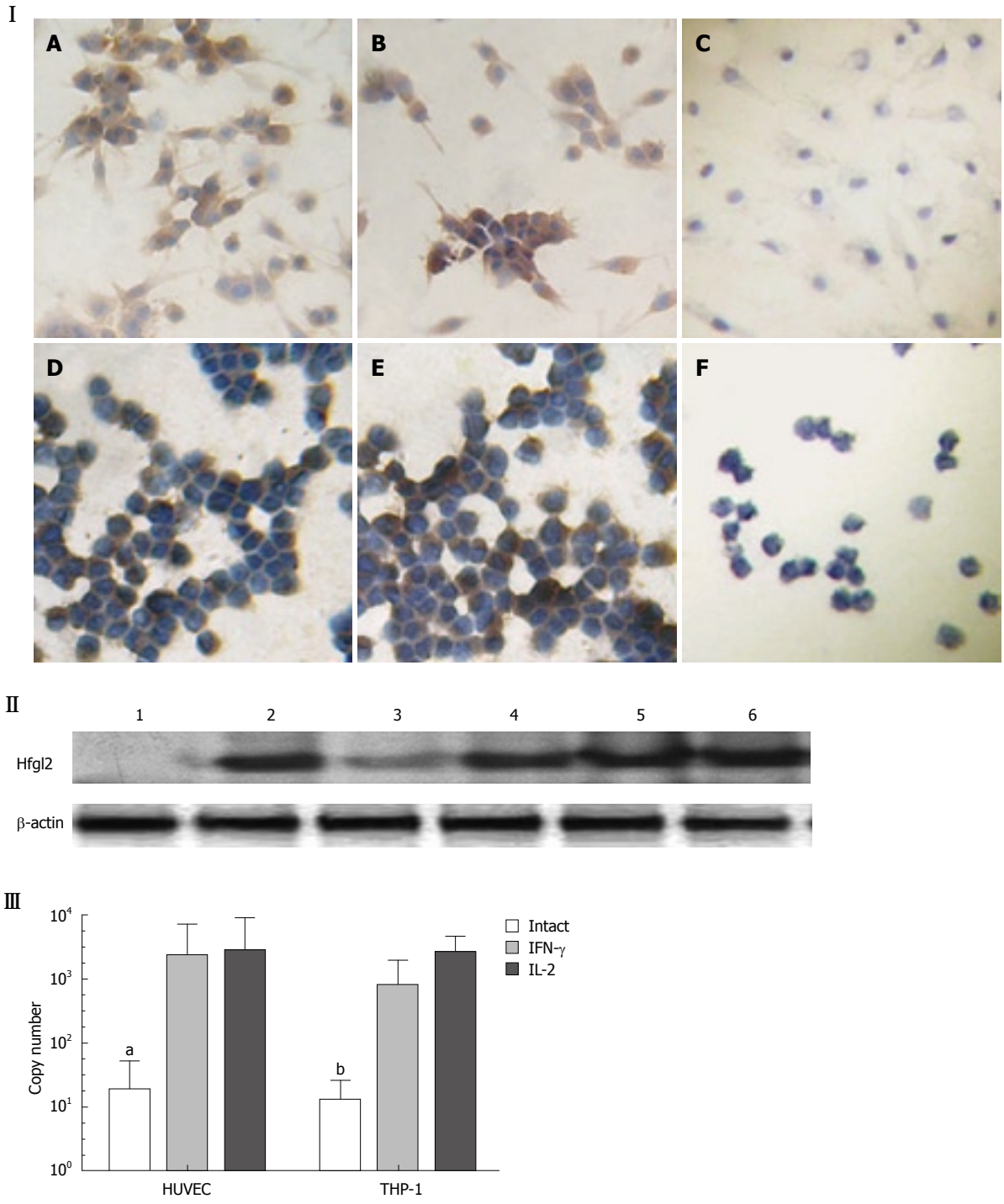


Figure 5 Increased expression of fgl2 after IFN-γ or IL-2 stimulation. Ⅰ: HUVEC and THP-1 cells were treated with IFN-γ (200 U/mL) and IL-2 (100 U/mL) and stained with antibodies specific for hfgl2. **A**: IFN-γ (200 U/mL, 12 h) + HUVEC (SP, x 200); **B**: IL-2 (100 U/mL, 8 h) + HUVEC (SP, x 200); **C**: NS +HUVEC (SP, x 200); **D**: IFN-γ (200 U/mL, 12 h) + THP-1 (SP, x 200); **E**: IL-2 (100 U/mL, 8 h) + THP-1(SP, x 200); **F**: NS + THP-1 (SP, x 200). Ⅱ: Western blotting analysis of hfgl2 expression after IFN-γ (200 U/mL) and IL-2 (100 U/mL) stimulation for 12 h and 8 h on HUVEC and THP-1 cells. 1: Intact HUVEC; 2: IFN-γ + HUVEC; 3: Intact THP-1; 4: IFN-γ + THP-1; 5: IL-2 + HUVEC; 6: IL-2 +THP-1. Ⅲ: Real-time PCR analysis of inducible hfgl2 mRNA after stimulation for 4 h. $^aP = 0.0329$, *vs* HUVEC alone; $^bP = 0.0059$, *vs* THP-1 alone.

VEGF receptors, TF, bFGF, and MMP-2[22-24]. These genes can create a number of pleiotropic responses, such as change in endothelial cell shape, increased vascular permeability, increased endothelial cell proliferation, and increased proteolysis, all of which contribute to increased tumor angiogenesis.

The pathogenic role of fgl2 is not entirely understood as only one pathway of fgl2 activation has been studied so far. In murine hepatitis viral infection, nucleocapsid protein induces transcription of fgl2 through the transcription factor hepatic nuclear factor 4α and its cognate receptor[25,26]. HBV X and core protein was shown to induce hfgl2 expression through a host factor c-Ets-2 and MAPK signal pathway[27]. In transplantation, fgl2 transcription appears to be regulated by cytokines. Macrophage induction of fgl2 is

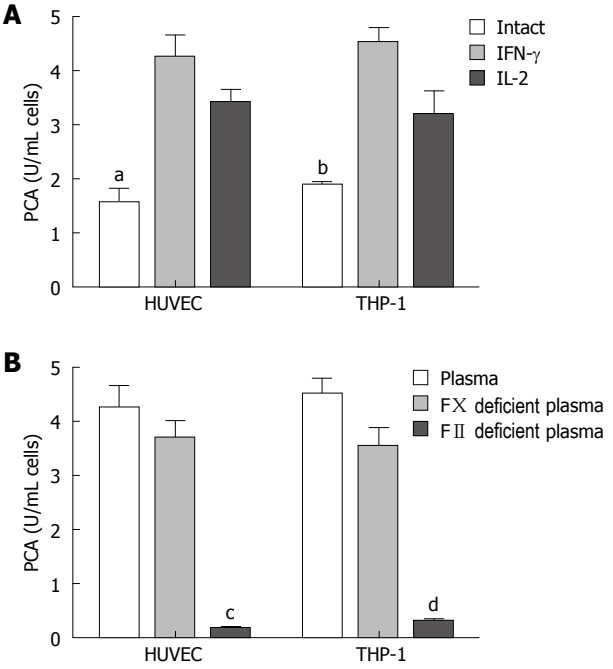


Figure 6 Increase of procoagulant activity (PCA) in HUVEC and THP-1 cells after IFN-γ and IL-2 stimulation dependent on FⅡ but not FX. HUVEC and THP-1 cells received IFN-γ (200 U/mL) and IL-2 (100 U/mL) for 12 h and the PCA was assayed. IFN-γ and IL-2 increased PCA and this effect is preserved in FX poor plasma whereas is absent in FⅡ poor plasma. [a]*P* = 0.0026, *vs* HUVEC alone; [b]*P* = 0.0037, *vs* THP-1 alone; [c]*P* = 0.0002, FⅡ deficient plasma group *vs* normal plasma group or FX deficient plasma group; [d]*P* = 0.0001, FⅡ deficient plasma group *vs* normal plasma group or FX deficient plasma group.

induced by IFN-γ, whereas preliminary data suggest that fgl2 transcription in endothelial cells occurs in response to TNF-α but not IFN-γ[28].

Our study has also shown that cultured HUVEC and THP-1 cells activated by IFN-γ or IL-2 demonstrated induction of hgl2 expression and enhanced activation of human prothrombin. The induced PCA activity was independent of factor X, but closely associated with factor Ⅱ. These results suggest that macrophages are attracted to invading tumors and subsequently release cytokines that later induce fgl2 expression in cancer. Increased fgl2 expression may activate thrombin, to exert its effect on tumor angiogenesis and metastasis through clotting-dependent and independent mechanisms. Additional studies in molecular pathways for induction of fgl2 in cancer are presently underway in our laboratory.

The fgl2 protein described here is a membrane bound prothrombinase. The recent discovery of a secreted form of fgl2 (sfgl2) produced by T regulatory cells has potent immune modulatory effects on the adaptive immune system. Sfgl2 was reported to prevent maturation of dendritic cells (DC) by inhibiting NF-κB nuclear translocation, expression of CD80 and MHCII, by inhibiting T cell proliferation in response to CD3/CD28, Concanavalin A, and allo-antigens. These observations have provided a potential explanation for many of the biological functions influenced by fgl2 protein[29-31] in our laboratory. fgl2 was also found in the extracellular matrix in malignant tumor tissue samples. This suggests the involvement of sfgl2 protein. Further studies are necessary to solve this conundrum.

In this study, we first reported the highly expressed fgl2 prothrombinase in a variety of tumor tissues both from patients and an animal model. Tumor related cytokines IFN-γ and IL-2 lead to the induction of hfgl2 expression and enhanced activation of human prothrombin. These observations suggest that fgl2 prothrombinase, in conjunction with thrombin and tissue factor, may contribute to tumor hypercoagulability and possibly to angiogenesis and metastasis. In turn, fgl2 may serve as a novel target for intervention of tumor development.

ACKNOWLEDGMENTS

The authors express their deepest appreciation to Dr. Gary Levy in University of Toronto for offering the reagents and critical comments for this study.

COMMENTS

Background

Fibrinogen-like protein 2 (fgl2)/fibroleukin, also called fg12 prothrombinase, has been found recently and belongs to fibrinogen-related protein superfamily. Fgl2 prothrombinase has serine protease activity. Human fgl2 gene is mapped at chromosome 7q11,23. Biological activity of the product of fgl2 prothrombinase, similar to coagulating factor Xa, can directly catalyze prothrombinase into activated thrombinase, initiating cascade coagulating reaction. Several studies abroad indicate that mouse fgl2 has been involved in MHV-3 induced fulminant hepatitis, spontaneous abortion and xenograft rejection by mediating "immune coagulation", fibrin deposition and microthrombus leading to the pathological changes.

Research frontiers

In addition to its primary role in hemostasis and blood coagulation, thrombin is a potent mitogen capable of inducing cellular functions. Thrombin can dramatically increase the growth and metastatic potential of tumor cells, thus it should be of great importance in the behavior of cancer. Both tissue factor (TF) and thrombin exert their influence on tumor angiogenesis and metastasis through clotting-dependent and clotting-independent mechanisms. Fgl2 functions as a novel immune coagulant with the ability to generate thrombin directly.

Innovations and breakthroughs

Fgl2 highly expressed in tumor cells and activated interstitial infiltrated cells, which may contribute to the characteristics of hypercoagulability and in turn induces tumor angiogenesis and metastasis.

Applications

In present study, the authors investigated hfgl2 expression and its histological localization in cancer, which will provide a new point of view on the characteristic hypercoagulability of cancer and efficacious anticoagulant therapy in cancer treatment.

Peer review

It has been proved that fgl2 functions as an immune coagulant with the ability to cleave prothrombin to thrombin directly and there are relationships between thrombosis and cancer. The aim of this study was to investigate the role of fgl2 in tumor development. They found that Hfgl2 was detected in tumor tissues from 127 out of 133 patients as well as tumor tissues collected from human HCC nude mice and IL-2 and IFN-γ could increase hfgl2 mRNA *in vitro*. It is an interesting subject and results were clearly described.

REFERENCES

1 **Levy GA**, Liu M, Ding J, Yuwaraj S, Leibowitz J, Marsden PA, Ning Q, Kovalinka A, Phillips MJ. Molecular and functional analysis of the human prothrombinase gene (HFGL2) and its role in viral hepatitis. *Am J Pathol* 2000; **156**:

1217-1225
2 **Marsden PA**, Ning Q, Fung LS, Luo X, Chen Y, Mendicino M, Ghanekar A, Scott JA, Miller T, Chan CW, Chan MW, He W, Gorczynski RM, Grant DR, Clark DA, Phillips MJ, Levy GA. The Fgl2/fibroleukin prothrombinase contributes to immunologically mediated thrombosis in experimental and human viral hepatitis. *J Clin Invest* 2003; **112**: 58-66
3 **Ding JW**, Ning Q, Liu MF, Lai A, Peltekian K, Fung L, Holloway C, Yeger H, Phillips MJ, Levy GA. Expression of the fgl2 and its protein product (prothrombinase) in tissues during murine hepatitis virus strain-3 (MHV-3) infection. *Adv Exp Med Biol* 1998; **440**: 609-618
4 **Zhu CL**, Yan WM, Zhu F, Zhu YF, Xi D, Tian DY, Levy G, Luo XP, Ning Q. Fibrinogen-like protein 2 fibroleukin expression and its correlation with disease progression in murine hepatitis virus type 3-induced fulminant hepatitis and in patients with severe viral hepatitis B. *World J Gastroenterol* 2005; **11**: 6936-6940
5 **Clark DA**, Arck PC, Chaouat G. Why did your mother reject you? Immunogenetic determinants of the response to environmental selective pressure expressed at the uterine level. *Am J Reprod Immunol* 1999; **41**: 5-22
6 **Maragoudakis ME**, Tsopanoglou NE, Andriopoulou P. Mechanism of thrombin-induced angiogenesis. *Biochem Soc Trans* 2002; **30**: 173-177
7 **Rickles FR**, Shoji M, Abe K. The role of the hemostatic system in tumor growth, metastasis, and angiogenesis: tissue factor is a bifunctional molecule capable of inducing both fibrin deposition and angiogenesis in cancer. *Int J Hematol* 2001; **73**: 145-150
8 **Yuwaraj S**, Ding J, Liu M, Marsden PA, Levy GA. Genomic characterization, localization, and functional expression of FGL2, the human gene encoding fibroleukin: a novel human procoagulant. *Genomics* 2001; **71**: 330-338
9 **Doolittle RF**. The structure and evolution of vertebrate fibrinogen: a comparison of the lamprey and mammalian proteins. *Adv Exp Med Biol* 1990; **281**: 25-37
10 **Schwartz BS**, Levy GA, Fair DS, Edgington TS. Murine lymphoid procoagulant activity induced by bacterial lipopolysaccharide and immune complexes is a monocyte prothrombinase. *J Exp Med* 1982; **155**: 1464-1479
11 **Ghanekar A**, Mendicino M, Liu H, He W, Liu M, Zhong R, Phillips MJ, Levy GA, Grant DR. Endothelial induction of fgl2 contributes to thrombosis during acute vascular xenograft rejection. *J Immunol* 2004; **172**: 5693-5701
12 **Mendicino M**, Liu M, Ghanekar A, He W, Koscik C, Shalev I, Javadi M, Turnbull J, Chen W, Fung L, Sakamoto S, Marsden P, Waddell TK, Phillips MJ, Gorczynski R, Levy GA, Grant D. Targeted deletion of Fgl-2/fibroleukin in the donor modulates immunologic response and acute vascular rejection in cardiac xenografts. *Circulation* 2005; **112**: 248-256
13 **Clark DA**, Foerster K, Fung L, He W, Lee L, Mendicino M, Markert UR, Gorczynski RM, Marsden PA, Levy GA. The fgl2 prothrombinase/fibroleukin gene is required for lipopolysaccharide-triggered abortions and for normal mouse reproduction. *Mol Hum Reprod* 2004; **10**: 99-108
14 **Knackstedt M**, Ding JW, Arck PC, Hertwig K, Coulam CB, August C, Lea R, Dudenhausen JW, Gorczynski RM, Levy GA, Clark DA. Activation of the novel prothrombinase, fg12, as a basis for the pregnancy complications spontaneous abortion and pre-eclampsia. *Am J Reprod Immunol* 2001; **46**: 196-210
15 **Knackstedt MK**, Zenclussen AC, Hertwig K, Hagen E, Dudenhausen JW, Clark DA, Arck PC. Th1 cytokines and the prothrombinase fgl2 in stress-triggered and inflammatory abortion. *Am J Reprod Immunol* 2003; **49**: 210-220
16 **Mu J**, Qu D, Bartczak A, Phillips MJ, Manuel J, He W, Koscik C, Mendicino M, Zhang L, Clark DA, Grant DR, Backx PH, Levy GA, Adamson SL. Fgl2 deficiency causes neonatal death and cardiac dysfunction during embryonic and postnatal development in mice. *Physiol Genomics* 2007; **31**: 53-62
17 **Li C**, Fung LS, Chung S, Crow A, Myers-Mason N, Phillips MJ, Leibowitz JL, Cole E, Ottaway CA, Levy G. Monoclonal antiprothrombinase (3D4.3) prevents mortality from murine hepatitis virus (MHV-3) infection. *J Exp Med* 1992; **176**: 689-697
18 **Zhu C**, Sun Y, Luo X, Yan W, Xi D, Ning Q. Novel mfgl2 antisense plasmid inhibits murine fgl2 expression and ameliorates murine hepatitis virus type 3-induced fulminant hepatitis in BALB/cJ mice. *Hum Gene Ther* 2006; **17**: 589-600
19 **Shoji M**, Hancock WW, Abe K, Micko C, Casper KA, Baine RM, Wilcox JN, Danave I, Dillehay DL, Matthews E, Contrino J, Morrissey JH, Gordon S, Edgington TS, Kudryk B, Kreutzer DL, Rickles FR. Activation of coagulation and angiogenesis in cancer: immunohistochemical localization in situ of clotting proteins and vascular endothelial growth factor in human cancer. *Am J Pathol* 1998; **152**: 399-411
20 **Contrino J**, Hair G, Kreutzer DL, Rickles FR. In situ detection of tissue factor in vascular endothelial cells: correlation with the malignant phenotype of human breast disease. *Nat Med* 1996; **2**: 209-215
21 **van Hinsbergh VW**, Collen A, Koolwijk P. Role of fibrin matrix in angiogenesis. *Ann N Y Acad Sci* 2001; **936**: 426-437
22 **Richard DE**, Vouret-Craviari V, Pouyssegur J. Angiogenesis and G-protein-coupled receptors: signals that bridge the gap. *Oncogene* 2001; **20**: 1556-1562
23 **Coughlin SR**. Thrombin signalling and protease-activated receptors. *Nature* 2000; **407**: 258-264
24 **Carmeliet P**. Biomedicine. Clotting factors build blood vessels. *Science* 2001; **293**: 1602-1604
25 **Ning Q**, Liu M, Kongkham P, Lai MM, Marsden PA, Tseng J, Pereira B, Belyavskyi M, Leibowitz J, Phillips MJ, Levy G. The nucleocapsid protein of murine hepatitis virus type 3 induces transcription of the novel fgl2 prothrombinase gene. *J Biol Chem* 1999; **274**: 9930-9936
26 **Ning Q**, Lakatoo S, Liu M, Yang W, Wang Z, Phillips MJ, Levy GA. Induction of prothrombinase fgl2 by the nucleocapsid protein of virulent mouse hepatitis virus is dependent on host hepatic nuclear factor-4 alpha. *J Biol Chem* 2003; **278**: 15541-15549
27 **Han MF**, Zhou YY, Xi D, Yan WM, Luo XP, Ning Q. Hepatitis B virus proteins induce activation of hfgl2 transcription through cEts2 transcription factor and MAPK signal pathway. *Hepatology* 2007; **46** Suppl: 641A
28 **Hancock WW**, Szaba FM, Berggren KN, Parent MA, Mullarky IK, Pearl J, Cooper AM, Ely KH, Woodland DL, Kim IJ, Blackman MA, Johnson LL, Smiley ST. Intact type 1 immunity and immune-associated coagulative responses in mice lacking IFN gamma-inducible fibrinogen-like protein 2. *Proc Natl Acad Sci USA* 2004; **101**: 3005-3010
29 **Chan CW**, Kay LS, Khadaroo RG, Chan MW, Lakatoo S, Young KJ, Zhang L, Gorczynski RM, Cattral M, Rotstein O, Levy GA. Soluble fibrinogen-like protein 2/fibroleukin exhibits immunosuppressive properties: suppressing T cell proliferation and inhibiting maturation of bone marrow-derived dendritic cells. *J Immunol* 2003; **170**: 4036-4044
30 **Marazzi S**, Blum S, Hartmann R, Gundersen D, Schreyer M, Argraves S, von Fliedner V, Pytela R, Ruegg C. Characterization of human fibroleukin, a fibrinogen-like protein secreted by T lymphocytes. *J Immunol* 1998; **161**: 138-147
31 **Ruegg C**, Pytela R. Sequence of a human transcript expressed in T-lymphocytes and encoding a fibrinogen-like protein. *Gene* 1995; **160**: 257-262

S- Editor Zhong XY **L- Editor** Ma JY **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5990

World J Gastroenterol 2008 October 21; 14(39): 5990-5995
World Journal of Gastroenterology ISSN 1007-9327

CLINICAL RESEARCH

Rectal administration of d-alpha tocopherol for active ulcerative colitis: A preliminary report

Seyed Amir Mirbagheri, Behtash Ghazi Nezami, Solmaz Assa, Mannan Hajimahmoodi

Seyed Amir Mirbagheri, Behtash Ghazi Nezami, Solmaz Assa, Amir-Alam hospital, Tehran University of Medical Sciences, Tehran 13145-784, Iran
Mannan Hajimahmoodi, Department of Drug and Food Control, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran 13145-784, Iran
Author contributions: Mirbagheri SA developed the main hypothesis of the study, performed endoscopic studies and clinical examinations, supervised and edited the paper; Mirbagheri SA, Nezami BG, and Assa S designed the research; Nezami BG and Assa S followed the patients and recorded the data; Hajimahmoodi M determined the plasma level of α-tocopherol in recruited patients; Nezami BG and Assa S analyzed the data and wrote the paper.
Supported by Research grant provided by the Tehran University of Medical Sciences
Correspondence to: Seyed Amir Mirbagheri, MD, Department of Internal Medicine, Amir-Alam Hospital, North Sa'adi Street, Tehran 13145-784, Iran. mirbagherimd@yahoo.com
Telephone: +98-216-6708688 Fax: +98-216-6704805
Received: June 1, 2008 Revised: August 11, 2008
Accepted: August 18, 2008
Published online: October 21, 2008

Abstract

AIM: To investigate the anti-oxidant and anti-neutrophil recruitment effects of rectal d-alpha (d-α) tocopherol administration on mild and moderately active ulcerative colitis (UC).

METHODS: Fifteen patients with mild and moderately active ulcerative colitis were enrolled in an open-label study of d-α tocopherol enema (8000 U/d) for 12 wk. All patients were receiving concomitant therapy with 5-aminosalicylic acid derivatives (5-ASA) and/or immunomodulator medications. Endoscopic evaluation was performed at baseline and after 4th and 12th weeks. Disease activity was measured with the Mayo disease activity index (DAI) and remission was defined as DAI of $\leqslant 2$ with no blood in stool. Clinical response was defined as a DAI reduction of $\geqslant 2$.

RESULTS: At the end of 12th week, the average DAI score significantly decreased compared to the beginning of the study (2.3 ± 0.37 *vs* 8 ± 0.48, $P < 0.0001$). One patient was withdrawn after 3 wk for being unavailable to follow-up. On the 4th week of therapy, 12 patients showed clinical response, 3 of whom (21.4%) achieving remission. After 12 wk, all 14 patients responded clinically to the therapy and remission was induced in 9 of them (64%). No patient reported adverse events or was hospitalized due to worsened disease activity.

CONCLUSION: This preliminary report suggests that rectal d-α tocopherol may represent a novel therapy for mild and moderately active UC. The observed results might be due to the anti-inflammatory and anti-oxidative properties of vitamin E.

Key words: Vitamin E; Ulcerative colitis; Inflammatory bowel disease; Enema; Activity index

Peer reviewer: Peter R Gibson, Professor, Department of Medicine, Monash University, Box Hill Hospital, Box Hill 3128, Australia

Mirbagheri SA, Nezami BG, Assa S, Hajimahmoodi M. Rectal administration of d-alpha tocopherol for active ulcerative colitis: A preliminary report. *World J Gastroenterol* 2008; 14(39): 5990-5995 Available from: URL: http://www.wjgnet.com/1007-9327/14/5990.asp DOI: http://dx.doi.org/10.3748/wjg.14.5990

INTRODUCTION

Inflammatory bowel disease (IBD) comprises a group of chronic, lifelong, immuno-inflammatory disorders, characterized by flare-ups due to increased inflammatory activity of the intestinal mucosa, interspersed with asymptomatic periods of remission[1]. Although the etiology of IBD remains unknown, it is believed that the generation of an exaggerated intestinal immune response to otherwise innocuous stimuli plays a key role in the pathophysiology of this intestinal disorder[2]. IBD is mainly characterized by upregulation of synthesis and release of different proinflammatory mediators, including reactive oxygen and nitrogen metabolites, eicosanoids, platelet-activating factor and cytokines. All of these mediators actively contribute to the pathogenic cascade that initiates and perpetuates the inflammatory response of the gut. Thus, the treatment strategy for IBD focuses on eliminating these causal inflammatory triggers and mediators.

Unfortunately no disease-specific treatment for IBD has yet emerged, and the best strategy to effectively downregulate the exacerbated immune response is likely

to interfere with multiple stages of the inflammatory cascade[3]. In fact, the drugs currently used for the management of human IBD, i.e. 5-aminosalicylic acid derivatives, immunosuppressives and systemic or local glucocorticoids, exert their beneficial effects through a combination of different mechanisms[4]. On the other hand, even the most effective drugs used in inflammatory bowel disease are only successful in about two-thirds of patients[5,6], while these drugs are not devoid of potentially serious side effects that limit their use in a further substantial proportion of patients[7,8].

It is now well established that vitamin E is a major lipophilic antioxidant in cellular membranes with excellent antioxidant activities[9,10] which protects membrane lipids from peroxidation[11,12] by scavenging not only chain carrying peroxyl radicals but also singlet oxygen and superoxide anion radicals[13]. This is especially interesting in the case of ulcerative colitis (UC), considering the pivotal role of oxygen free radicals in the genesis of mucosal damage. Additionally, the production of reactive oxygen species increases by the colonic mucosa in patients with UC[14-16]; and inhibition of lipid peroxidation or scavenging of oxygen free radicals produces valuable preventive and therapeutic strategies for IBD[12].

Thus, given the recent evidence suggesting anti-inflammatory properties for vitamin E[17,18], d-alpha (d-α) tocopherol, as the dominant vitamin E isomer in plasma with the highest biopotency, may be expected to reduce the development of tissue injury in IBD. In support of this hypothesis, we report the preliminary results of an ongoing open-label case series study on clinical and endoscopic changes of disease severity in patients with active UC who received daily rectal doses of d-α tocopherol for at least 12 wk.

MATERIALS AND METHODS

Inclusion and exclusion criteria

We recruited 15 volunteer UC patients (5 males, 10 females) between February 2006 and February 2008, seen in our university-based gastroenterology practice. The study protocol was submitted to the university ethics committee and written informed consent was obtained from all participating patients. All enrolled patients had active disease, limited to sigmoid at the beginning of the study. In order to minimize observation errors, all diagnoses were made on the basis of a combination of clinical, endoscopic and histological criteria by a single faculty member gastroenterologist. Only patients with mild and moderately active UC despite a minimum of 4 wk of therapy with at least 3 g/d of an oral 5-ASA compound were eligible for inclusion to whom we permitted concomitant therapy with azathioprine or 6-mercaptopurine as long as the patient had been receiving the medication for a minimum of 24 wk and was on a stable dose for a minimum of 12 wk before enrolment. No patient received concomitant therapy with corticosteroids or rectally administered therapies of any kind. Use of anti-diarrheal medications was not permitted and no patient had taken additional non-steroidal anti-inflammatory drugs (NSAIDs) and/or antibiotics during the 3 mo preceding their enrolment.

Patients were excluded if they had evidence of infectious colitis, history of an active malignancy, previous surgical procedure except for cholecystectomy or appendicitis, and contraindication to flexible sigmoidoscopy or biopsy. Further exclusion criteria included present or recent pregnancy, and concomitant serious illness such as history of diabetes mellitus, hypertension, severe liver disease and cardiac failure.

Study design

Disease activity was assessed using Mayo Disease Activity Index (DAI)[19]. This index is calculated by summing the scores of four factors, each of which are graded on a scale from 0 to 3. The four features of disease activity are stool frequency, bleeding, physician's assessment of disease activity, and mucosal appearance. The maximum potential score is 12 points, with mild and moderate disease activity defined as a score below 10. Only patients with mild or moderate active UC based on DAI score were selected.

A primary clinical and colonoscopic evaluation was performed in all cases. Patients were then assessed clinically every 2 wk, and the following endoscopic evaluations were performed after 4th and 12th weeks. Therefore, it was not possible to calculate a DAI score at each time point. However, we computed a modified DAI (mDAI) score that includes all components of the full DAI score except the endoscopic appearance of the bowel. The maximum potential score in the mDAI is 9.

Meticulous laboratory evaluations were conducted for all participants at the time of recruitment, including determination of the hematocrit value, white blood cell and platelet count, liver function tests, ERS, stool exam and culture, and serum level of vitamin E. Serum samples were stored at -80℃ until analysis. Vitamin E was measured, after extraction with methanol, by HPLC, with UV detection at 294 nm. Methanol, deionized water and butanol (90:4:6) were used as mobile phase and the column was Eurospher 100 C_8 (4.6 mm × 25 cm). Re-evaluation of these tests was done each month until completion of study[20].

All patients were trained to rectally apply the sufficient amount of liquid vitamin E (0912, NOW Foods, IL, USA) equivalent to 8000 IU (15 mL) using specific instrument for enema (Enema irrigator disposable, Model: D-201, Taiwan Snatch Co., Taiwan; Figure 1) every night at home and were advised to lie for at least 15 min in left supine position after administration. During the first 12 wk all patients were given diary sheets containing multiple choice questions on stool characteristics including the number of defecation per day, stool consistency and type of blood excretion at each defecation episode. Patients were asked to complete each sheet on its day and bring them back at next visit (every 2 wk). After the first 12 wk we stopped obtaining diary sheets from patients and continued the follow-up by monthly visits only and endoscopic

Figure 1 Specific instrument for enema (Enema irrigator disposable). Fifteen mL of the liquid vitamin E was rectally administered in our patients using this disposable enema irrigator.

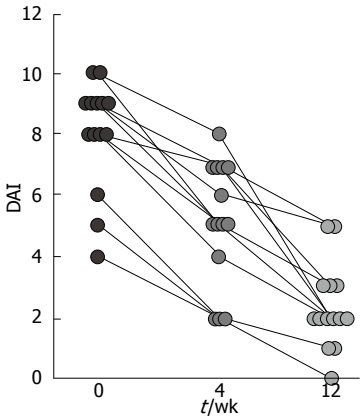


Figure 2 Individual changes in disease activity from week 0 to 12, assessed by the Mayo Disease Activity Index, in subjects who received d-α Tocopherol enema. One patient who was withdrawn early from the study is not included. There was a significant decrease in the mean DAI score for patients after 4th and 12th weeks of therapy ($P < 0.0001$).

studies every 3 mo. This second phase of study is still in progress.

Definition of outcomes

Patients with a final DAI score of $\leqslant 2$ points were considered to have achieved remission. A clinical response was defined as a reduction in the DAI of $\geqslant 2$ points. Clinical relapse was defined as the occurrence or worsening of symptoms, accompanied by an increase in the DAI score to 4, necessitating a change in therapy (addition of rectal therapies such corticosteroids or 5-ASA, surgery, *etc*). Refractory patients were those who had no significant improvement from their baseline, despite 12 wk of drug application.

Statistical analysis

Descriptive data are reported as percentages and medians and ranges. For statistical analysis, the 1-way analysis of variance (ANOVA) followed by Tukey post hoc was used. When appropriate, The Student *t* distribution was employed to compare 2 groups. Average DAI and mDAI scores at different time points were compared using the Mann-Whitney *U* test. All analyses used two sided tests of statistical significance with a significance level of 0.05.

RESULTS

Fifteen patients (5 males) were enrolled in the study

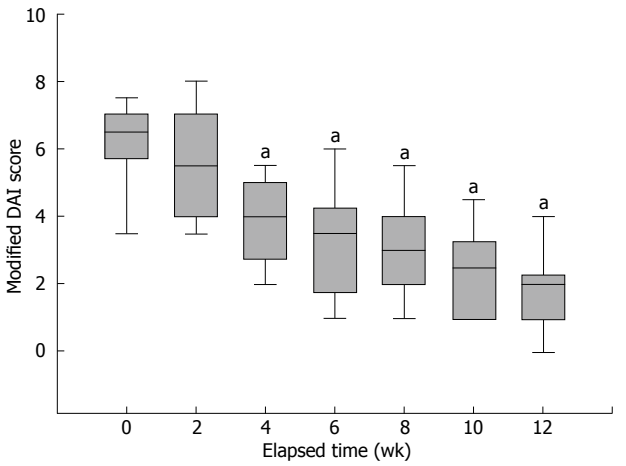


Figure 3 Change in the modified DAI. A modified DAI score, including all components of the complete DAI other than the endoscopic appearance is calculated at each observation point. By week 4 the mean modified DAI score significantly decreased compared to baseline (week 0) scores. [a]$P < 0.0001$ *vs* baseline.

and one patient left the study due to permanent change of his city of residence. Therefore the analysis was performed on 14 patients. The age of patients was 33 ± 9.6 years (mean ± SE) ranging from 21 to 55 years old, and the duration of UC was 4.7 ± 1.6 years. At enrolment, all patients had active disease with mild and moderate disease activity restricted to sigmoid (30 cm maximum involvement). All patients were receiving treatment at the time of entry into the study, consisting of sulphasalazine in 4 cases and mesalazine in the others. Two patients were also receiving azathioprine. No patient was on corticosteroids, antimicrobials or NSAIDS. At enrollment, the median Mayo DAI score was 8 (range, 4 to 10). Only 3 patients had mild disease activity, while the other 11 patients had moderate UC. On the 12th week, 9 patients had disease of mild severity and 5 had moderately active disease (Figure 2).

As summarized in Figure 2, clinical response was observed in 12 patients on the 4th week while clinical remission was induced only in 3 patients. After 12 wk, all patients responded clinically to therapy, with 9 of them going to clinical remission. In a secondary analysis, we examined the change in the DAI and mDAI scores over time (Figures 2 and 3). The mean DAI score before and on the 4th and 12th week of therapy were 8 ± 0.48, 5.1 ± 0.54 and 2.3 ± 0.37, respectively. Mann-Whitney *U* test revealed significant decrease in the mean DAI scores on 4th and 12th week of therapy comparing to the baseline ($P = 0.01$ and $P < 0.0001$, respectively) as shown in Figure 2. The average mDAI score started to decrease significantly on second week (Figure 3). As shown in Figure 4, mDAI score was significantly lower than at baseline for patients by week 4 and remained significantly lower for the remainder of the study ($P < 0.0001$ at weeks 4, 6, 8, 10 and 12, respectively). During the course of study there was no case of worsening disease activity or report of serious adverse event.

At the end of 12 wk, 12 patients elected to continue to receive d-α tocopherol, while 2 patients left the

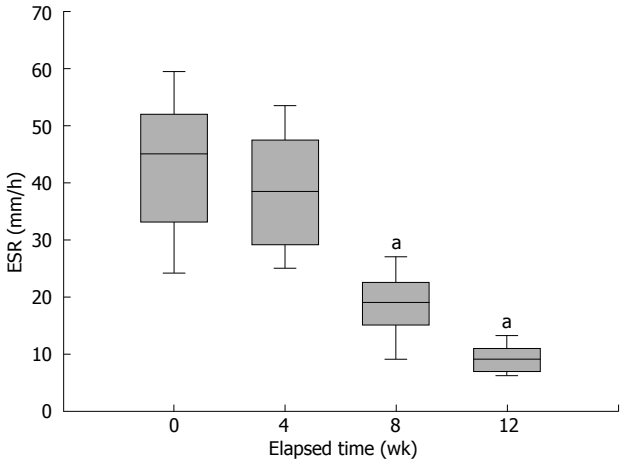


Figure 4 Individual changes in ESR from week 0 to 12, in our patients. There was a significant decrease in the mean ESR on the 8th and the 12th week of therapy compared to the beginning of the study. $^{a}P < 0.0001$ *vs* baseline (0 wk).

survey due to personal reasons despite achieving a desirable response and satisfaction with therapy. All patients are assessed monthly for symptoms of disease activity and undergo endoscopic studies every 3 mo. The overall follow-up of patients in our study is approaching its mean of 8 mo by the time of composing this article. Interestingly, there have been no recurrences in patients who remained on therapy, while 2 flare-ups occurred in patients who left the study, after 4 wk and 7 wk.

As shown in Figure 4 the mean value of baseline ESR was 42.26 ± 11.7 while it was 9.33 mm/h on 12th week, showing a significant decrease ($P < 0.0001$). Eleven patients had moderate anemia at baseline (defined as haematocrit < 35 in men and < 40 in women) for which supplemental iron and folic acids were prescribed. At the end of the 12 wk, a low haematocrit was detected in only 3 patients, and it increased from 35.3 ± 6 to 43.9 ± 6 after 12 wk. The serum level of α-tocopherol did not change significantly after 12 wk compared to that of baseline (7.2 ± 4.2 and 6.7 ± 1.1, respectively), and all patients had serum levels less than or within the normal range (5-12 μg/dL).

DISCUSSION

This case series provides evidence suggesting that local administration of d-α tocopherol might exert protective effects against UC in patients with mild and moderately active disease. In our study, rectal administration of d-α tocopherol significantly reduced the clinical disease activity indices and eliminated further need to corticosteroid therapy in otherwise non-responsive UC patients.

Recent studies demonstrated that plasma levels of α-tocopherol decrease after infection, trauma, burns, and inflammatory reactions, indicating that this antioxidant is exhausted during acute tissue injury[21,22]. Similar concept exists for inflammatory bowel disease, indicating that anti-oxidants are intimately involved in the process of active IBD, for example ingested high doses of vitamin E as an anti-oxidant along with other anti-oxidants reduce the extent of tissue damage in IBD, and tissue antioxidant levels are shown to be decreased in UC patients[23-25]. Systemic α-tocopherol in conjunction with selenium is also shown to reduce the severity of IBD in chemically induced colitis in rats[26]. Therefore, it is tempting to hypothesize that increasing the exposure of affected intestinal mucosa to this anti-oxidant might strengthen the defense capacity of the affected gut.

Vitamin E, particularly α-tocopherol, exerts a number of non-anti-oxidant functions, some associated with inhibition of protein kinase C (PKC) such as inhibition of platelet aggregation, and others independent of PKC such as the expression of intercellular adhesion molecule-1 (ICAM-1, CD54), integrins and CD36[27,28]. α-tocopherol also inhibits respiratory burst in human macrophages via a mechanism involving PKC inhibition, followed by attenuation of p47 (phox) phosphorylation and membrane translocation[29].

Increased NF-κB activity is found in inflamed intestinal mucosa; and factors implicated in IBD, such as TNF-α, LPS, and IL-1, are potent activators of NF-κB[30]. On the other hand, reactive oxygen species, the hypothetical mediators of IBD flare-ups, have been implicated in the stimulation of the signal transduction pathway involving NF-κB. Many current therapies for IBD act at least in part through the inhibition of NF-κB or through inhibition of signals that activate NF-κB. Interestingly, α-tocopherol is proved to inhibit NF-κB activation in rat Kupffer cells and a human monocytic cell line[31,32].

One of the proinflammatory genes regulated by NF-κB is ICAM-1 (cell surface glycoprotein, playing a critical role in mediating leukocyte-endothelial and a marker of active inflammation). Gulubova *et al*[33] demonstrated a marked upregulation of endothelial E-selectin, ICAM-1, and VCAM-1 expression in the inflamed colonic mucosa and submucosa in active UC. Infiltration of neutrophils into colonic mucosa is a central event in the acute phase of UC for which cell adhesion molecules are necessary[34]. Accordingly, by blocking the activity of NF-κB, which leads to decreased production of proinflammatory cytokines, anti-inflammatory properties of α-tocopherol might be explained. Therefore, α-tocopherol in high local doses might also affect the binding affinity between the neutrophil and the endothelial cell by decreasing the expression of adhesion molecules on the endothelial cells.

Systemic markers of inflammation, such as ESR, CRP, platelet count and white blood cell count are commonly used in clinical practice, but correlation to ongoing intestinal inflammation is poor[35]. Therefore, we monitored the consecutive changes in ESR (Figure 4) and complete blood count (CBC) of our patients. These results show a significant conversion of ESR and quantitative increase in haematocrit.

To the best of our knowledge, this is the first data on remission inducing properties of vitamin E. This study also confirms the feasibility and acceptability of rectal administration of vitamin E in patients with IBD. Except for one patient who left the study due to difficulty for

follow-up, all patients completed the three month course of study required for primary assessments. Furthermore, 12 patients are still taking medication and participate in the regular evaluations. By the time of composing this article, the average course of treatment is approaching 8 mo. Disease activity was rated as inactive (clinical remission) in 64%. Comparing these results with the beginning of study demonstrates a significant reduction in disease severity of UC patients ($P < 0.0001$) starting after the 4th week of therapy. As shown in Figure 3, symptoms started to improve after 3 wk. This result is comparable with conventional therapies for IBD such as mesalazine or sulfasalazine. Overall satisfaction of patients despite the uncomfortable method of nightly rectal administration of d-α tocopherol reflects that the desirable outcomes outweigh its difficulty in application.

Elevated and toxic levels of plasma vitamin E were a concern in our study, since there was no previous study measuring the systemic absorption of vitamin E when applied intrarectally. Thus, plasma levels of vitamin E were measured to eliminate concerns about vitamin E overdosage and results revealed that the mean vitamin E level was not significantly different from that of before the study. This shows that despite the high doses of rectal vitamin E administered every night, little is absorbed and no concern remains about the overdosage of vitamin E in patients.

In the setting of a case series study we only recruited UC patients with strictly defined criteria. Our hypothesis was the increased local effects of vitamin E in UC, in which the pathology is mainly restricted to mucosal layer. In addition to the substantial physical and financial burden of IBD on patients, it is often difficult to continue treatment due to decreased therapeutic effects or adverse reactions over time[7,8]. In this regard, use of vitamin E may expand the choices available for treatment of IBD, simplifying prescription or therapeutic technique.

The nature of our study casts limitations to implicate definite clinical results from this report, since case series cannot measure and do not eliminate the placebo effect. However, the best expected placebo effect is reported to be 16%-52% in rectal therapies of IBD which is still far lower than our results[36]. Best UC therapies only have 60% to 80% success rates, while we gained 100% clinical response with over 60% remission induction in our patients on the 12th week. This is especially important considering that these results are achieved without prescribing any synthetic agent, and we eliminated the need to corticosteroids in patients otherwise resistance to therapy.

Taken together, case series like this study are best used as a source of hypotheses for investigation by stronger study designs. Thus, future researchs should aim at testing the efficacy of natural vitamin E enema in a well controlled study to measure its exact effect on reducing risk of flare-ups with the minimum confounding factors. This evidence suggests that vitamin E reduced the development of colon inflammation. The observed effect seems to be due to antioxidative and anti-inflammatory effects of vitamin E which is potently taking effect by local administration. Based on our preliminary results vitamin E might show considerable promise as a new therapeutic modality for IBD.

ACKNOWLEDGMENTS

The personnel of Pars Hospital, Tehran, are thanked for the collaboration. Authors thank Dr. Taha Gholipour (Tehran University of Medical Sciences) for kindly accepting to review the manuscript. This work was supported by a research grant provided by the Tehran University of Medical Sciences.

COMMENTS

Background

The exact etiology of inflammatory bowel disease (IBD) is not yet understood, however it is believed that the generation of an exaggerated intestinal immune response to otherwise innocuous stimuli plays a key role in the pathophysiology of this intestinal disorder. IBD is mainly characterized by upregulation of synthesis and release of different pro-inflammatory mediators all of which actively contribute to the pathogenic cascade that initiates and perpetuates the inflammatory response of the gut. Thus, the current treatment strategy for IBD focuses on eliminating these causal inflammatory triggers and mediators.

Research frontiers

Vitamin E is a major lipophilic antioxidant in cellular membranes with excellent antioxidant activities which protects membrane lipids from peroxidation by scavenging not only chain carrying peroxyl radicals but also singlet oxygen and superoxide anion radicals. This is especially interesting in case of ulcerative colitis (UC), considering the pivotal role of oxygen free radicals in the genesis of mucosal damage. Given the recent evidence suggesting anti-inflammatory properties for Vitamin E, d-alpha (d-α) tocopherol, as the dominant vitamin E isomer in plasma with the highest biopotency, may be expected to reduce the development of tissue injury in UC.

Innovations and breakthroughs

This case series provides evidence for the first time that local administration of d-α tocopherol might exert protective effects against UC. The authors have carefully followed the serial alterations of patients' disease activity index, along with few other markers of disease severity and have shown that rectal administration of d-α tocopherol significantly reduces the clinical disease activity indices which eliminated further need to corticosteroid therapy in otherwise non-responsive UC patients with mild and moderately active disease. The observed effect seems to be due to antioxidant and anti-inflammatory effects of vitamin E which is potently taking effect by local administration.

Applications

The results of this interesting study suggests that natural antioxidants like d-α tocopherol might show considerable promise as new therapeutic modalities for IBD, with apparently lower side effects and complications compared to the current therapies.

Peer review

This is the first study to address the immunoregulatory effects of d-α tocopherol in a dominant Th1 response disease. The outstanding results demonstrate that natural isomer of vitamin E, reduces the extent of macroscopic mucosal damage and clinical severity of related syndromes in UC patients, when applied intra-rectally for as short as 4 wk.

REFERENCES

1 **Keefer L**, Keshavarzian A. Feasibility and acceptability of gut-directed hypnosis on inflammatory bowel disease: a brief communication. *Int J Clin Exp Hypn* 2007; **55**: 457-466

2 **Camuesco D**, Galvez J, Nieto A, Comalada M, Rodriguez-Cabezas ME, Concha A, Xaus J, Zarzuelo A. Dietary olive oil supplemented with fish oil, rich in EPA and DHA (n-3) polyunsaturated fatty acids, attenuates colonic inflammation in rats with DSS-induced colitis. *J Nutr* 2005; **135**: 687-694

3 **Kho YH**, Pool MO, Jansman FG, Harting JW. Pharmacotherapeutic options in inflammatory bowel disease: an update. *Pharm World Sci* 2001; **23**: 17-21
4 **Belluzzi A**. Polyunsaturated fatty acids (n-3 PUFAs) and inflammatory bowel disease (IBD): pathogenesis and treatment. *Eur Rev Med Pharmacol Sci* 2004; **8**: 225-229
5 **Kamm MA**. Review article: maintenance of remission in ulcerative colitis. *Aliment Pharmacol Ther* 2002; **16** Suppl 4: 21-24
6 **Xu CT**, Meng SY, Pan BR. Drug therapy for ulcerative colitis. *World J Gastroenterol* 2004; **10**: 2311-2317
7 **Stein RB**, Hanauer SB. Comparative tolerability of treatments for inflammatory bowel disease. *Drug Saf* 2000; **23**: 429-448
8 **Nakajima H**, Munakata A, Yoshida Y. Adverse effects of sulfasalazine and treatment of ulcerative colitis with mesalazine. *J Gastroenterol* 1995; **30** Suppl 8: 115-117
9 **Burton GW**, Traber MG. Vitamin E: antioxidant activity, biokinetics, and bioavailability. *Annu Rev Nutr* 1990; **10**: 357-382
10 **Hajiani M**, Golestani A, Shariftabrizi A, Rastegar R, Payabvash S, Salmasi AH, Dehpour AR, Pasalar P. Dose-dependent modulation of systemic lipid peroxidation and activity of anti-oxidant enzymes by vitamin E in the rat. *Redox Rep* 2008; **13**: 60-66
11 **Burton GW**, Joyce A, Ingold KU. First proof that vitamin E is major lipid-soluble, chain-breaking antioxidant in human blood plasma. *Lancet* 1982; **2**: 327
12 **Isozaki Y**, Yoshida N, Kuroda M, Takagi T, Handa O, Kokura S, Ichikawa H, Naito Y, Okanoue T, Yoshikawa T. Effect of a novel water-soluble vitamin E derivative as a cure for TNBS-induced colitis in rats. *Int J Mol Med* 2006; **17**: 497-502
13 **Fukuzawa K**, Gebicki JM. Oxidation of alpha-tocopherol in micelles and liposomes by the hydroxyl, perhydroxyl, and superoxide free radicals. *Arch Biochem Biophys* 1983; **226**: 242-251
14 **Lih-Brody L**, Powell SR, Collier KP, Reddy GM, Cerchia R, Kahn E, Weissman GS, Katz S, Floyd RA, McKinley MJ, Fisher SE, Mullin GE. Increased oxidative stress and decreased antioxidant defenses in mucosa of inflammatory bowel disease. *Dig Dis Sci* 1996; **41**: 2078-2086
15 **McKenzie SJ**, Baker MS, Buffinton GD, Doe WF. Evidence of oxidant-induced injury to epithelial cells during inflammatory bowel disease. *J Clin Invest* 1996; **98**: 136-141
16 **Yamada T**, Grisham MB. Role of neutrophil-derived oxidants in the pathogenesis of intestinal inflammation. *Klin Wochenschr* 1991; **69**: 988-994
17 **Fox ES**, Brower JS, Bellezzo JM, Leingang KA. N-acetylcysteine and alpha-tocopherol reverse the inflammatory response in activated rat Kupffer cells. *J Immunol* 1997; **158**: 5418-5423
18 **Yoshida N**, Yoshikawa T, Manabe H, Terasawa Y, Kondo M, Noguchi N, Niki E. Vitamin E protects against polymorphonuclear leukocyte-dependent adhesion to endothelial cells. *J Leukoc Biol* 1999; **65**: 757-763
19 **Naber AH**, de Jong DJ. Assessment of disease activity in inflammatory bowel disease; relevance for clinical trials. *Neth J Med* 2003; **61**: 105-110
20 **Combs GF Jr**. The Vitamins: Fundamental Aspects in Nutrition and Health. Second ed. San Diego: Academic Press, 1998: 618
21 **Suntres ZE**, Shek PN. Treatment of LPS-induced tissue injury: role of liposomal antioxidants. *Shock* 1996; **6** Suppl 1: S57-S64
22 **Demling R**, LaLonde C, Ikegami K, Picard L, Nayak U. Alpha-tocopherol attenuates lung edema and lipid peroxidation caused by acute zymosan-induced peritonitis. *Surgery* 1995; **117**: 226-231
23 **D'Odorico A**, Bortolan S, Cardin R, D'Inca' R, Martines D, Ferronato A, Sturniolo GC. Reduced plasma antioxidant concentrations and increased oxidative DNA damage in inflammatory bowel disease. *Scand J Gastroenterol* 2001; **36**: 1289-1294
24 **Koch TR**, Yuan LX, Stryker SJ, Ratliff P, Telford GL, Opara EC. Total antioxidant capacity of colon in patients with chronic ulcerative colitis. *Dig Dis Sci* 2000; **45**: 1814-1819
25 **Buffinton GD**, Doe WF. Depleted mucosal antioxidant defences in inflammatory bowel disease. *Free Radic Biol Med* 1995; **19**: 911-918
26 **Ademoglu E**, Erbil Y, Tam B, Barbaros U, Ilhan E, Olgac V, Mutlu-Turkoglu U. Do vitamin E and selenium have beneficial effects on trinitrobenzenesulfonic acid-induced experimental colitis. *Dig Dis Sci* 2004; **49**: 102-108
27 **Azzi A**, Ricciarelli R, Zingg JM. Non-antioxidant molecular functions of alpha-tocopherol (vitamin E). *FEBS Lett* 2002; **519**: 8-10
28 **Breyer I**, Azzi A. Differential inhibition by alpha- and beta-tocopherol of human erythroleukemia cell adhesion: role of integrins. *Free Radic Biol Med* 2001; **30**: 1381-1389
29 **Cachia O**, Benna JE, Pedruzzi E, Descomps B, Gougerot-Pocidalo MA, Leger CL. alpha-tocopherol inhibits the respiratory burst in human monocytes. Attenuation of p47(phox) membrane translocation and phosphorylation. *J Biol Chem* 1998; **273**: 32801-32805
30 **Ekstrand-Hammarstrom B**, Osterlund C, Lilliehook B, Bucht A. Vitamin E down-modulates mitogen-activated protein kinases, nuclear factor-kappaB and inflammatory responses in lung epithelial cells. *Clin Exp Immunol* 2007; **147**: 359-369
31 **Blackwell TS**, Blackwell TR, Holden EP, Christman BW, Christman JW. In vivo antioxidant treatment suppresses nuclear factor-kappa B activation and neutrophilic lung inflammation. *J Immunol* 1996; **157**: 1630-1637
32 **Naraghi M**, Deroee AF, Ebrahimkhani M, Kiani S, Dehpour A. Nitric oxide: a new concept in chronic sinusitis pathogenesis. *Am J Otolaryngol* 2007; **28**: 334-337
33 **Gulubova MV**, Manolova IM, Vlaykova TI, Prodanova M, Jovchev JP. Adhesion molecules in chronic ulcerative colitis. *Int J Colorectal Dis* 2007; **22**: 581-589
34 **Strober W**, Kelsall B, Fuss I, Marth T, Ludviksson B, Ehrhardt R, Neurath M. Reciprocal IFN-gamma and TGF-beta responses regulate the occurrence of mucosal inflammation. *Immunol Today* 1997; **18**: 61-64
35 **Cellier C**, Sahmoud T, Froguel E, Adenis A, Belaiche J, Bretagne JF, Florent C, Bouvry M, Mary JY, Modigliani R. Correlations between clinical activity, endoscopic severity, and biological parameters in colonic or ileocolonic Crohn's disease. A prospective multicentre study of 121 cases. The Groupe d'Etudes Therapeutiques des Affections Inflammatoires Digestives. *Gut* 1994; **35**: 231-235
36 **Meyers S**, Janowitz HD. The "natural history" of ulcerative colitis: an analysis of the placebo response. *J Clin Gastroenterol* 1989; **11**: 33-37

S- Editor Zhong XY **L- Editor** Negro F **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.5996

World J Gastroenterol 2008 October 21; 14(39): 5996-6003
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Dietary glycine blunts liver injury after bile duct ligation in rats

Matthias Froh, Zhi Zhong, Peter Walbrun, Mark Lehnert, Susanne Netter, Reiner Wiest, Lars Conzelmann, Erwin Gäbele, Claus Hellerbrand, Jürgen Schölmerich, Ronald G Thurman

Matthias Froh, Peter Walbrun, Susanne Netter, Reiner Wiest, Erwin Gäbele, Claus Hellerbrand, Jürgen Schölmerich, Department of Internal Medicine I, University of Regensburg, Regensburg 93042, Germany
Matthias Froh, Zhi Zhong, Mark Lehnert, Lars Conzelmann, Erwin Gäbele, Ronald G Thurman, Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States
Zhi Zhong, Department of Pharmaceutical Sciences, University of South Carolina, Charleston, South Carolina 29425, United States
Mark Lehnert, Department of Surgery, University of Frankfurt, Frankfurt am Main 60325, Germany
Lars Conzelmann, Department of Cardiothoracic and Vascular Surgery, University of Mainz, Mainz 55099, Germany
Author contributions: Froh M, Zhong Z, Schölmerich J and Thurman RG designed research; Froh M, Zhong Z, Walbrun P, Lehnert M and Netter S performed research; Froh M, Zhong Z, Conzelmann L and Gäbele E analyzed data; and Froh M, Hellerbrand C and Schölmerich J wrote the paper.
Supported by Grants from the National Institute of Alcohol Abuse and Alcoholism (NIAAA) and by a grant from the Deutsche Forschungsgemeinschaft, No. FR 1644/4-1
Correspondence to: Matthias Froh, Department of Internal Medicine I, University of Regensburg, Regensburg 93042, Germany. froh.science@mac.com
Telephone: +49-941-9447012 Fax: +49-941-9447011
Received: January 5, 2008 Revised: July 13, 2008
Accepted: July 20, 2008
Published online: October 21, 2008

Abstract

AIM: To investigate the effects of (dietary) glycine against oxidant-induced injury caused by bile duct ligation (BDL).

METHODS: Either a diet containing 5% glycine or a standard diet was fed to male Sprague-Dawley (SD) rats. Three days later, BDL or sham-operation was performed. Rats were sacrificed 1 to 3 d after BDL. The influence of deoxycholic acid (DCA) in the presence or absence of glycine on liver cells was determined by measurement of calcium and chloride influx in cultivated Kupffer cells and lactate dehydrogenase (LDH) activity was determined in the supernatant of cultivated hepatocytes.

RESULTS: Serum alanine transaminase levels increased to about 600 U/L 1 d after BDL. However, enzyme release was blunted by about two third in rats receiving glycine. Release of the alkaline phosphatase and aspartate aminotransferase was also blocked significantly in the group fed glycine. Focal necrosis was observed 2 d after BDL. Glycine partially blocked the histopathological changes. Incubation of Kupffer cells with DCA led to increased intracellular calcium that could be blocked by incubation with glycine. However, systemic blockage of Kupffer cells with gadolinium chloride had no effects on transaminase release. Incubation of isolated hepatocytes with DCA led to a significant release of LDH after 4 h. This release was largely blocked when incubation with glycine was performed.

CONCLUSION: These data indicate that glycine significantly decreased liver injury, most likely by a direct effect on hepatocytes. Kupffer cells do not appear to play an important role in the pathological changes caused by cholestasis.

Key words: Glycine; Bile duct ligation; Cholestasis; Kupffer cells; Serum alanine transaminase; Deoxycholic acid

Peer reviewer: Tom H Karlsen, MD, Institute of Immunology, Rikshospitalet University Hospital, Oslo N-0027, Norway

Froh M, Zhong Z, Walbrun P, Lehnert M, Netter S, Wiest R, Conzelmann L, Gäbele E, Hellerbrand C, Schölmerich J, Thurman RG. Dietary glycine blunts liver injury after bile duct ligation in rats. *World J Gastroenterol* 2008; 14(39): 5996-6003 Available from: URL: http://www.wjgnet.com/1007-9327/14/5996.asp DOI: http://dx.doi.org/10.3748/wjg.14.5996

INTRODUCTION

Chronic cholestasis liver diseases lead to liver injury and ultimately progress to portal fibrosis, cirrhosis, and end-stage liver disease requiring liver transplantation. They include primary sclerosing cholangitis, primary biliary cirrhosis, extrahepatic biliary atresia, idiopathic adulthood ductopenia, idiopathic neonatal hepatitis, Byler's disease, and arteriohepatic dysplasia[1-4]. Various drugs, total parenteral nutrition, sarcoidosis, chronic liver transplant rejection, and graft-versus-host disease may also cause chronic cholestasis[5-7]. Currently the most promising therapy for chronic cholestatic liver diseases is ursodeoxycholic acid[8], that may delay liver disease progression, but cannot prevent liver injury or fibrosis[9].

The pathophysiology of cholestasis induced liver injury and fibrosis remains unclear. One possible mechanism is that hepatic accumulation of hydrophobic bile acids causes oxidative stress in the liver[10]. Previous studies showed that hepatic mitochondria generate reactive oxygen species when isolated hepatocytes are exposed to hydrophobic bile acids[10,11]. This mitochondrial free radical production may be an important mechanism of cholestatic liver injury. However, the major source of free radicals remains unclear. One possible cell type responsible for the generation of free radicals could be the Kupffer cells, the resident macrophages of the liver. They are involved in disease states, such as endotoxin shock[12], alcoholic liver diseases[13], and other toxicant-induced liver injury by releasing eicosanoids, inflammatory cytokines (IL-1, IL-6, TNF-α), and free radical species[14].

Glycine, a simple nonessential amino acid, is a well-known inhibitory neurotransmitter in the central nervous system that acts *via* a glycine-gated chloride channel and has been shown to be protective against hypoxia, ischemia, and various cytotoxic substances[15-17]. Furthermore, it was demonstrated that dietary glycine protected both, the lung and the liver against lethal doses of endotoxin in the rat[18] and improved graft survival after liver transplantation[19].

Based on pharmacological data[15-17], a glycine-gated chloride channel was detected in Kupffer cells and other macrophages[20] that influenced the activation process of these cells. Glycine binds to and opens a chloride channel at the cell membrane, causing cell hyperpolarization that subsequently blocks calcium influx[20]. Thus it prevents the activation of intracellular signaling cascades.

Accordingly, we hypothesized in this study that dietary glycine has a protective effect in liver injury after bile duct ligation (BDL) by preventing activation of Kupffer cells.

MATERIALS AND METHODS

Animal husbandry and diet treatment

Adult male Sprague-Dawley (SD) rats (200-250 g) were housed four to a cage in a facility approved by the Association for the Accreditation and Assessment of Laboratory Animal Care International. Three days before surgery, rats were randomly assigned to two experimental groups and fed either a semisynthetic powdered diet (Teklad test diets, Madison, WI, USA) containing 5% glycine and 15% casein (glycine group) or 20% casein (control group). After surgery, each rat continued to receive its assigned diet throughout the entire experimental period. All animals received humane care in compliance with guidelines approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

BDL

Rats underwent BDL and transection or sham operation under ether anesthesia, as described elsewhere[21]. Briefly, the common bile duct was located through a midline abdominal incision, double ligated near the liver, and transected between ligatures. Control rats received sham operation underwent the same procedure except that the bile duct was only gently manipulated, but not ligated or sectioned. Some rats were given gadolinium chloride ($GdCl_3$; 20 mg/kg body weight iv 24 h before BDL) to selectively deplete Kupffer cells. Rats were sacrificed 1 to 3 d after BDL or sham operation for further investigations (n = 5-6 per group).

Clinical chemistry and histology

Blood samples were collected from the tail veins at times indicated. Serum alkaline phosphatase (ALP), alanine aminotransaminase (ALT), aspartate aminotransaminase (AST), and bilirubin were measured using analytic kits from Sigma (St. Louis, MO, USA). On the day of death, each rat was anesthetized with pentobarbital sodium (75 mg/kg ip), the abdomen was opened, and the portal vein was cannulated with a 20-gauge cannula. The liver was rinsed using a syringe containing 10 mL physiological saline, followed by slow infusion of 5 mL 10% buffered formaldehyde (VWR International, West Chester, PA, USA). After 48 h in fixative, paraffin sections were prepared and stained with hematoxylin-eosin. Liver pathology was scored in a blinded manner based on a scoring system described by Nanji *et al*[22] (inflammation and necrosis: 1 focus per low-power field: 1+; 2 or more foci: 2+).

Preparation and culture of Kupffer cells and hepatocytes

Kupffer cells were isolated by collagenase digestion and differential centrifugation using Percoll (Sigma, Taufkirchen, Germany) as described elsewhere[23] with slight modifications[24]. Briefly, the liver was perfused through the portal vein with Ca^{2+}- and Mg^{2+}-free HBSS at 37℃ for 10 min at a flow rate of 20 mL/min. Subsequently, perfusion was with HBSS containing 0.02% collagenase Ⅳ (Sigma) at 37℃ for 10 min. After the liver was digested, it was excised and cut into small pieces in collagenase buffer. The suspension was filtered through nylon gauze and the filtrate was centrifuged two times at 70 *g* for 3 min at 4℃ to remove parenchymal cells. The nonparenchymal cell fraction (mostly Kupffer cells) in the supernatant was washed with buffer and centrifuged at 650 *g* for 7 min at 4℃. Cell pellets were suspended in buffer and centrifuged on a density cushion of Percoll (25% and 50%) at 1800 *g* for 15 min at 4℃. The Kupffer cell fraction was collected, centrifuged at 650 *g* for 7 min and suspended again in buffer. Viability of cells was determined by Trypan blue exclusion. Purity (> 90%) of Kupffer cell cultures was evaluated by morphological observation and by phagocytic uptake of FITC-labeled 1 μm latex-beads. Kupffer cells were cultured in RPMI-1640 medium (Sigma) supplemented with 10% FCS and antibiotics/antimycotics (100 U/mL of penicillin G, 100 μg/mL of streptomycin sulfate, and 0.25 μg/mL amphotericin B; Sigma) at 37℃ in a 10% CO_2-containing atmosphere. Nonadherent cells were removed after 30 min by replacing the culture medium.

The parenchymal cell fraction (mostly hepatocytes) was also isolated by Percoll (50%) centrifugation as described previously[25] and cultured at 1×10^6 cells/well in RPMI 1640 medium (Sigma) containing 10% heat-inactivated fetal bovine serum and antibiotics as described above. Cells were cultured for 24 h before used for further experiments.

Measurement of intracellular Ca^{2+} concentration in Kupffer cells

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured fluorometrically using the fluorescent calcium indicator dye fura-2. KC (1×10^6 cells/plate) were incubated in modified HBSS (mHBSS; in mmol/L): 110 NaCl, 5 KCl, 0.3 Na_2HPO_4, 0.4 KH_2PO_4, 5.6 glucose, 0.8 $MgSO_4 \cdot 7H_2O$, 4 $NaHCO_3$, 1.26 $CaCl_2$, 15 HEPES, pH 7.4 containing 5 μmol/L fura-2 AM (Molecular Probes, Eugene, OR, USA) at room temperature for 45 min. Coverslips plated with Kupffer cells were rinsed and placed in chambers with mHBSS at room temperature. Changes in fluorescence intensity of fura-2 at excitation wavelengths of 340 and 380 nm and emission at 510 nm were monitored in individual Kupffer cells. A Nikon inverted fluorescent microscope interfaced with dual-wavelength fluorescent photometer (Intracellular Imaging, Cincinnati, OH, USA) was used to ratiometrically determine $[Ca^{2+}]_i$. Data were collected and analyzed using InCyt software (Intracellular Imaging).

Determination of lactate dehydrogenase (LDH) release in hepatocytes

Twenty-four hours after isolation, hepatocytes were stimulated with deoxycholic acid (DCA; 0.1 mmol/L; Sigma) or normal saline in the presence or absence of glycine (1 mmol/L; Sigma). After 4 h of culture, supernatant was collected and LDH assays were performed *via* standard enzymatic techniques as described elsewhere[26].

Measurement of radiolabelled chloride influx by Kupffer cells

Assays for uptake of ^{36}Cl used an adaptation of a method described for neurons by Schwartz *et al*[27] and modified by Morrow and Paul[28]. Briefly, 2×10^6 Kupffer cells were plated on coverslips in 60 mm^2 culture dishes and incubated as described above. After 24 h, media was replaced with HEPES buffer (20 mmol/L HEPES, 118 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L $MgSO_4$, and 2.5 mmol/L $CaCl_2$, pH 7.4) and allowed to equilibrate for 10 min at room temperature. Coverslips were gently blotted dry and incubated in a petri dish with 2 mL of buffer containing 2 mCi/mL ^{36}Cl in the presence of glycine (1 mmol/L) and/or DCA (0.01 and 0.1 mmol/L) for 5 s. Chloride influx was linear between 2-10 s; thus, a 5 s incubation time was chosen for all experiments. Chloride influx was terminated by washing the coverslip with ice-cold buffer for 3 s followed by a second wash for 7 s[28]. Coverslips were placed in scintillation vials, and protein was solubilized by adding 1.6 mL NaOH (0.2 mol/L) for 2 h. An aliquot (0.16 mL) was collected for determination of protein by the method of Lowry *et al*[29]. Ecolume (10 mL) was added and radioactivity was determined by standard scintillation spectroscopy.

Statistical analysis

Data are presented as mean ± SD. ANOVA and the Student-Newman-Keuls post hoc tests were used for the determination of statistical significance between treatment groups, and $P < 0.05$ was selected before the study as the level of significance.

Boxplots illustrate median values and interquartile distance. The error bars represent the 5th and 95th percentiles.

RESULTS

Effects of dietary glycine on serum enzymes and histology after BDL

In untreated rats fed a standard chow diet, serum alanine transaminase (ALT) levels average 40 U/L and were not significantly altered by sham operation (data not shown). One day after BDL, ALT increased to 541 U/L (Figure 1A), and remained elevated at day 2 and 3 after BDL with 599 U/L and 543 U/L, respectively. When rats were treated with dietary glycine, ALT levels increased to 248 U/L one day after BDL (Figure 1A). On day 2 and 3, ALT levels were also significantly decreased compared to bile duct-ligated rats fed a control diet (232 U/L on day 2 and 161 U/L on day 3). Serum aspartate aminotransferase (AST) and alkaline phosphatase (AP) levels, which were also measured at day 1, 2, and 3 after BDL, revealed similar results as ALT (Figure 1B and C).

Normal liver architecture was observed in rats on a control and a glycine diet after sham operation (Figure 2A). Two days after BDL focal necrosis and white blood cell infiltration were detected in livers of rats receiving a standard diet (Figure 2B). These pathological changes were partially blocked in rats receiving dietary glycine (Figure 2C).

Influence of DCA on calcium and chloride influx in cultivated Kupffer cells in the presence or absence of glycine

Intracellular calcium concentration ($[Ca^{2+}]_i$) in cultured Kupffer cells was determined fluorometrically with the calcium indicator fura-2 as described in MATERIALS AND METHODS. After the addition of 0.01 or 0.1 nmol/L DCA, $[Ca^{2+}]_i$ levels increased as expected over the investigated time period of 10 min (Figure 3). Glycine (1 mmol/L) added 3 min before DCA inhibited this increase/induction in $[Ca^{2+}]_i$. Glycine alone had no detectable effect on $[Ca^{2+}]_i$ (data not shown).

The glycine-gated chloride channel mediates the influx of chloride and hyperpolarizes the cells[30] thereby preventing DCA-induced increases of $[Ca^{2+}]_i$ (Figure 3). Indeed, glycine (1 mmol/L) caused a significant, about 4-fold influx of radiolabeled chloride in the presence of DCA (0.01 or 0.1 nmol/L) (Figure 4).

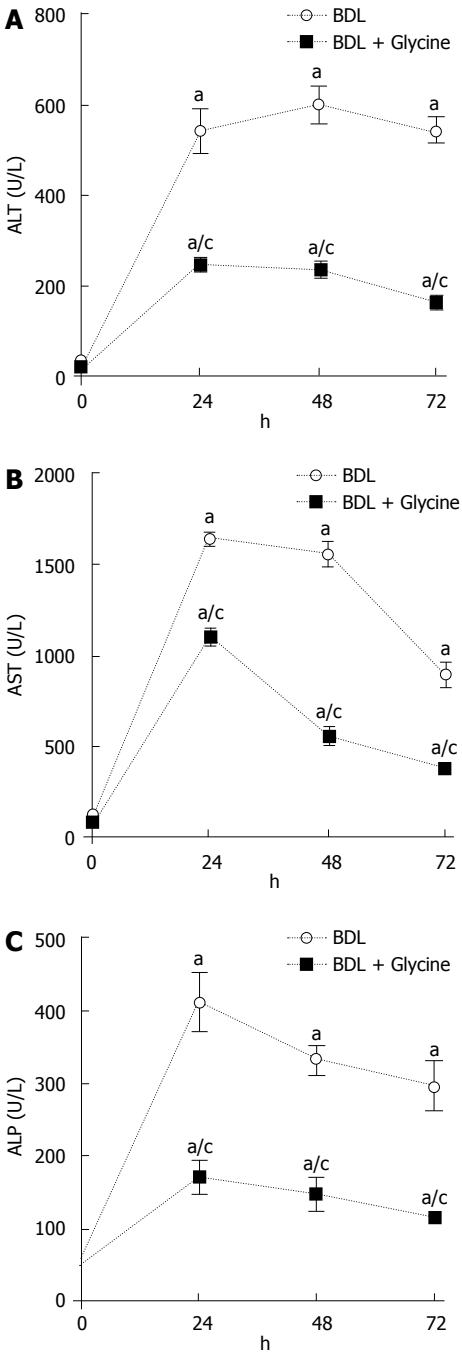


Figure 1 Dietary glycine blunts increased liver enzyme release after BDL. Blood was collected at the time points indicated. Serum alanine aminotransferase (**A**), aspartate aminotransferase (**B**), and alkaline phosphatase (**C**) activity was determined using commercial kits. Values are mean ± SD (n = 5-6 in each group). $^{a}P < 0.05$ *vs* sham operation; $^{c}P < 0.05$ *vs* bile duct-ligated rats fed control diet.

This effect of glycine was significantly reduced by the classical glycine-gated chloride channel antagonist strychnine (data not shown).

Effects of Kupffer cell elimination on serum enzymes after BDL

To investigate whether Kupffer cells play an important role in cholestatic liver injury, rats were treated with $GdCl_3$ that selectively depletes Kupffer cells, before BDL. Suppression of Kupffer cells with $GdCl_3$ neither

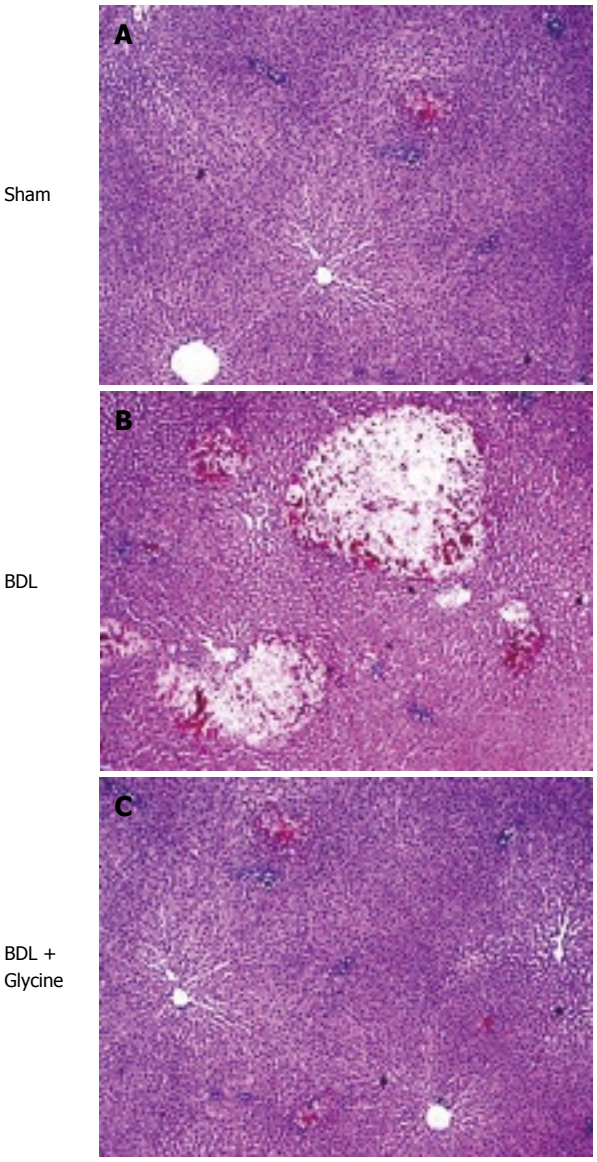


Figure 2 BDL induced focal necrosis that was blunted by dietary glycine. Shown are representative images (× 75). **A**: Sham operation with control diet; **B**: BDL with control diet; **C**: BDL with dietary glycine.

blunted ALT release (Figure 5) nor attenuated focal necrosis after BDL (data not shown), confirming our previously published data[31]. In detail, ALT levels increased up to 779 U/L (± 53) 24 h after BDL. Pretreatment with $GdCl_3$ one day before BDL had no effect on this transaminase release after BDL (870 ± 78 U/L).

Influence of DCA on LDH release in isolated hepatocytes in the presence or absence of glycine

Incubation of isolated hepatocytes with DCA (0.1 mmol/L) led to a specific release of LDH (Figure 6) over the investigated time period (1 h incubation time: 101 ± 8 U/L; 2 h incubation time: 112 ± 7 U/L; 3 h incubation time: 119 ± 8 U/L; 4 h incubation time: 149 ± 18 U/L). This release was significantly blocked when glycine was simultaneously added (1 h incubation time: 42 ± 7 U/L; 2 h incubation time: 77 ± 7 U/L; 3 h incubation time: 92 ± 8 U/L; 4 h incubation time:

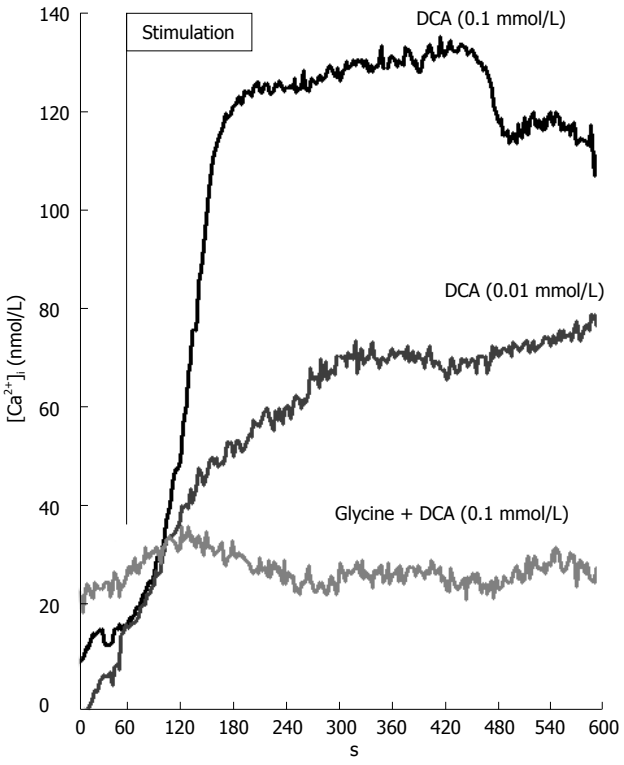


Figure 3 Glycine prevents DCA-induced rise of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in Kupffer cells. $[Ca^{2+}]_i$ was measured fluorometrically. Data are representative of 6 independent experiments.

79 ± 6 U/L). Incubation with glycine or saline alone had almost no effect on the release of LDH (Figure 6).

DISCUSSION

Chronic cholestatic liver diseases are one of the leading indications for liver transplantation in children and adults[1,2]. Therefore, new strategies to reduce the pathological changes caused by (chronic) cholestasis are needed, because current therapies, such as ursodeoxycholic acid[9], do not prevent liver injury. Oxidative stress and activation of Kupffer cells are probably involved in the pathogenesis of liver injury caused by cholestasis. Glycine has been shown to be an anti-inflammatory amino acid acting *via* inhibitory effects on several white blood cells, including Kupffer cells[15-19]. Glycine activates a chloride channel, leading to cell hyperpolarization and a concomitant blocking of calcium influx *via* a voltage dependent calcium channel[20]. Accordingly, we hypothesized that the activation of Kupffer cells and the associated free radical formation after BDL could be blocked by glycine, thus leading to a decreased liver injury.

Dietary glycine blunts liver injury due to BDL

In confirmation of previous work from our and several other laboratories[21,31-35], BDL caused hepatic enzyme release (Figure 1) and focal cell necrosis (Figure 2), as expected. However, hepatic enzyme release (ALT, AST, AP) was significantly blunted and histopathological changes were partially blocked in the group receiving

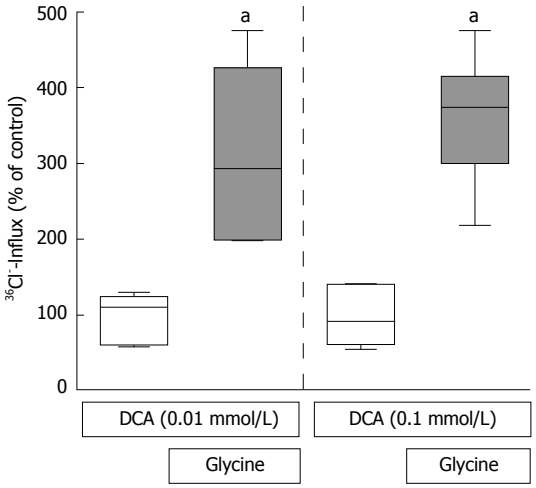


Figure 4 Glycine stimulates influx of radiolabeled chloride in Kupffer cells. Data are expressed as % of control. Values are mean ± SD and are representative of at least 6 individual experiments. $^aP < 0.05$ *vs* DCA (= control) group.

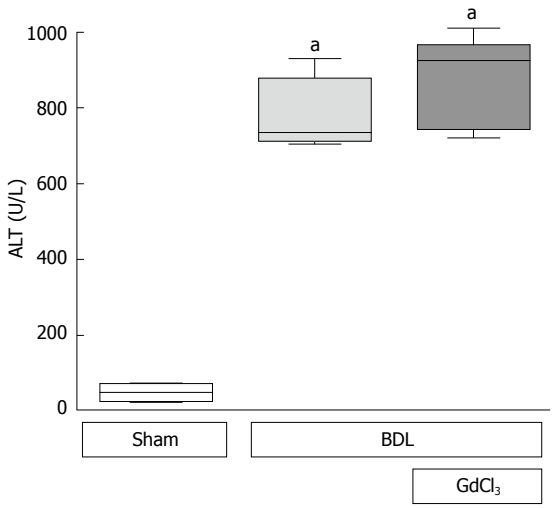


Figure 5 Elimination of Kupffer cells does not prevent elevated ALT release after BDL. Rats were given gadolinium chloride ($GdCl_2$; 20 mg/kg body weight) or saline 24 h before BDL or sham opration was performed. Values are mean ± SD (n = 5-6 in each group). $^aP < 0.05$ *vs* sham-operated group.

dietary glycine. Incubation of Kupffer cells with DCA led to increased intracellular calcium that was inhibited by incubation with glycine (Figure 3), most likely thru a glycine stimulated influx of chloride (Figure 4). However, systemic blockage of Kupffer cells with gadolinium chloride had no effect on transaminase release (Figure 5), indicating a minor, if any role of Kupffer cells in the pathophysiology of experimental cholestasis. Incubation of hepatocytes with DCA *in vitro* led to a significant release of LDH that was reduced by glycine (Figure 6).

How does dietary glycine decrease cholestasis-induced liver injury?

The protective effects of glycine are probably due to its direct effect on target cells or mediated by inhibition of inflammatory cell activation. Glycine appears to exert several protective effects, including anti-inflammatory, immunomodulatory and direct cytoprotective actions.

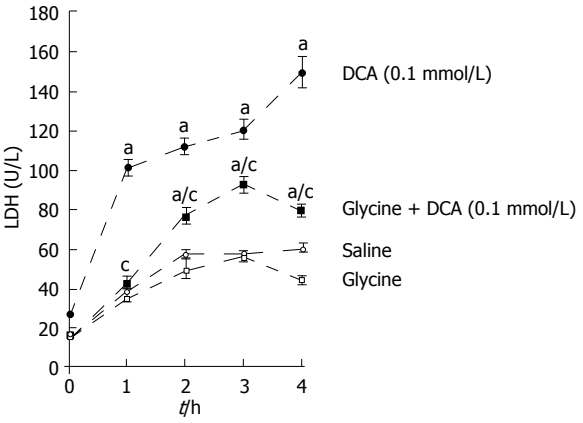


Figure 6 Glycine reduces DCA-induced release of LDH in isolated hepatocytes. Cells were isolated and stimulated with DCA (0.1 mmol/L) or saline in the presence or absence of glycine (1 mmol/L). Values are mean ± SD (n = 5-6 in each group). $^{a}P < 0.05$ *vs* saline or glycine group; $^{c}P < 0.05$ *vs* DCA treated hepatocytes.

The underlying mechanisms are not completely understood. Glycine decreases oxidative stress[15-19] by different and partly indirect mechanisms that prevent reactive oxygen species formation. Furthermore, glycine protects renal tubular cells, hepatocytes and endothelial cells against injury from hypoxia, ischemia-reperfusion and ATP depletion[16,36-39]. Most studies show that glycine protects plasma membrane integrity, but does not restore ATP levels or affect intracellular pH[16,38-40].

Activation of the glycine-gated chloride channel is another widely postulated mechanism for the effects of glycine. The glycine receptor exists in a wide variety of cells, beside its typical occurrence at the postsynaptic neuronal membranes of the spinal cord. Besides endothelial cells and renal proximal tubular cells[17,41], cells involved in inflammatory and immune responses, such as macrophages, monocytes, neutrophils, and T lymphocytes, express a glycine receptor[18,20,42-44]. Glycine acts thru its receptor on inflammatory cells, such as Kupffer cells, to suppress activation of transcription factors and the formation of free radicals and inflammatory cytokines. In the plasma membrane, glycine appears to activate a chloride channel[20,30] that stabilizes or hyperpolarizes the plasma membrane potential (Figure 4). As a consequence, agonist-induced opening of L-type voltage dependent calcium channels and the resulting increases in intracellular calcium ions are suppressed (Figure 3), which may account for the immunomodulatory and anti-inflammatory effects of glycine. By preventing Kupffer cell activation, a decreased formation of inflammatory and fibrogenic mediators may be achieved. However, the role of Kupffer cells in fibrosis is controversial. Destruction of Kupffer cells attenuated liver fibrosis caused by carbon tetrachloride[45]. By contrast, in a rat model of reversible biliary obstruction, inactivation of Kupffer cells impaired collagen metabolism and inhibited the resolution of fibrosis[46]. Kupffer cells release many mediators, like TNF-α, TGF-β, human growth factor, PDGF, and reactive oxygen species[47,48] that activate stellate cells leading to fibrosis. TNF-α production and NF-κB activation increase during cholestasis[49,50]. Activation of NF-κB, probably due to oxidative stress, could lead to expression of TNF-α. However, suppression of Kupffer cell function with $GdCl_3$, a treatment that blocks carbon tetrachloride-induced fibrosis, did not attenuate injury caused by cholestasis (Figure 5) confirming previous work from our laboratory[31]. This finding indicates that Kupffer cells most likely do not play a prominent role in cholestasis-induced fibrosis *in vivo* and that glycine does not work exclusively by inhibiting Kupffer cell activation.

Recent work suggested that liver parenchymal cells at least contain a glycine dependent receptor. In isolated hepatocytes, glycine blocks the increase in intracellular calcium due to PGE2 and phenylephrine, an a1-type adrenergic receptor agonist[51]. Low-dose strychnine partially reverses the inhibition by glycine. When extracellular chloride is omitted, glycine is much less effective in preventing increases in intracellular calcium due to PGE2. These data suggested that hepatoprotection by glycine is, in part, due to its direct effect on hepatocytes *via* regulating of intracellular calcium[51]. Consistent with these earlier findings, a direct effect of glycine on LDH release in isolated hepatocytes was observed in the present study after DCA challenge (Figure 6). Nevertheless, the effect of the conjugation of glycine and the used "secondary bile acid" DCA on the ability to lyse cells directly and solubilize cellular and membrane components should be also considered.

In conclusion, we demonstrated that hepatic injury, due to BDL, is significantly reduced by dietary glycine. Moreover, the data indicate that glycine decreases liver injury under the conditions of experimental cholestasis thru a direct effect on hepatocytes. Surprisingly, Kupffer cells do not appear to play a major role in the pathological changes caused by cholestasis.

COMMENTS

Background

Chronic cholestasis leads to liver injury and will ultimately progress to portal fibrosis, cirrhosis and end-stage liver disease requiring liver transplantation. Oxidative stress and activation of Kupffer cells are probably involved in liver injury caused by cholestasis. The nonessential amino acid glycine has been shown to be anti-inflammatory in several injury models, acting *via* inhibitory effects on several white blood cells, including Kupffer cells. Additionally, it activates a chloride channel, leading to cell hyperpolarization and a concomitant blocking of calcium influx into the cell *via* a voltage dependent calcium channel.

Research frontiers

Ursodeoxycholic acid is currently the most promising therapy for chronic cholestatic liver diseases; however, it cannot prevent fibrosis. How cholestasis induces liver injury and fibrosis remains unclear. One possible mechanism is that accumulation of hydrophobic bile acids causes oxidative stress in the liver, leading to tissue injury, fibrosis and finally liver cirrhosis. One possible cell type responsible for the generation of free radicals could be the Kupffer cells, the resident macrophages of the liver. It is known that destruction of Kupffer cells by gadolinium chloride or transduction of Kupffer cells by recombinant adenovirus can protect the liver against injury. However, the role of Kupffer cells in fibrosis is controversial. Destruction of Kupffer cells attenuated liver fibrosis caused by carbon tetrachloride. By contrast, in a rat model of reversible biliary obstruction, inactivation of Kupffer cells impaired collagen metabolism and inhibited the resolution of fibrosis.

Innovations and breakthroughs

Recent studies demonstrated that dietary glycine protected both the lung and

liver against lethal doses of endotoxin in the rat and improved graft survival after liver transplantation. Based on pharmacological data a glycine-gated chloride channel could be detected in Kupffer cells and other macrophages that influence the activation process of these cells by preventing the activation of intracellular signaling cascades.

Applications

The aim of this study was to investigate the effects of (dietary) glycine against oxidant-induced injury caused by bile duct ligation (BDL). The findings suggested that glycine significantly decreased liver injury, most likely by a direct effect on hepatocytes. Kupffer cells do not appear to play an important role in the pathological changes caused by cholestasis.

Terminology

Glycine, a simple nonessential amino acid, is a well-known inhibitory neurotransmitter in the central nervous system that acts *via* a glycine-gated chloride channel and has been shown to be protective against hypoxia, ischemia, and various cytotoxic substances. Kupffer cells, which are derived from monocyte/macrophage cell lineage, are the resident macrophages of the liver. Although they represent about 80% of the total fixed macrophage population, they are less than 5% of the total hepatic cell population. Kupffer cells play a critical role in the pathogenesis of several disease states, including endotoxin shock and alcoholic liver disease, because they release physiologically active substances such as eicosanoids, inflammatory cytokines, and many free radical species leading to localized tissue injury.

Peer review

This is an interesting study. It investigated the effects of (dietary) glycine against oxidant-induced injury caused by BDL.

REFERENCES

1 **Starzl TE**, Demetris AJ, Van Thiel D. Liver transplantation (1). *N Engl J Med* 1989; **321**: 1014-1022
2 **Whitington PF**, Balistreri WF. Liver transplantation in pediatrics: indications, contraindications, and pretransplant management. *J Pediatr* 1991; **118**: 169-177
3 **Poupon R**, Chazouilleres O, Poupon RE. Chronic cholestatic diseases. *J Hepatol* 2000; **32**: 129-140
4 **Kim WR**, Ludwig J, Lindor KD. Variant forms of cholestatic diseases involving small bile ducts in adults. *Am J Gastroenterol* 2000; **95**: 1130-1138
5 **Engelhardt JF**, Ye X, Doranz B, Wilson JM. Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. *Proc Natl Acad Sci USA* 1994; **91**: 6196-6200
6 **Tyler DD**. Polarographic assay and intracellular distribution of superoxide dismutase in rat liver. *Biochem J* 1975; **147**: 493-504
7 **Qureshi WA**. Intrahepatic cholestatic syndromes: pathogenesis, clinical features and management. *Dig Dis* 1999; **17**: 49-59
8 **Stiehl A**, Rudolph G, Raedsch R, Moller B, Hopf U, Lotterer E, Bircher J, Folsch U, Klaus J, Endele R. Ursodeoxycholic acid-induced changes of plasma and urinary bile acids in patients with primary biliary cirrhosis. *Hepatology* 1990; **12**: 492-497
9 **Skulina D**, Owczarek D, Ciecko-Michalska I, Szczepanski W, Tetnowski J, Garlicka M, Janas-Skulina U. [The influence of ursodeoxycholic acid on some biochemical, immunologic and histopathologic parameters in patients with primary biliary cirrhosis] *Przegl Lek* 1999; **56**: 201-204
10 **Sokol RJ**, Winklhofer-Roob BM, Devereaux MW, McKim JM Jr. Generation of hydroperoxides in isolated rat hepatocytes and hepatic mitochondria exposed to hydrophobic bile acids. *Gastroenterology* 1995; **109**: 1249-1256
11 **Pastor A**, Collado PS, Almar M, Gonzalez-Gallego J. Antioxidant enzyme status in biliary obstructed rats: effects of N-acetylcysteine. *J Hepatol* 1997; **27**: 363-370
12 **Chensue SW**, Terebuh PD, Remick DG, Scales WE, Kunkel SL. In vivo biologic and immunohistochemical analysis of interleukin-1 alpha, beta and tumor necrosis factor during experimental endotoxemia. Kinetics, Kupffer cell expression, and glucocorticoid effects. *Am J Pathol* 1991; **138**: 395-402
13 **Nolan JP**, Leibowitz A, Vladatin AL. Influence of alcohol on Kupffer cell function and possible significance in liver injury. In: Liehr H, Green M, eds. The reticuloendothelial system and pathogenesis of liver disease. Amsterdam: Elsevier, 1980: 125-136
14 **Ogle CK**, Wu JZ, Mao X, Szczur K, Alexander JW, Ogle JD. Heterogeneity of Kupffer cells and splenic, alveolar, and peritoneal macrophages for the production of TNF, IL-1, and IL-6. *Inflammation* 1994; **18**: 511-523
15 **Zhong Z**, Connor HD, Yin M, Moss N, Mason RP, Bunzendahl H, Forman DT, Thurman RG. Dietary glycine and renal denervation prevents cyclosporin A-induced hydroxyl radical production in rat kidney. *Mol Pharmacol* 1999; **56**: 455-463
16 **Venkatachalam MA**, Weinberg JM, Patel Y, Saikumar P, Dong Z. Cytoprotection of kidney epithelial cells by compounds that target amino acid gated chloride channels. *Kidney Int* 1996; **49**: 449-460
17 **Miller GW**, Schnellmann RG. A putative cytoprotective receptor in the kidney: relation to the neuronal strychnine-sensitive glycine receptor. *Life Sci* 1994; **55**: 27-34
18 **Wheeler MD**, Rose ML, Yamashima S, Enomoto N, Seabra V, Madren J, Thurman RG. Dietary glycine blunts lung inflammatory cell influx following acute endotoxin. *Am J Physiol Lung Cell Mol Physiol* 2000; **279**: L390-L398
19 **Bachmann S**, Caldwell-Kenkel JC, Currin RT, Tanaka Y, Takei Y, Marzi I, Thurman RG, Lemasters JJ. Ultrastructural correlates of liver graft failure from storage injury: studies of graft protection by Carolina rinse solution and pentoxifylline. *Transplant Proc* 1993; **25**: 1620-1624
20 **Froh M**, Thurman RG, Wheeler MD. Molecular evidence for a glycine-gated chloride channel in macrophages and leukocytes. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G856-G863
21 **Symeonidis A**, Trams EG. Morphologic and functional changes in the livers of rats after ligation or excision of the common bile duct. *Am J Pathol* 1957; **33**: 13-27
22 **Nanji AA**, Khettry U, Sadrzadeh SM. Lactobacillus feeding reduces endotoxemia and severity of experimental alcoholic liver (disease). *Proc Soc Exp Biol Med* 1994; **205**: 243-247
23 **Pertoft H**, Smedsrod B. Separation and characterization of liver cells. In: Pretlow TG, Pretlow TP, eds. Cell separation: methods and selected applications. New York: Academic Press, 1987: 1-24
24 **Froh M**, Kono A, Thruman RG. Isolation of liver Kupffer cells. In: Costa LG, Hodgson E, Lawrence DA, Reed DJ, eds. Current Protocols in Toxicology. New York: John Wiley & Sons, 2002: 14.04.01-14.04.12
25 **Garcia-Ruiz C**, Morales A, Ballesta A, Rodes J, Kaplowitz N, Fernandez-Checa JC. Effect of chronic ethanol feeding on glutathione and functional integrity of mitochondria in periportal and perivenous rat hepatocytes. *J Clin Invest* 1994; **94**: 193-201
26 **Bergmeyer HU**, Bernt E. Lactate dehydrogenase UV assay with pyruvate and NADPH. In: Bergmeyer HU, ed. Methods of Enzymatic Analysis. New York: Academic Press, 1974: 574-579
27 **Schwartz RD**, Paul SM, Majewska MD. Factors modulating the sensitivity of the GABA receptor-gated chloride ion channel. *Clin Neuropharmacol* 1986; **9** Suppl 4: 389-391
28 **Morrow AL**, Paul SM. Benzodiazepine enhancement of gamma-aminobutyric acid-mediated chloride ion flux in rat brain synaptoneurosomes. *J Neurochem* 1988; **50**: 302-306
29 **Lowry OH**, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193**: 265-275
30 **Ikejima K**, Qu W, Stachlewitz RF, Thurman RG. Kupffer cells contain a glycine-gated chloride channel. *Am J Physiol* 1997; **272**: G1581-G1586
31 **Zhong Z**, Froh M, Lehnert M, Schoonhoven R, Yang L, Lind

H, Lemasters JJ, Thurman RG. Polyphenols from Camellia sinenesis attenuate experimental cholestasis-induced liver fibrosis in rats. *Am J Physiol Gastrointest Liver Physiol* 2003; **285**: G1004-G1013

32 **Froh M**, Conzelmann L, Walbrun P, Netter S, Wiest R, Wheeler MD, Lehnert M, Uesugi T, Scholmerich J, Thurman RG. Heme oxygenase-1 overexpression increases liver injury after bile duct ligation in rats. *World J Gastroenterol* 2007; **13**: 3478-3486

33 **Parola M**, Leonarduzzi G, Robino G, Albano E, Poli G, Dianzani MU. On the role of lipid peroxidation in the pathogenesis of liver damage induced by long-standing cholestasis. *Free Radic Biol Med* 1996; **20**: 351-359

34 **Sokol RJ**, Devereaux M, Khandwala RA. Effect of dietary lipid and vitamin E on mitochondrial lipid peroxidation and hepatic injury in the bile duct-ligated rat. *J Lipid Res* 1991; **32**: 1349-1357

35 **Zhong Z**, Froh M, Wheeler MD, Smutney O, Lehmann TG, Thurman RG. Viral gene delivery of superoxide dismutase attenuates experimental cholestasis-induced liver fibrosis in the rat. *Gene Ther* 2002; **9**: 183-191

36 **Marsh DC**, Vreugdenhil PK, Mack VE, Belzer FO, Southard JH. Glycine protects hepatocytes from injury caused by anoxia, cold ischemia and mitochondrial inhibitors, but not injury caused by calcium ionophores or oxidative stress. *Hepatology* 1993; **17**: 91-98

37 **Nichols JC**, Bronk SF, Mellgren RL, Gores GJ. Inhibition of nonlysosomal calcium-dependent proteolysis by glycine during anoxic injury of rat hepatocytes. *Gastroenterology* 1994; **106**: 168-176

38 **Qian T**, Nieminen AL, Herman B, Lemasters JJ. Mitochondrial permeability transition in pH-dependent reperfusion injury to rat hepatocytes. *Am J Physiol* 1997; **273**: C1783-C1792

39 **Weinberg JM**, Davis JA, Abarzua M, Kiani T. Relationship between cell adenosine triphosphate and glutathione content and protection by glycine against hypoxic proximal tubule cell injury. *J Lab Clin Med* 1989; **113**: 612-622

40 **Frank A**, Rauen U, de Groot H. Protection by glycine against hypoxic injury of rat hepatocytes: inhibition of ion fluxes through nonspecific leaks. *J Hepatol* 2000; **32**: 58-66

41 **Yamashina S**, Konno A, Wheeler MD, Rusyn I, Rusyn EV, Cox AD, Thurman RG. Endothelial cells contain a glycine-gated chloride channel. *Nutr Cancer* 2001; **40**: 197-204

42 **Ikejima K**, Iimuro Y, Forman DT, Thurman RG. A diet containing glycine improves survival in endotoxin shock in the rat. *Am J Physiol* 1996; **271**: G97-103

43 **Li X**, Bradford BU, Wheeler MD, Stimpson SA, Pink HM, Brodie TA, Schwab JH, Thurman RG. Dietary glycine prevents peptidoglycan polysaccharide-induced reactive arthritis in the rat: role for glycine-gated chloride channel. *Infect Immun* 2001; **69**: 5883-5891

44 **Wheeler M**, Stachlewitz RF, Yamashina S, Ikejima K, Morrow AL, Thurman RG. Glycine-gated chloride channels in neutrophils attenuate calcium influx and superoxide production. *FASEB J* 2000; **14**: 476-484

45 **Rivera CA**, Bradford BU, Hunt KJ, Adachi Y, Schrum LW, Koop DR, Burchardt ER, Rippe RA, Thurman RG. Attenuation of CCl(4)-induced hepatic fibrosis by GdCl(3) treatment or dietary glycine. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G200-G207

46 **Roggin KK**, Papa EF, Kurkchubasche AG, Tracy TF Jr. Kupffer cell inactivation delays repair in a rat model of reversible biliary obstruction. *J Surg Res* 2000; **90**: 166-173

47 **Friedman SL**. Cytokines and fibrogenesis. *Semin Liver Dis* 1999; **19**: 129-140

48 **Alcolado R**, Arthur MJ, Iredale JP. Pathogenesis of liver fibrosis. *Clin Sci* (Lond) 1997; **92**: 103-112

49 **Fox ES**, Kim JC, Tracy TF. NF-kappaB activation and modulation in hepatic macrophages during cholestatic injury. *J Surg Res* 1997; **72**: 129-134

50 **Bemelmans MH**, Gouma DJ, Greve JW, Buurman WA. Cytokines tumor necrosis factor and interleukin-6 in experimental biliary obstruction in mice. *Hepatology* 1992; **15**: 1132-1136

51 **Qu W**, Ikejima K, Zhong Z, Waalkes MP, Thurman RG. Glycine blocks the increase in intracellular free Ca2+ due to vasoactive mediators in hepatic parenchymal cells. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G1249-G1256

S- Editor Li DL **L- Editor** Rippe RA **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6004

World J Gastroenterol 2008 October 21; 14(39): 6004-6011
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Facilitating effects of berberine on rat pancreatic islets through modulating hepatic nuclear factor 4 alpha expression and glucokinase activity

Zhi-Quan Wang, Fu-Er Lu, San-Hua Leng, Xin-Sheng Fang, Guang Chen, Zeng-Si Wang, Li-Ping Dong, Zhong-Qing Yan

Zhi-Quan Wang, Fu-Er Lu, San-Hua Leng, Xin-Sheng Fang, Guang Chen, Zeng-Si Wang, Li-Ping Dong, Zhong-Qing Yan, Institute of Integrated Traditional Chinese and Western Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China
Author contributions: Lu FE and Leng SH designed the research; Wang ZQ, Fang XS, Chen G, Wang ZS, Dong LP and Yan ZQ performed the research; Wang ZQ and Fang XS analyzed the data; and Wang ZQ wrote the paper.
Supported by The National Natural Science Foundation of China, No. 30500685
Correspondence to: Dr. Fu-Er Lu, Institute of Integrated Traditional Chinese and Western Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China. felu@tjh.tjmu.edu.cn
Telephone: +86-27-83663237 Fax: +86-27-83663237
Received: June 23, 2008 Revised: September 20, 2008
Accepted: September 27, 2008
Published online: October 21, 2008

Abstract

AIM: To observe the effect of berberine on insulin secretion in rat pancreatic islets and to explore its possible molecular mechanism.

METHODS: Primary rat islets were isolated from male Sprague-Dawley rats by collagenase digestion and treated with different concentrations (1, 3, 10 and 30 μmol/L) of berberine or 1 μmol/L Glibenclamide (GB) for 24 h. Glucose-stimulated insulin secretion (GSIS) assay was conducted and insulin was determined by radioimmunoassay. 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate cytotoxicity. The mRNA level of hepatic nuclear factor 4 alpha (*HNF4α*) was determined by reverse transcription polymerase chain reaction (RT-PCR). Indirect immunofluorescence staining and Western blot analysis were employed to detect protein expression of HNF4α in the islets. Glucokinase (GK) activity was measured by spectrophotometric method.

RESULTS: Berberine enhanced GSIS rather than basal insulin secretion dose-dependently in rat islets and showed no significant cytotoxicity on islet cells at the concentration of 10 μmol/L. Both mRNA and protein expressions of HNF4α were up-regulated by berberine in a dose-dependent manner, and GK activity was also increased accordingly. However, GB demonstrated no regulatory effects on HNF4α expression or GK activity.

CONCLUSION: Berberine can enhance GSIS in rat islets, and probably exerts the insulinotropic effect *via* a pathway involving HNF4α and GK, which is distinct from sulphonylureas (SUs).

Key words: Berberine; Sulphonylureas; Hepatocyte nuclear factor 4 alpha; Glucokinase; Pancreatic islet

Peer reviewer: Giovanni Tarantino, MD, Professor, Department of Clinical and Experimental Medicine, Federico Ⅱ University Medical School, VIA S. PANSINI, 5, Naples 80131, Italy

Wang ZQ, Lu FE, Leng SH, Fang XS, Chen G, Wang ZS, Dong LP, Yan ZQ. Facilitating effects of berberine on rat pancreatic islets through modulating hepatic nuclear factor 4 alpha expression and glucokinase activity. *World J Gastroenterol* 2008; 14(39): 6004-6011 Available from: URL: http://www.wjgnet.com/1007-9327/14/6004.asp DOI: http://dx.doi.org/10.3748/wjg.14.6004

INTRODUCTION

Type 2 diabetes mellitus is a complex and heterogeneous disorder caused by the interaction of hereditary and environmental factors, pathophysiologically characterized by insulin resistance and functional defects in insulin release from pancreatic β cells.

Sulphonylureas (SUs) are the most commonly prescribed insulin secretagogues. These drugs act *via* augmentation of insulin secretion from pancreatic β cells. The SU receptor-1 on the ATP-sensitive potassium channels (K_{ATP} channels) is occupied by SU leading to closure of the potassium channels and subsequent opening of calcium channels, resulting in exocytosis of insulin granules[1]. Yet, the maintenance of satisfactory long-term glycaemic control in patients undergoing SU therapy is usually restricted by increased risk of

hypoglycemia coupled with declined insulinotropic activity due to desensitized β cells to the agents[2,3]. Therefore, new types of insulinotropic substances with an alternative action profile are in demand.

Berberine, the major active constituent of Chinese herb *Rhizoma Coptidis*, is being used to treat diabetes for decades, showing obvious therapeutic actions with few reported side effects. Previous studies have demonstrated that berberine modulates cholesterol through increasing low-density lipoprotein receptor mRNA stability[4], reduces body adiposity and increases insulin sensitivity partly through activating AMP-activated protein kinase[5], and improves glucose metabolism *via* induction of glycolysis[6], implying a promising future for berberine in the therapy of diabetes. Moreover, a new research further revealed that berberine also possessed insulinotropic property in isolated pancreatic islets[7]. However, the underlying mechanism is not fully understood.

Recently, the hepatocyte nuclear factors (HNFs) transcriptional regulatory networks were identified in pancreatic islet tissue, providing insight into the molecular basis of abnormal β cell function. It was deemed that three members of HNFs family, HNF4α, HNF1α and HNF6, were at the center of the connected network, operating cooperatively to regulate numerous developmental and metabolic functions in human pancreatic islets. It was also revealed that HNF4α was bound to about 11% of the genes represented on the DNA microarray in pancreatic islets[8,9]. The occupancy by HNF4α of a substantial fraction of expressed genes suggests that HNF4α is a widely acting transcription factor and crucial for development and proper secretary function of pancreatic β cells. Furthermore, Bartoov-Shifman *et al*[10] found that HNF4α could activate insulin gene directly, through a previously unrecognized cis element. Clinical researches have indicated that mutations in gene encoding HNF4α result in maturity-onset diabetes of the young type 1, characterized by autosomal dominant inheritance, early onset and impairment of glucose-stimulated insulin secretion (GSIS)[11]. And two independent laboratory studies demonstrated that β-cell-specific HNF4α knock-out mice exhibited impaired GSIS and deficient intracellular calcium response to glucose or SU[12,13].

Increasing evidences suggest an essential role for HNF4α in the maintenance of proper secretary function of pancreatic β cell and glucose metabolism. In this study, we hypothesize that berberine facilitates insulin secretion through a pathway involving HNF4α in pancreatic islets. We introduce Glibenclamide (GB) as a control to compare the action profile of berberine with SUs, and to explore the possible molecular mechanism.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats weighing 250-300 g [Grade SPF, Certificate No. SCXK (E2004-0007)] were purchased from the Experimental Animal Center, Tongji Medical College, Huazhong University of Science and Technology. The rats were housed at 22℃, and 60%-70% relative humidity, with 12 h light/dark cycle. The rats were given free access to food and tap water. All rats received humane care in compliance with the institutional animal care guidelines approved by the Experimental Animal Ethical Committee of Tongji Medical College, Huazhong University of Science and Technology.

Drug preparation

Berberine hydrochloride (Sigma, St Louis, MO, USA) and GB (Alexis CO, San Diego, CA, USA) were dissolved in dimethyl sulfoxide (DMSO, Amresco, TX, USA), with a final concentration of DMSO 0.01% (v/v) in the culture medium.

Islet isolation and culture conditions

Primary pancreatic islets were isolated as previously reported[14,15]. In brief, rats were anesthetized with intramuscular pentobarbital injection, 8-10 mL ice-cold Hanks' balanced salt solution (HBSS) containing 0.75 mg/mL type V collagenase (Sigma, St Louis, MO, USA) was injected *via* pancreatic duct, extended pancreas was removed and digested in a 38℃ water bath for 8-10 min. Then digestion was terminated by 30 mL ice-cold HBSS with 10% fetal bovine serum (Gibco, USA), and the suspension was filtered through a 600 μm screen to discard the undigested tissue. After twice washes with HBSS, islets were purified by Ficoll-400 (Amersham Pharmacia Biotech, Uppsala, Sweden) discontinuous gradient centrifugation at 800 × *g* for 20 min at 4℃, and hand-picked under dissecting microscope. About 250-400 islets were yielded from each pancreas. The purity of islets was evaluated by dithizone (DTZ, Sigma, St Louis, MO, USA) staining[16], and the viability was assessed according to the acridine orange/propidium iodide (AO/PI, Sigma, St Louis, MO, USA) fluorescent staining method[17]. Freshly isolated islets were first cultured overnight at 37℃ in a 50 mL/L CO_2-950 mL/L air atmosphere in serum-free RPMI 1640 (Hyclone, Gaithersburg, MD, USA) containing 2% (w/v) bovine serum albumin fraction V (BSA, Amresco, TX, USA), 11.1 mmol/L glucose, 5 mmol/L glutamine, 1 mmol/L sodium pyruvate, 100 IU/mL pennicillin, 100 μg/mL streptomycin and 15 mmol/L HEPES. Then islets were cultured for 24 h in various experimental media containing 1, 3, 10 and 30 μmol/L berberine or 1 μmol/L GB. Normal control was also set by incubating islets with medium in the absence of berberine or GB.

GSIS assay

For evaluation of insulin secretion, islets were washed twice with Krebs-Ringers Bicarbonated HEPES [KRBH, containing 120 mmol/L NaCl, 4.8 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 25 mmol/L $NaHCO_3$, 10 mmol/L HEPES, 2.8 mmol/L glucose, 0.5% BSA (w/v), pH 7.4] at the end of the incubation. Batches of 10 size-matched islets (six replicas per condition) were transferred into

1.5 mL Eppendorf tubes, and pre-incubated for 30 min at 37℃ in KRBH with 2.8 mmol/L glucose. Subsequently, islets were incubated in KRBH supplemented with either 2.8 mmol/L or 16.7 mmol/L glucose for 1 h at 37℃. Aliquots of supernatant were collected after gentle centrifugation and stored at -20℃ for insulin determination by radioimmunoassay kit (Beijing Institute of Atomic Energy, China).

3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay

Cytotoxicity of berberine and GB on islet cells was tested by a colorimetric assay that detected the conversion of MTT (Sigma, St Louis, MO, USA) into the formazan by the mitochondrial enzyme succinate dehydrogenase in viable cells[18]. After *in vitro* treatment, islets were dissociated into single cells by incubation in Ca^{2+}/Mg^{2+}-free KRBH containing 5 mmol/L EDTA and 0.25 mg/mL trypsin for 10 min at 37℃ with gentle shaking, and then resuspended in RPMI 1640. Islet cells were cultured in a 96-well plate supplemented with 0.5 mg/mL MTT. After 4 h incubation, the insoluble formazan crystals within islet cells were extracted by DMSO, and absorbance was measured by ELX800 Universal Microplated Reader (BioTek Instruments Inc, USA) at wavelength of 630 nm.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

Semi-quantitative RT-PCR was performed to determine the mRNA level of HNF4α in the islets. Total RNA was extracted from about 300 islets by the Trizol Reagent Kit (Gibco, USA) according to the manufacturer's instructions. Two μg of RNA from each sample was then reverse-transcribed into first-strand cDNA in 25 μL solution using Oligo (dT) Primers and MMLV reverse transcriptase (Promega, Madison, WI, USA). PCR reaction was performed in a standard 25 μL reaction solution contained 3 μL cDNA, 0.5 μL each of sense and anti-sense primers. Sequence-specific primers for cDNA amplification were as follows: *HNF4α* (product 464 bp, sense 5'-GCAGTGCGTGGTAGACAAAGA TA-3'; anti-sense 5'-AGTGCCGAGGGACGATGTA G-3') and the housekeeping gene *β-actin* (product 213 bp, sense 5'-AGATCTGGCACCACACCTTCTAC-3'; anti-sense 5'-TCAGGATCTTCATGAGGTAGTCT-3'). Reaction conditions for *HNF4α* were as follows: predenaturing at 95℃ for 5 min , denaturing at 95℃ for 1 min, annealing at 60℃ for 50 s, extending at 72℃ for 1 min, 35 cycles, with final extending at 72℃ for 10 min. Amplification of *β-actin* was performed by predenaturing at 94℃ for 5 min, denaturing at 94℃ for 1 min, annealing at 55℃ for 50 s, extending at 72℃ for 50 s, 30 cycles and final extending at 72℃ for 10 min. PCR products were electrophoresed through 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet illumination. Band intensity was calculated densitometrically using the SensiAnsys software (Shanghai PeiQing Science & Technology, China).

Immunofluorescence

Indirect immunofluorescence assay was performed as previously described with a few modifications[19,20]. In brief, islets were placed into 1.5 mL Eppendorf tubes, fixed in 4% paraformaldehyde/10 mmol/L PBS for 30 min, followed by a 3 h permeabilization with 0.3% Triton-X100/10 mmol/L PBS. Subsequently, islets were blocked with 5% fetal bovine serum/0.2 Triton-X100/10 mmol/L PBS overnight at 4℃ and then equilibrated in antibody dilution buffer (1% BSA/0.2% Triton X-100/10 mmol/L PBS) twice for 20 min at room temperature. Primary antibodies used were as follows: goat anti-rat HNF4α IgG (1:300, Santa Cruz, San Diego, CA, USA), Guinea pig anti-insulin antibody (1:300, Sigma, St Louis, MO, USA), and the incubation was carried out for 16 h at 4℃. The secondary antibodies were Cy3 conjugated rabbit anti-goat IgG (1:400, Sigma, St Louis, MO, USA) and FITC conjugated rabbit anti-guinea pig IgG (1:250, Sigma, St Louis, MO, USA), incubation was performed for 1 h at room temperature. Finally, islets were mounted with 50% glycerol/10 mmol/L PBS, smeared onto glass slides and subjected to Confocal Laser Scanning Microscope (Olympus FV500, Japan). The excitation wavelengths for Cy3 and FITC were 552 nm and 488 nm, and the emission wavelengths were 565 nm and 525 nm, respectively. To avoid variability in fluorescent intensity caused by depth-related changes and wide range of islet diameters, a single section image was taken at the depth 1/3 the distance between the upper islet surface and its maximum diameter. Ten areas from each cover slip were randomly selected and analyzed by the HMIAS-2000 Imaging System (Champion Medical Imaging Co., Wuhan, China).

Western blot analysis

Primary pancreatic islets treated with the various experimental conditions were lysated in buffer containing 25 mmol/L Tris, 1% SDS, 5% β-mercaptoethanol, 10 mmol/L EDTA, 20 mmol/L PMSF, 10 mg/L aprotinin, 10 mg/L leupeptin, 10 mg/L antipain, 8 mol/L urea. Homogenates were centrifuged at 12 000 × *g* for 15 min at 4℃, supernatants were harvested. Protein concentrations were measured by the method of Bradford[21], with Coomassie brilliant blue staining kit (Jiancheng Biology Institute, Nanjing, China). And 50 μg total protein for each group was boiled for 5 min in sample buffer [50 mmol/L Tris·Cl (pH 6.8), 100 mmol/L DTT, 2% SDS, 0.1% bromchlorphenol blue, 10% glycerol] and separated by 12% SDS-PAGE. Thereafter, proteins were transferred electrophoretically onto a polyvinylidine fluoride membrane. Before immunostaining, the membranes were blocked with 5% non-fat milk in Tris-buffered saline and 0.1% Tween (TBST) overnight at 4℃, followed by incubation with appropriate dilutions of the primary specific antibody goat anti-rat HNF4α IgG (1:500, Santa Cruz Biotechnology Inc, CA, USA) in 5%BSA/TBST at 4℃ for 16 h. The secondary antibody was horseradish

peroxidase (HRP) conjugated rabbit anti-goat IgG (1:2000, Sigma, St Louis, MO, USA), incubation was carried out at room temperature for 2 h. GAPDH was probed with anti-GAPDH IgG as a loading control. Immunodetection was performed using an enhanced chemiluminescence detection kit (Pierce, Rockford, IL). Protein bands on films (Eastman Kodak, Rochester, NY, USA) were analyzed by densitometry (Bio-Rad, Hercules, USA) using "Quantity One" quantitation analysis software program.

Glucokinase (GK) activity assay

GK activity was measured by spectrophotometric assay as previously described[22]. Briefly, islet were washed twice with PBS, approximately 150 islets from each group were homogenized (30 strokes) in 200 μL lysis buffer containing 20 mmol/L K_2HPO_4, 5 mmol/L dithiothreitol, 1 mmol/L EDTA, and 110 mmol/L KCl, followed by sonication (20 KHz, 60 W) for 3 × 10 s on ice. The homogenate was then centrifuged at 12000 × *g* for 10 min, and the supernatant fraction was used for GK determination. Then 10 μL of the supernatant was added to 100 μL reaction buffer, containing 50 mmol/L HEPES/HCl (pH 7.6), 100 mmol/L KCl, 7.4 mmol/L $MgCl_2$, 15 mmol/L β-mercaptoethanol, 0.5 mmol/L NAD^+, 0.05% BSA (w/v), 2 IU/mL glucose-6-phosphate dehydrogenase, and 5 mmol/L ATP. The assay was conducted for 1 h at 30℃, and reaction was stopped by adding 1 mL of 500 mmol/L $NaHCO_3$ buffer (pH 9.4). In each assay, blanks were obtained by incubating 0.5 or 100 mmol/L glucose in the absence of ATP. Absorbance was measured at 340 nm, correction for hexokinase activity was applied by substracting the activity measured at 0.5 mmol/L glucose from the activity measured at 100 mmol/L glucose. Protein concentrations were determined by the Bradford assay.

Statistical analysis

All the data were expressed as mean ± SD, and analyzed with SPSS 13.0 software by one-way analysis of variance (ANOVA) LSD-*t* and SNK-*q*. $P < 0.05$ was considered statistically significant.

RESULTS

Purity and viability of freshly isolated islets

The purity of the freshly isolated islets was estimated by the percentage of DTZ-positive islets (crimson red) in the preparation. According to the method, an approximate 95% purity was assessed. For viability evaluation, islets were exposed to AO/PI, and subjected to fluorescent microscopy (Nikon ECLIPSE TE2000-U, Japan) with viable cells stained green while nonviable cells bright red. The viability exceeded 90% as assessed by the method (Figure 1).

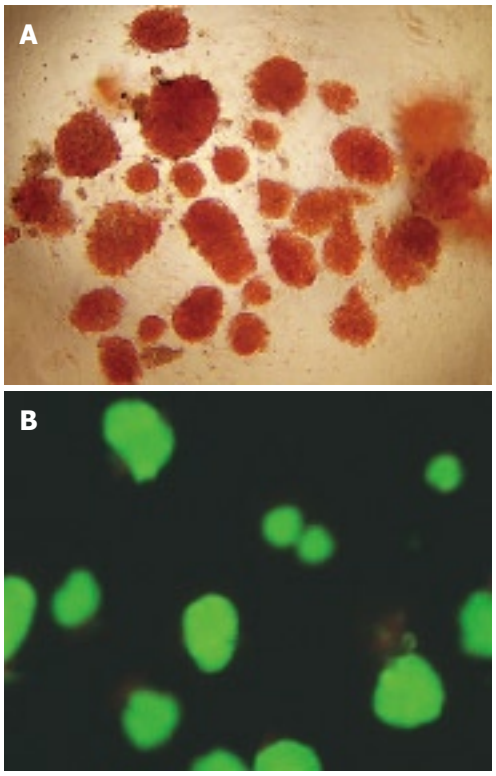


Figure 1 Quality assessment of freshly isolated islets. **A**: DTZ staining of islets under an inverted microscope (x 10); **B**: AO/PI double fluorescence staining of islets under a fluorescent microscopy (x 10).

Effects of berberine and GB on insulin secretion

In the experiment, we employed static incubation assay to further examine the facilitative effect of berberine on islet insulin secretion. Islets were first incubated with 2.8 mmol/L glucose, and then challenged with 16.7 mmol/L glucose. All samples were determined by immunoradioassay. As Figure 2A depicted, islets in control group exhibited a normal response to glucose stimulation, with insulin secretion of 7.21 ± 1.43 *vs* 30.50 ± 5.17 (mIU/L per hour per 10 islets). Treatment of islets with 1 μmol/L GB potently elevated basal insulin secretion ($P < 0.01$), while inhibited GSIS by about 3 folds relative to normal control ($P < 0.05$). In contrast, although none of the four berberine groups showed any promoting effects on basal insulin secretion, treatment with 1, 3, 10 μmol/L berberine resulted in dose-dependently increased GSIS ($P < 0.05$ or $P < 0.01$), still no enhancement was observed in islets of 30 μmol/L berberine group.

Cytotoxicity of berberine and GB on islet cells

We used MTT assay to analyze cytotoxicity caused by berberine and GB. Results were expressed as percentage of formazan absorbance relative to control value. It was observed that, 30 μmol/L berberine inhibited formazan absorbance by about 2 folds compared to the control, indicating significant cytotoxicity on islet cells ($P < 0.01$). None of the other groups demonstrated significantly diminished absorbance (Figure 2B).

Effects of berberine and GB on HNF4α gene expression

In RT-PCR experiment, it was observed that at concentrations lower than 10 μmol/L, berberine

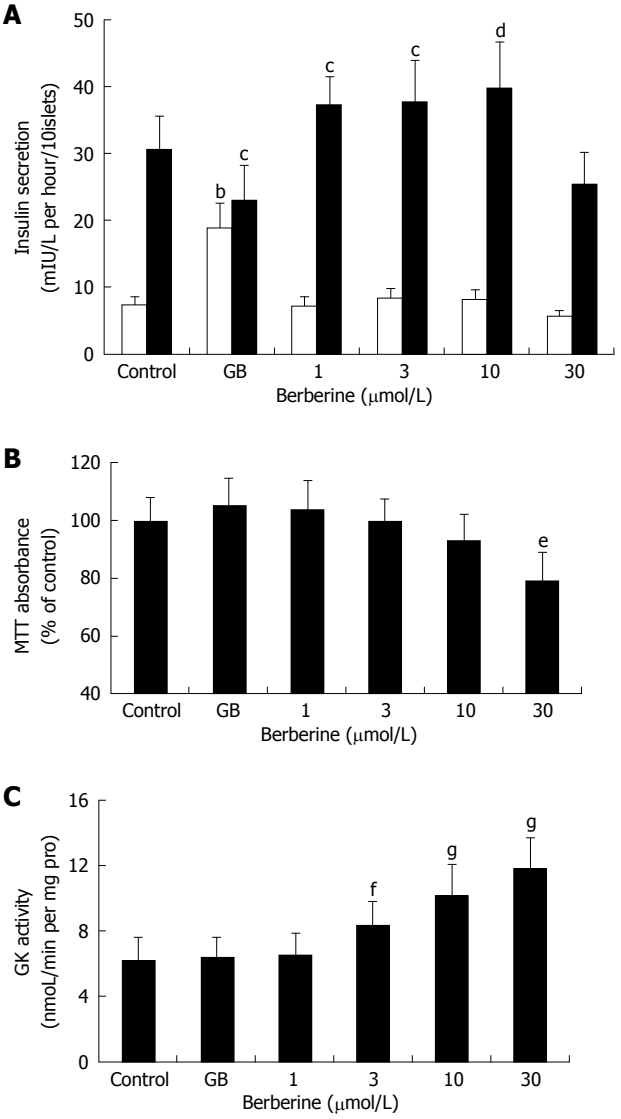


Figure 2 Pharmacological effects of berberine and GB on pancreatic islets. **A**: Effects of berberine and GB on basal (2.8 mmol/L Glucose) and GSIS (16.7 mmol/L Glucose) by pancreatic islets. Control and GB represent islets of untreated and 1 μmol/L GB treated groups, while 1, 3, 10 and 30 represent berberine groups of indicated concentrations. $^{b}P < 0.01$ *vs* control at 2.8 mmol/L glucose; $^{c}P < 0.05$, $^{e}P < 0.01$ *vs* control at 16.7 mmol/L glucose; **B**: Cytotoxicity of berberine and GB on islet cells assessed by MTT assay. Results were expressed as the percentage of absorbance to the control value. Data are presented as mean ± SD ($n = 6$). $^{e}P < 0.01$ *vs* control. **C**: Effects of berberine and GB on islet GK activity. $^{f}P < 0.05$, $^{g}P < 0.01$ *vs* control.

treatment induced a general dose-dependent increase relative to control in *HNF4α* mRNA expression ($P < 0.05$ or $P < 0.01$). However, no significant difference was observed in islets incubated with either 30 μmol/L berberine or 1 μmol/L GB compared with the control (Figure 3).

Immunofluorescence and confocal microscopy

Confocal images showed apparent co-localization of insulin (green) and HNF4α (red) in the islets. As Figure 4 depicts, islets exhibited a normal architecture, featuring predominant distribution of insulin throughout the entire β cell cytoplasm as well as typical nuclear localization of HNF4α in both β cell and peripheral

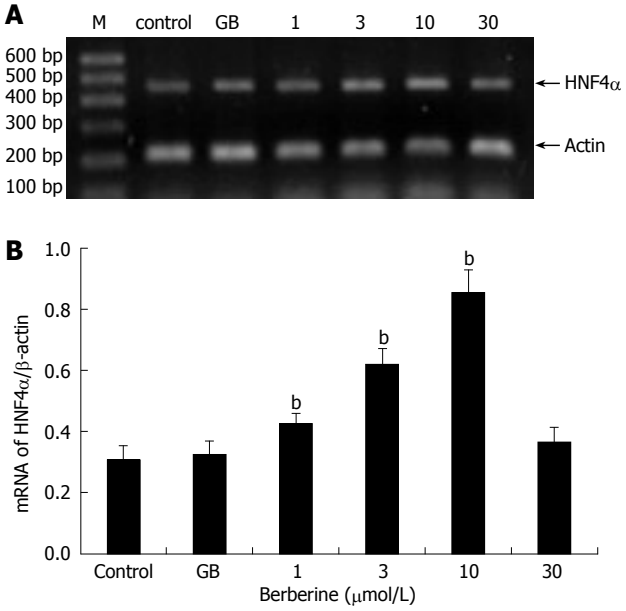


Figure 3 Effects of berberine and GB on HNF4α mRNA expression. **A**: A representative gel electrophresis profile of DNA fragment; **B**: Semi-quantitative mRNA determination, results were normalized to β-actin and expressed as arbitrary unit. M: DNA Marker, control and GB represent islets of untreated and 1 μmol/L GB treated groups; 1, 3, 10 and 30 represent berberine groups of indicated concentrations. Data are presented as mean ± SD ($n = 6$), $^{b}P < 0.01$ *vs* control.

α cell. It was noteworthy that islets of berberine treated groups demonstrated more intense and distinct fluorescence of HNF4α than control group, with the strongest red fluorescence emitted from the 10 μmol/L berberine treated group. However, no distinguishable change of HNF4α staining was observed in GB group compared with control group.

Effects of berberine and GB on HNF4α protein expression

To further clarify the correlation of berberine's insulinotropic effect with HNF4α expression, we quantified the protein level of HNF4α by Western blot. Similar to the result of immunofluorescence, protein expression of HNF4α also demonstrated a dose-dependent increase in the berberine treated islets ($P < 0.05$ or $P < 0.01$), with maximum expression in 10 μmol/L berberine group. Still no significant difference was found between GB group and control group (Figure 5).

Effects of berberine and GB on islet GK activity

In the experiment, GK activity was determined in the islet homogenates. As shown in Figure 2C, compared to the control, treatment with 3, 10 and 30 μm/L berberine significantly activated islet GK activity ($P < 0.05$ or $P < 0.01$) in a dose-dependent manner, while no enhancement of GK activity was observed in the islets incubated with 1 μmol/L berberine or 1 μmol/L GB.

DISCUSSION

The SUs are a family of oral hypoglycemic agents used

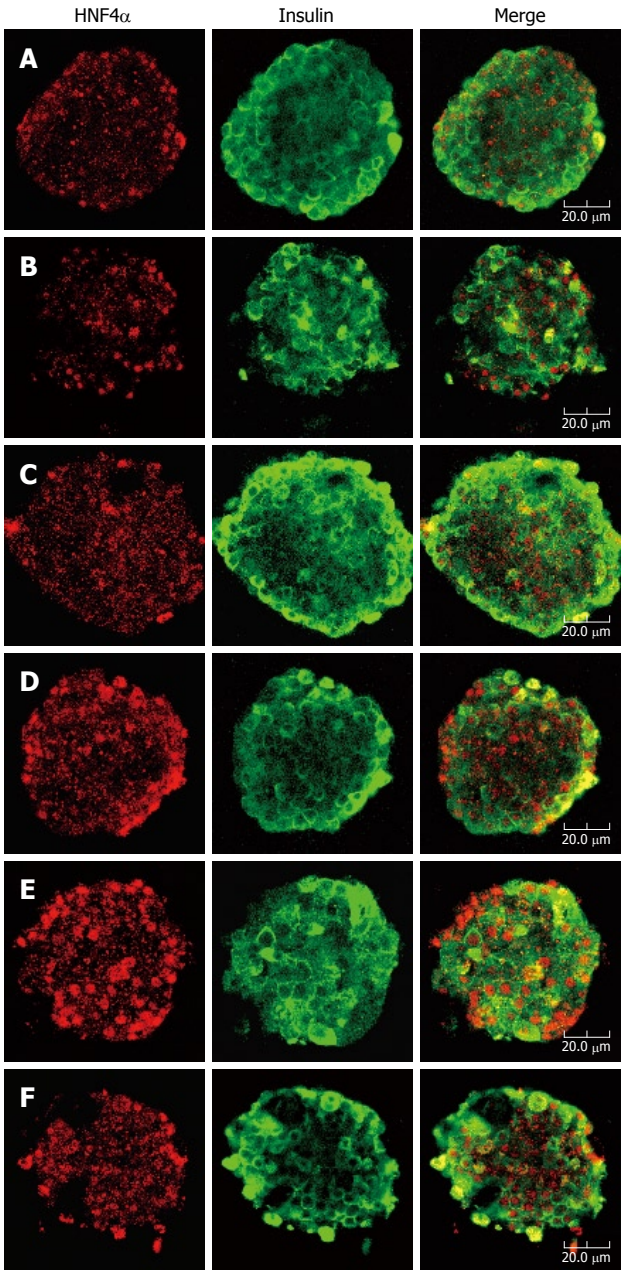


Figure 4 Double immunofluorescence staining for HNF4α (red) and insulin (green) of rat pancreatic islets. After treatment with indicated concentrations of berberine or GB for 24 h, islets were fixed and stained with anti-HNF4α and anti-insulin antibodies. Images of islets were taken at the corresponding depth by confocal laser microscopy (x 40). **A**: Control, **B**: 1 μmol/L GB; **C** to **F**: Represent 1, 3, 10 and 30 μmol/L berberine. Bar in the figure indicates 20 μm. Apparent nuclear localization of HNF4α could be observed in all groups of islets, while insulin fluorescence was diffusely distributed in cytoplasma of β cells. In the islets treated with various concentrations of berberine, the red fluorescence emitted was comparatively intense, suggesting up-regulated expression of HNF4α, while no obvious difference of HNF4α staining was found between control and GB treated islets.

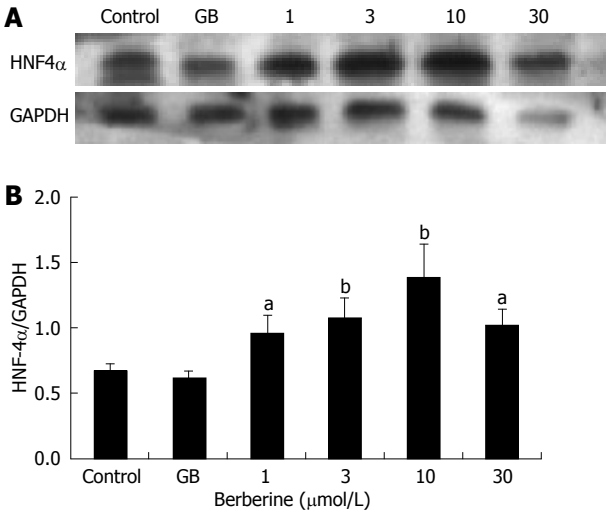


Figure 5 Effects of berberine and GB on protein expression of HNF4α. **A**: A representative Western blot of HNF4α; **B**: Quantification of HNF4α protein, results were adjusted to GAPDH and expressed as relative density units. Control and GB represent islets of untreated and 1 μmol/L GB treated groups, 1, 3, 10 and 30 represent berberine groups of indicated concentrations. Data are presented as mean ± SD (n = 3). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control.

extensively for the treatment of type 2 diabetes. They mediate the insulinotropic effect *via* blocking β cell K_{ATP} channels and depolarizing the membrane. Nonetheless, because the potent insulin stimulating property is independent of glucose challenge, they enhance insulin secretion even at basal glucose levels. Therefore, patients receiving SUs therapy are at high risk of hypoglycemia. Moreover, chronic SUs therapy may lead to a selective desensitization of pancreatic β cells to SUs[1,2].

In the present study, the insulinotropic effect of berberine on rat islets was compared with GB, a classical SU derivative. It was demonstrated that 1 μmol/L GB acutely promoted basal insulin secretion by approximately 250% in isolated islets, while markedly reduced GSIS in the presence of 16.7 mmol/L glucose. This observation is in good agreement with previous reports[2,23]. The suppressed GSIS might partly result from the reduction of K_{ATP} channel activity induced by chronic exposure to GB and/or the depletion of insulin stores, reflecting the controversy of β-cell desensitization *vs* β-cell exhaustion[3,24]. In contrast to the action profile of GB, berberine induced no significant changes in basal insulin secretion but increased GSIS dose-dependently at the concentrations of 1-10 μmol/L. This result is consistent with a previous research by Ko *et al*[25], which showed that berberine exerted no stimulatory effect on basal insulin secretion (2 mmol/L glucose), but increased GSIS at the concentration of 5 and 50 μmol/L in MIN6 cells.

To define the underlying mechanism for the completely different action profile of GB and berberine, we first examined the cytotoxicity caused by the drugs. Our results indicated that only 30 μmol/L berberine demonstrated statistically significant toxicity on islet cell. Hence, it could not be the distinctions of cell metabolism that account for the different action modalities of the two agents. Accordingly, we concluded berberine probably stimulated insulin secretion *via* a mechanism distinct from SUs! In the following experiments, we determined the gene and protein expressions of HNF4α, a transcription factor confirmed to play an essential role in GSIS, attempting to explore if HNF4α a potential target underling the facilitating effect of berberine on GSIS. Our data conspicuously showed

that both the mRNA and protein expressions increased in a dose-dependent manner, reaching their maximum at 10 μmol/L berberine then declining to the levels parallel those of control. No significant changes were observed in the islets treated with 1 μmol/L GB compared with the control. These results strongly suggest the potential involvement of HNF4α in berberine's insulinotropic action but not GB-induced insulin secretion. The significant cytotoxicity caused by 30 μmol/L berberine might offer a plausible explanation for the reduction of HNF4α expression.

The question remains as to how HNF4α mediate the stimulatory effects of berberine on GSIS in pancreatic islets, as few evidences to date substantiate that HNF4α regulates GSIS directly in pancreatic islets. For elucidating this, we further investigated the effect of berberine on GK activity in rat islets. It is generally acknowledged that GK plays the crucial role of "glucose sensor" in pancreatic β-cell, due to its specific kinetic properties that include low affinity and positive cooperativity for glucose and a lack of inhibition by its product glucose-6-phosphate. GK regulates GSIS by modulation of the glycolytic pathway and controlling the rate of its subsequent metabolism[26,27]. It is also deemed that GK is one of the downstream targets of the HNFs transcription regulatory circuit in pancreatic islets[9]. Therefore, there is a good possibility that berberine exerts the facilitating effect on GSIS through direct action of GK! As expected, our results showed that berberine also elevated GK activity dose-dependently in islets, exhibiting the strongest effect at the concentration of 30 μmol/L. Yet, still no significant difference was observed between islets treated with 1 μmol/L GB and the control, which agrees with a previous report indicating that GB exerted no regulatory effect on GK activity in isolated islets[28]. Thus, our data further support the hypothesis that GK plays a role in the stimulatory effect of berberine on GSIS.

It appears puzzling that discrepancies also existed despite the conspicuous correlation among insulin secretion and HNF4α expression and GK activity in general. It was demonstrated that 1 μmol/L berberine significantly increased insulin secretion, HNF4α gene and protein expressions, however, no enhancement of GK activity was observed. In contrast, 30 μmol/L berberine significantly increased GK activity, while both insulin secretion and HNF4α gene expression were at a normal level. As mentioned above, HNFs form a network, function solely or cooperatively to regulate the expression of multiple target genes that are important in the maintenance of metabolism homeostasis. It is conceivable, therefore, that not only HNF4α but also other HNFs such as HNF1α or HNF6 might participate in the modulation of GK activity. However, these need to be clarified further.

Taken together, our results suggest that berberine might exert its insulinotropic effect in isolated rat islets by up-regulating the expression of HNF4α, which probably acts solely or together with other HNFs to modulate GK activity, rendering β cell more sensitive to glucose fluctuation and response more effectively to glucose challenge. Interestingly, Ko *et al*[25] revealed that berberine facilitated GSIS in MIN6 cells partly *via* an enhanced insulin/insulin-like growth factor-1 (IGF-1) signaling cascade, which seems discrepant from the pathway we proposed. However, it is speculated that insulin signaling could interact with HNF-regulated transcription in beta cells, and insulin or IGF-1 act as potential upstream inductive signals regulating the HNFs and their target genes[9]. This might at least in part provide plausible explanation for the controversy.

In conclusion, our findings indicate that berberine enhances GSIS, rather than basal insulin secretion dose-dependently in isolated rat islets. This might partly be attributable to the up-regulation of HNF4α expression and GK activity by berberine. It is also suggested that HNF4α and GK might not participate in GB-induced insulin secretion. Berberine would be a promising insulin secretagogue which works through a mechanism distinct from SUs.

ACKNOWLEDGMENTS

We thank the staff of the Institute of Integrated Traditional and Western Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology for their help with this study.

COMMENTS

Background

Berberine, a main constituent isolated from Chinese herb *Rhizoma coptidis*, is gaining increasing attention, especially for its anti-diabetic properties, including improving insulin resistance, lowing blood glucose and modulating lipid metabolism. Recent researches further reveal that berberine also possesses insulinotropic action, yet, the molecular mechanism remains unclear.

Research frontiers

The transcriptional regulatory circuit of hepatic nuclear factors (HNFs) has recently been identified in pancreatic islets. And numerous evidences suggest an essential role for hepatic nuclear factor 4 alpha (HNF4α) in the proper secretary function of β cells. This study aims to elucidate if HNF4α underlies the mechanism of berberine's facilitating effect on insulin secretion.

Innovations and breakthroughs

In this study, the authors found that berberine could promote glucose-stimulated insulin secretion (GSIS) rather than basal insulin secretion in primary rat islets. Furthermore, they revealed that berberine might exert the insulinotropic effect through a mechanism involving HNF4α and glucokinase (GK), which is absolutely distinct from that of the widely used sulphonylureas (SUs).

Applications

The data suggest that berberine might be a promising insulin secretagogue which works *via* a unique mechanism in diabetes treatment.

Terminology

HNF4α is a transcriptional factor belonging to the hepatocyte nuclear factor family. It has been demonstrated that HNF4α is expressed mainly in liver and pancreatic islet tissue, regulating the transcription of multiple target genes implicated in glucose metabolism and insulin secretion. GK is known as "glucose sensor", modulating insulin secretion by controlling the rate of glycolysis in pancreatic β cells.

Peer review

It is a simple and elegant study. Authors observed the effect of berberine on insulin secretion by rat pancreatic islets and explored its possible molecular mechanism.

REFERENCES

1 **Groop LC**. Sulfonylureas in NIDDM. *Diabetes Care* 1992; **15**: 737-754
2 **Rabuazzo AM**, Buscema M, Vinci C, Caltabiano V, Vetri M, Forte F, Vigneri R, Purrello F. Glyburide and tolbutamide induce desensitization of insulin release in rat pancreatic islets by different mechanisms. *Endocrinology* 1992; **131**: 1815-1820
3 **Kawaki J**, Nagashima K, Tanaka J, Miki T, Miyazaki M, Gonoi T, Mitsuhashi N, Nakajima N, Iwanaga T, Yano H, Seino S. Unresponsiveness to glibenclamide during chronic treatment induced by reduction of ATP-sensitive K+ channel activity. *Diabetes* 1999; **48**: 2001-2006
4 **Kong W**, Wei J, Abidi P, Lin M, Inaba S, Li C, Wang Y, Wang Z, Si S, Pan H, Wang S, Wu J, Wang Y, Li Z, Liu J, Jiang JD. Berberine is a novel cholesterol-lowering drug working through a unique mechanism distinct from statins. *Nat Med* 2004; **10**: 1344-1351
5 **Lee YS**, Kim WS, Kim KH, Yoon MJ, Cho HJ, Shen Y, Ye JM, Lee CH, Oh WK, Kim CT, Hohnen-Behrens C, Gosby A, Kraegen EW, James DE, Kim JB. Berberine, a natural plant product, activates AMP-activated protein kinase with beneficial metabolic effects in diabetic and insulin-resistant states. *Diabetes* 2006; **55**: 2256-2264
6 **Yin J**, Gao Z, Liu D, Liu Z, Ye J. Berberine improves glucose metabolism through induction of glycolysis. *Am J Physiol Endocrinol Metab* 2008; **294**: E148-E156
7 **Leng SH**, Lu FE, Xu LJ. Therapeutic effects of berberine in impaired glucose tolerance rats and its influence on insulin secretion. *Acta Pharmacol Sin* 2004; **25**: 496-502
8 **Odom DT**, Zizlsperger N, Gordon DB, Bell GW, Rinaldi NJ, Murray HL, Volkert TL, Schreiber J, Rolfe PA, Gifford DK, Fraenkel E, Bell GI, Young RA. Control of pancreas and liver gene expression by HNF transcription factors. *Science* 2004; **303**: 1378-1381
9 **Kulkarni RN**, Kahn CR. Molecular biology. HNFs--linking the liver and pancreatic islets in diabetes. *Science* 2004; **303**: 1311-1312
10 **Bartoov-Shifman R**, Hertz R, Wang H, Wollheim CB, Bar-Tana J, Walker MD. Activation of the insulin gene promoter through a direct effect of hepatocyte nuclear factor 4 alpha. *J Biol Chem* 2002; **277**: 25914-25919
11 **Yamagata K**, Furuta H, Oda N, Kaisaki PJ, Menzel S, Cox NJ, Fajans SS, Signorini S, Stoffel M, Bell GI. Mutations in the hepatocyte nuclear factor-4alpha gene in maturity-onset diabetes of the young (MODY1). *Nature* 1996; **384**: 458-460
12 **Gupta RK**, Vatamaniuk MZ, Lee CS, Flaschen RC, Fulmer JT, Matschinsky FM, Duncan SA, Kaestner KH. The MODY1 gene HNF-4alpha regulates selected genes involved in insulin secretion. *J Clin Invest* 2005; **115**: 1006-1015
13 **Miura A**, Yamagata K, Kakei M, Hatakeyama H, Takahashi N, Fukui K, Nammo T, Yoneda K, Inoue Y, Sladek FM, Magnuson MA, Kasai H, Miyagawa J, Gonzalez FJ, Shimomura I. Hepatocyte nuclear factor-4alpha is essential for glucose-stimulated insulin secretion by pancreatic beta-cells. *J Biol Chem* 2006; **281**: 5246-5257
14 **Lacy PE**, Kostianovsky M. Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes* 1967; **16**: 35-39
15 **Gotoh M**, Maki T, Satomi S, Porter J, Bonner-Weir S, O'Hara CJ, Monaco AP. Reproducible high yield of rat islets by stationary in vitro digestion following pancreatic ductal or portal venous collagenase injection. *Transplantation* 1987; **43**: 725-730
16 **Latif ZA**, Noel J, Alejandro R. A simple method of staining fresh and cultured islets. *Transplantation* 1988; **45**: 827-830
17 **Chen XB**, Li YX, Jiao Y, Dong WP, Li G, Chen J, Tan JM. Influence of heme oxygenase-1 gene transfer on the viability and function of rat islets in in vitro culture. *World J Gastroenterol* 2007; **13**: 1053-1059
18 **Janjic D**, Wollheim CB. Islet cell metabolism is reflected by the MTT (tetrazolium) colorimetric assay. *Diabetologia* 1992; **35**: 482-485
19 **de Vargas LM**, Sobolewski J, Siegel R, Moss LG. Individual beta cells within the intact islet differentially respond to glucose. *J Biol Chem* 1997; **272**: 26573-26577
20 **Brissova M**, Fowler MJ, Nicholson WE, Chu A, Hirshberg B, Harlan DM, Powers AC. Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy. *J Histochem Cytochem* 2005; **53**: 1087-1097
21 **Bradford MM**. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248-254
22 **Aalinkeel R**, Srinivasan M, Kalhan SC, Laychock SG, Patel MS. A dietary intervention (high carbohydrate) during the neonatal period causes islet dysfunction in rats. *Am J Physiol* 1999; **277**: E1061-E1069
23 **Chen J**, Jeppesen PB, Abudula R, Dyrskog SE, Colombo M, Hermansen K. Stevioside does not cause increased basal insulin secretion or beta-cell desensitization as does the sulphonylurea, glibenclamide: studies in vitro. *Life Sci* 2006; **78**: 1748-1753
24 **Rustenbeck I**, Wienbergen A, Bleck C, Jorns A. Desensitization of insulin secretion by depolarizing insulin secretagogues. *Diabetes* 2004; **53** Suppl 3: S140-S150
25 **Ko BS**, Choi SB, Park SK, Jang JS, Kim YE, Park S. Insulin sensitizing and insulinotropic action of berberine from Cortidis rhizoma. *Biol Pharm Bull* 2005; **28**: 1431-1437
26 **German MS**. Glucose sensing in pancreatic islet beta cells: the key role of glucokinase and the glycolytic intermediates. *Proc Natl Acad Sci USA* 1993; **90**: 1781-1785
27 **Matschinsky F**, Liang Y, Kesavan P, Wang L, Froguel P, Velho G, Cohen D, Permutt MA, Tanizawa Y, Jetton TL. Glucokinase as pancreatic beta cell glucose sensor and diabetes gene. *J Clin Invest* 1993; **92**: 2092-2098
28 **Patane G**, Piro S, Anello M, Rabuazzo AM, Vigneri R, Purrello F. Exposure to glibenclamide increases rat beta cells sensitivity to glucose. *Br J Pharmacol* 2000; **129**: 887-892

S- Editor Li DL **L- Editor** Ma JY **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6012

World J Gastroenterol 2008 October 21; 14(39): 6012-6017
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Comparison of the chloride channel activator lubiprostone and the oral laxative Polyethylene Glycol 3350 on mucosal barrier repair in ischemic-injured porcine intestine

Adam J Moeser, Prashant K Nighot, Birgit Roerig, Ryuji Ueno, Anthony T Blikslager

Adam J Moeser, Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh NC 27606, United States
Prashant K Nighot, Anthony T Blikslager, Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh NC 27606, United States
Birgit Roerig, Ryuji Ueno, Sucampo Pharmaceuticals, Inc, Bethesda MD 20814, United States
Author contributions: Moeser AJ and Blikslager AT wrote the paper; Moeser AJ and Nighot PK performed the research; Roerig B and Ueno R contributed new reagents and interpreted data; Moeser AJ and Blikslager AT designed the research.
Correspondence to: Dr. Anthony T Blikslager, College of Veterinary Medicine, North Carolina State University, 4700 Hillsborough Street, Raleigh NC 27606, United States. anthony_blikslager@ncsu.edu
Telephone: +1-919-5137725 Fax: +1-919-5136336
Received: June 30, 2008 Revised: August 18, 2008
Accepted: August 25, 2008
Published online: October 21, 2008

Abstract

AIM: To investigate the effects of lubiprostone and Polyethylene Glycol 3350 (PEG) on mucosal barrier repair in ischemic-injured porcine intestine.

METHODS: Ileum from 6 piglets (approximately 15 kg body weight) was subjected to ischemic conditions by occluding the local mesenteric circulation for 45 min *in vivo*. Ileal tissues from each pig were then harvested and mounted in Ussing chambers and bathed in oxygenated Ringer's solution *in vitro*. Intestinal barrier function was assessed by measuring transepithelial electrical resistance (TER) and mucosal-to-serosal fluxes of ^{3}H-mannitol and ^{14}C-inulin. Statistical analyses of data collected over a 120-min time course included 2-way ANOVA for the effects of time and treatment on indices of barrier function.

RESULTS: Application of 1 μmol/L lubiprostone to the mucosal surface of ischemic-injured ileum *in vitro* induced significant elevations in TER compared to non-treated tissue. Lubiprostone also reduced mucosal-to-serosal fluxes of ^{3}H-mannitol and ^{14}C-inulin. Alternatively, application of a polyethylene laxative (PEG, 20 mmol/L) to the mucosal surface of ischemic tissues significantly increased flux of ^{3}H-mannitol and ^{14}C-inulin.

CONCLUSION: This experiment demonstrates that lubiprostone stimulates recovery of barrier function in ischemic intestinal tissues whereas the PEG laxative had deleterious effects on mucosal repair. These results suggest that, unlike osmotic laxatives, lubiprostone stimulates repair of the injured intestinal barrier.

Key words: Intestinal ischemia; Barrier function; Permeability; Laxative; Polyethylene Glycol

Peer reviewer: Henrike Hamer, PhD, Department of Internal Medicine, Division of Gastroenterology (Box 46), Maastricht University, PO Box 616, 6200 MD Maastricht, The Netherlands

Moeser AJ, Nighot PK, Roerig B, Ueno R, Blikslager AT. Comparison of the chloride channel activator lubiprostone and the oral laxative Polyethylene Glycol 3350 on mucosal barrier repair in ischemic-injured porcine intestine. *World J Gastroenterol* 2008; 14(39): 6012-6017 Available from: URL: http://www.wjgnet.com/1007-9327/14/6012.asp DOI: http://dx.doi.org/10.3748/wjg.14.6012

INTRODUCTION

Ischemic intestinal disorders including intestinal volvulus, thromboembolic disease, and low flow states associated with shock, have a high mortality rate due to the rapid onset of sepsis and multiple organ failure[1-3]. Intestinal ischemic lesions are characterized by sloughing of the apical villus epithelium and rapid breakdown of mucosal barrier function[2,4,5], accompanied by increased intestinal permeability and subsequent bacterial translocation, sepsis, and multiple organ dysfunction syndrome (MODS)[6-8]. Rapid restoration of the compromised intestinal barrier is critical for patient survival. However, limited treatment options are available that target mucosal barrier repair[9].

Lubiprostone, an FDA-approved laxative (Amitiza, Sucampo Pharmaceuticals, Inc.) was previously shown to stimulate rapid repair of intestinal barrier function in ischemic-injured porcine ileum[10]. Lubiprostone activates ClC-2 Cl^- channels resulting in luminal Cl^- secretion and water movement responsible for its laxative properties[11-15]. ClC-2 Cl^- channel activation

by lubiprostone is also the predominant mechanism by which this compound stimulates repair of the tight junctions and mucosal barrier repair in ischemic tissues[10]. The mechanism for this action may relate to co-localization of ClC-2 with tight junction proteins such as occludin[16,17]. Other commercially available laxative agents such as high molecular weight Polyethylene Glycol (PEG 3350) also induce fluid movement into the lumen *via* different mechanisms. Polyethylene laxatives are composed of high molecular weight PEG which triggers an osmotic gradient in the lumen serving to draw ions and water from the paracellular space. In addition to its laxative effects, PEG agents have been shown to confer mucosal protective effects in various gastrointestinal injury animal models including 2, 4, 6-trinitrobenzene sulphonic acid (TNBS)-induced colitis[18] and bacterial translocation and sepsis induced by surgical stress[19].

Given the potential alternative beneficial roles of these two oral laxatives in intestinal injury and repair, the objective of this study was to compare the effects of lubiprostone and PEG 3350 on repair of mucosal barrier function in ischemic-injured intestine.

MATERIALS AND METHODS

Compounds

Lubiprostone was obtained from R-Tech Ueno (Sanda, Japan). PEG 3350 (Miralax, Schering-Plough Health Care Products, Inc. Kenilworth NJ) was obtained from the North Carolina State University, College of Veterinary Medicine pharmacy. ^{3}H-mannitol and ^{14}C-inulin were obtained from Sigma Chemical (St. Louis, MO).

Experimental animal surgeries

All studies were approved by the North Carolina State University Institutional Animal Care and Use Committee. Six to eight-week-old Yorkshire crossbred pigs of either sex were housed individually, and maintained on a commercial pelleted feed. Pigs were fasted for 24 h prior to experimental surgery. General anesthesia was induced with xylazine (1.5 mg/kg, IM), ketamine (11 mg/kg, IM), and 5% isoflurane vaporized in 1000 mL/L O_2 and was maintained with 2% isoflurane delivered *via* an endotracheal tube. Pigs were placed on a heating pad and ventilated with 1000 mL/L O_2 using a volume-limited, time-cycled ventilator (Hallowell, Pittsfield, MA). Lactated Ringers solution was administered iv at a maintenance rate of 15 mL/kg per hour. The ileum was approached *via* a ventral midline incision. Ileal segments were delineated by ligating the intestine at 10-cm intervals, and subjected to ischemia by occluding the local mesenteric blood supply for 45 min.

Using chamber studies

Following the 45-min ischemic period, tissues were harvested from the pig and the mucosa was stripped from the seromuscular layer in oxygenated (950 mL/L O_2/50 mL/L CO_2) Ringer's solution (mmol/L: Na^+, 154; K^+, 6.3; Cl^-, 137; HCO_3^-, 24; pH 7.4) containing 5 μmol/L indomethacin to prevent endogenous prostaglandin production during the stripping procedure. Tissues were then mounted in 1.14 cm^2 aperture Ussing chambers, as described in previous studies. For Ussing chamber experiments, ileal tissues from one pig were mounted on multiple Ussing chambers and subjected to different *in vitro* treatments. Data means are representative of 6 Ussing chamber experiments (n = 6 animals). Tissues were bathed on the serosal and mucosal sides with 10 mL Ringer's solution. The serosal bathing solution contained 10 mmol/L glucose, and was osmotically balanced on the mucosal side with 10 mmol/L mannitol. Bathing solutions were oxygenated (950 mL/L O_2/50 mL/L CO_2) and circulated in water-jacketed reservoirs. The spontaneous potential difference (PD) was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through Ag-AgCl electrodes using a voltage clamp that corrected for fluid resistance. Transepithelial electrical resistance (TER) ($\Omega.cm^2$) was calculated from the spontaneous PD and short-circuit current (I_{sc}). If the spontaneous PD was between -1.0 and 1.0 mV, tissues were current-clamped at ± 100 μA for 5 s and the PD recorded. I_{sc} and PD were recorded at 15-min intervals over a 120-min experiment.

Experimental treatments

After tissues were mounted on Ussing chambers, tissues were allowed to acclimate for 30 min to achieve stable baseline measurements after which experimental treatments were added. Lubiprostone (1 μmol/L) or PEG 3350 (20 mmol/L) were added to the mucosal side of tissues and TER and I_{sc} were measured at 15-min intervals over a 120-min recovery period. The PEG 3350 dose was selected as it is the recommended oral dosage for laxative properties and thus would approximate the luminal concentrations attained *in vivo*.

Mucosal-to-serosal fluxes of radiolabeled paracellular probes

To assess mucosal permeability after experimental treatments, 0.2 μCi/mL ^{3}H-labeled mannitol (180 kDa) and 0.2 μCi/mL, ^{14}C-labeled inulin (5000 kDa) were added to the mucosal side of tissues mounted in Ussing chambers. After a 15-min equilibration period, standards were taken from the mucosal side of each chamber and a 60-min flux period was established by taking 0.5 mL samples from the serosal compartment. The presence of ^{3}H and ^{14}C was established by measuring β-emission in a liquid-scintillation counter (LKB Wallac, Model 1219 Rack Beta, Perkin Elmer Life and Analytical Sciences, Inc., Boston, MA). Unidirectional mannitol fluxes from mucosa-to-serosa were determined using standard equations.

Histological examination

Tissues were taken at 0 and 120 min for routine histological evaluation. Tissues were sectioned (5 μm)

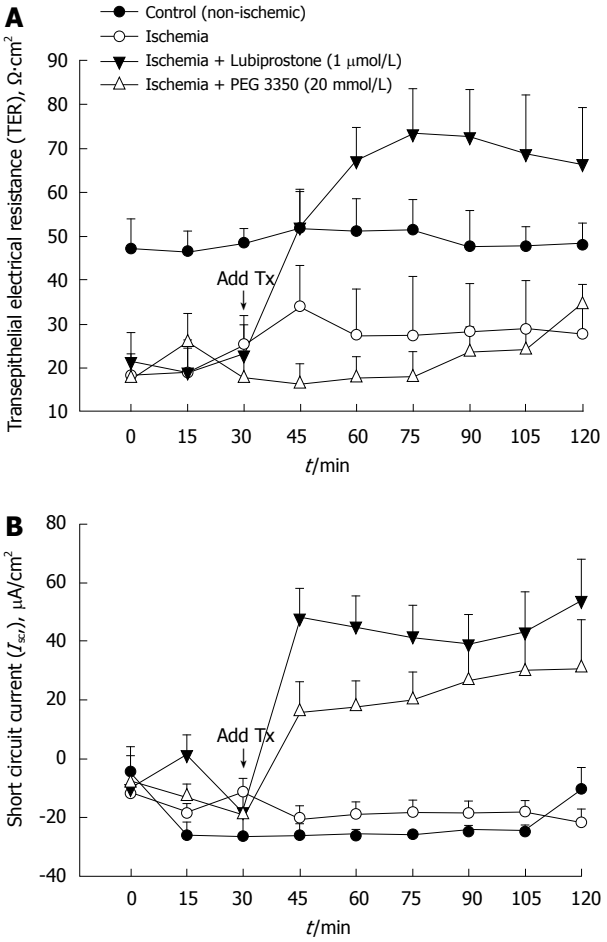


Figure 1 TER (**A**) and short circuit current (I_{sc}) (**B**) in ischemic porcine ileum treated with lubiprostone and PEG 3350.

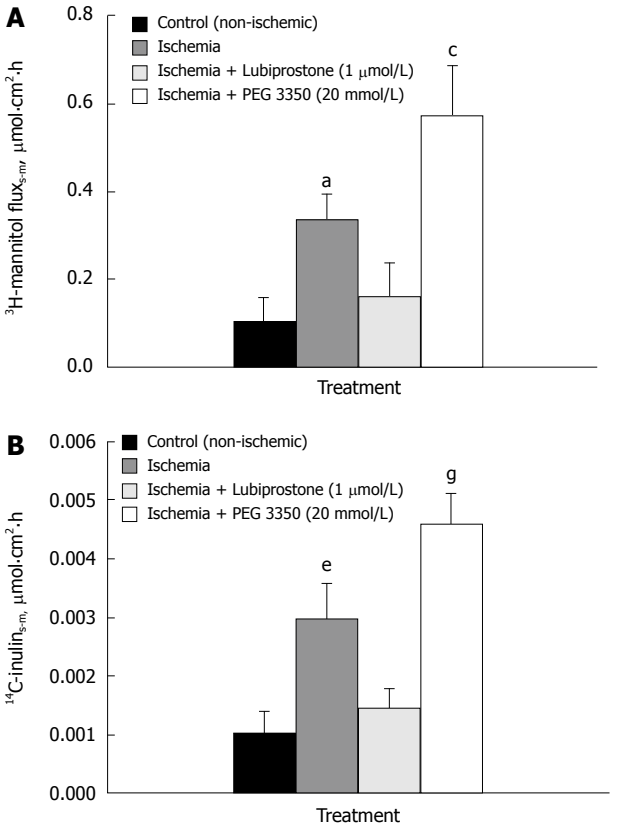


Figure 2 **A**: Mucosal-to-serosal flux of ^{3}H-mannitol in porcine ileum. $^aP < 0.05$ ischemia *vs* control, ischemia/lubiprostone; $^cP < 0.05$ ischemia + PEG3350 *vs* control, ischemia, ischemia + lubiprostone; **B**: Mucosal-to-serosal flux of ^{14}C-inulin in porcine ileum. $^eP < 0.05$ ischemia *vs* control, ischemia/lubiprostone; $^gP < 0.05$ ischemia + PEG3350 *vs* control, ischemia, ischemia/lubiprostone.

and stained with hematoxylin and eosin. For each tissue, 3 sections were evaluated. Four well-oriented villi and crypts were identified in each section. Villus length was obtained using a micrometer in the eye piece of a light microscope.

Statistical analysis

All data were reported as mean ± SE. TER and I_{sc} data were analyzed by using an ANOVA for repeated measures. Radiolabeled flux data was analyzed by using a standard one-way ANOVA (Sigmastat, Jandel Scientific, San Rafael, CA). A Tukey's test was used to determine differences between treatments following ANOVA.

RESULTS

TER and I_{sc} in ischemia-injured porcine ileal mucosa treated with lubiprostone or PEG 3350

Porcine ileum was subjected to 45 min of acute mesenteric ischemia and mounted on Ussing chambers for measurement of TER and I_{sc} over a 120-min recovery period. Ischemic-injured mucosa had significantly lower starting TER values (by approximately 40%) compared with non-ischemic control tissue (Figure 1), indicating significant impairment of intestinal barrier function induced by ischemia. Application of 1 μmol/L lubiprostone to the mucosal side of ischemic-injured mucosa induced rapid elevations in TER that attained non-ischemic control tissues within 15 min of its addition and TER continued to increase 45 min post-treatment. Lubiprostone stimulated rapid elevations in I_{sc} (an index of electrogenic ion transport) that peaked at 15 min post-treatment (peak ΔI_{sc} = 66 μA/cm^2) and remained elevated throughout the remainder of the experiment. Mucosal addition of 20 mmol/L PEG 3350 stimulated a transient increase in TER measured 15 min after treatment; however, TER returned to ischemic control levels within 30 min post-treatment. PEG 3350 stimulated significant elevations in I_{sc} (peak ΔI_{sc} = 35 μA/cm^2) compared with ischemic control tissues. In non-ischemic ileal tissues, lubiprostone and PEG 3350 induced similar elevations in TER (ΔTER = 30% ± 7% and 36% ± 8% in lubiprostone and PEG 3350-treated tissues) (data not shown).

Mucosal-to-serosal flux of paracellular probes

Mucosal-to-serosal flux of both medium molecular weight (^{3}H-mannitol, 180 kDa) and large molecular weight (^{14}C-Inulin, 5000 kDa) paracellular probes in ileal tissues mounted in Ussing chambers were conducted as an alternative measurement of mucosal permeability. In line with TER responses, ischemic tissues had greater (P < 0.01) serosal-to-mucosal fluxes of both ^{3}H-mannitol and ^{14}C-inulin (Figure 2). Lubiprostone treatment significantly

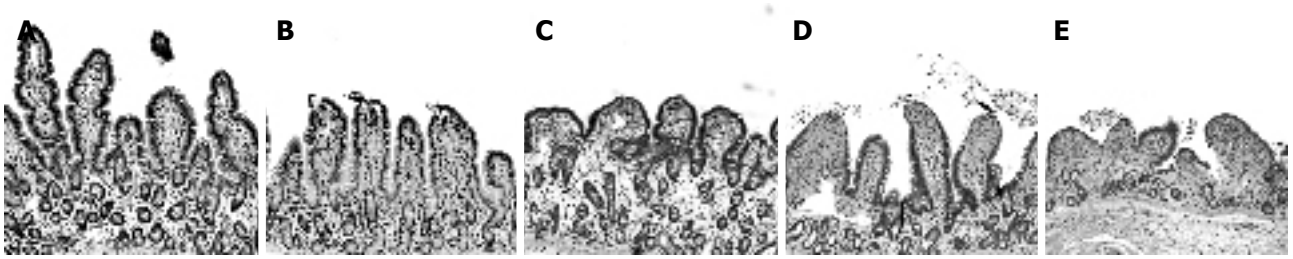


Figure 3 Light microscopic evaluation of ischemic intestinal tissues. **A**: Control (non-ischemic); **B**: Time 0 post-ischemia; **C**: Time 120-min post ischemia; **D**: Lubiprostone-time 120 min post-ischemia; **E**: -PEG 3350-time 120-min post-ischemia.

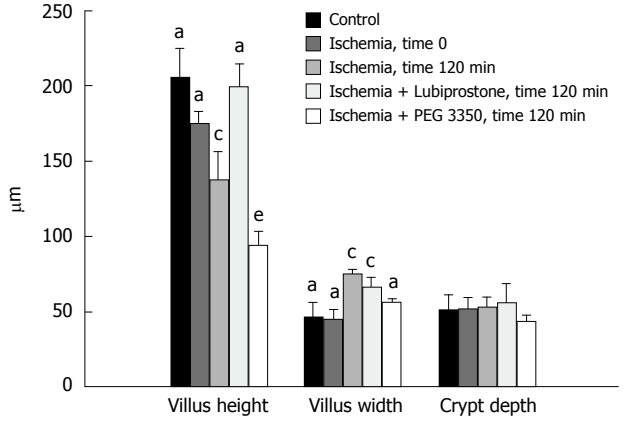


Figure 4 Morphometric evaluations of ischemic intestinal tissues. Villus height: $^{a}P < 0.05$ control, ischemia time 0 min, lubiprostone *vs* ischemia time 120 min, PEG 3350; $^{c}P < 0.05$ ischemia time 120 min *vs* all other groups; $^{e}P < 0.05$ PEG 3350 *vs* all other groups; Villus width: $^{a}P < 0.05$ control, ischemia time 0 min *vs* ischemia time 120 min, lubiprostone; $^{c}P < 0.05$ ischemia time 120 min, lubiprostone *vs* all other groups.

decreased the fluxes of ^{3}H-mannitol and ^{14}C-inulin ($P < 0.05$). Alternatively, ischemic tissues treated with PEG 3350 displayed significantly increased fluxes of permeability markers compared with all other treatments ($P < 0.05$).

Histological evaluation of ischemic-injured tissues

Control (non-ischemic) tissues treated with lubiprostone or PEG 3350 had no identifiable histopathological findings compared with non-treated control tissues (not shown). Ileal tissues subjected to ischemia displayed classic histological ischemic lesions characterized by sloughing of the surface epithelium of the apical villus (Figure 3). The epithelial layer was completely restituted after 60 min of mounting tissues on Ussing chambers (not shown); this effect has been demonstrated in our previous studies[4,5]. At 120 min post-ischemia on Ussing chambers, villus contraction was evident demonstrated by villi that were reduced in height and increased in width compared with non-ischemic control tissues (Figure 4). Laxative agents had a significant effect on intestinal villous length in ischemic-injured tissues in this study. Lubiprostone-treated tissues had greater villous lengths compared with ischemic-injured controls when measured at 120 min post-ischemia. In contrast, PEG 3350-treated ischemic tissues had significantly reduced villous height compared with ischemic-injured control tissues.

DISCUSSION

Results from the present study demonstrate marked differences in the ability of two oral laxatives, lubiprostone and PEG 3350, to stimulate repair of intestinal barrier function in ischemic-injured porcine ileum. Lubiprostone stimulated rapid repair of mucosal barrier function in ischemic ileal mucosa as defined by rapid elevations in TER and reductions in the mucosal-to-serosal flux of ^{3}H-mannitol and ^{14}C-inulin. PEG 3350 failed to induce significant changes in TER and had a detrimental influence on mucosal barrier repair evidenced by enhanced mucosal permeability to ^{3}H-mannitol and ^{14}C-inulin in ischemic-injured tissues treated with PEG 3350.

Oral lubiprostone and PEG 3350 both have laxative properties *via* different mechanisms. Lubiprostone activates ClC-2 Cl^- secretion promoting Na^+ and water movement into the lumen[20,21], whereas PEG 3350 induces luminal osmotic effects drawing electrolytes and water into the lumen. This is the reason that both compounds induced significant increases in short circuit current in the Ussing chambers, an indirect measure of ionic movement across the mucosa. Alternatively, there was no change in short circuit current in untreated tissues. Lubiprostone also stimulated rapid repair of mucosal barrier function in ischemic ileal tissues. Previous studies showed that lubiprostone's influence on intestinal permeability is due to its ability to activate ClC-2 channels[10]. Although exact signaling events triggered by ClC-2 activation that lead to intestinal repair are not well understood, lubiprostone treatment was shown to trigger rapid recruitment of the tight junction protein occludin to the apical intercellular space, an event critical for the re-establishment of mucosal electrical resistance. In the present study, lubiprostone increased baseline TER in non-injured control tissues, suggesting that lubiprostone's effect is not restricted to injured mucosa.

To our knowledge, the influence of PEG 3350 on intestinal mucosal repair has not been directly investigated. However, PEG compounds have been shown to be protective against different forms of intestinal injury. Videla *et al*[18] demonstrated that oral PEG 4000 was protective against TNBS-induced colitis in rats. In a study by Wu *et al*[19], luminal administration of a high molecular weight PEG prevented increases in intestinal permeability induced by *P. aerugenosa* in Caco-2 monolayers and prevented lethal sepsis *in vivo*

induced by *P. aerugenosa* following surgical stress. In the latter study, PEG 4000 treatment resulted in increased mucosal hydrophobicity and reductions in baseline mucosal permeability. The reduction in permeability seen with PEG is likely attributable to its osmotic effects which may draw water from the paracellular space resulting in collapse of the tight junctions. Madara JL demonstrated that mucosal osmotic loads of 600 mosM induced rapid elevations in TER in guinea pig jejunum in Ussing chambers, an effect mediated by decreased cation selectivity of the tight junctions and alterations in the cytoskeleton[22]. In the present study, PEG 3350 induced increases in TER (by 36% ± 8%) in control (non-injured) ileal tissues. However, in the present study PEG 3350 was ineffective in improving TER in ischemic tissues and further increased mucosal permeability in ischemic-injured tissues. This suggests intact barrier function is required for PEG to stimulate increases in TER. In ischemic tissues, it is likely that PEG 3350 would freely traverse the damaged epithelium and equilibrate with the serosal compartment failing to produce an osmotic gradient in tissues.

Villous contraction in response to ischemic injury is a protective mechanism that aids in reducing the surface area of the denuded basement membrane, allowing epithelial cells adjacent to the injury to migrate and cover the epithelial defect and restore epithelial continuity[23-26]. In the present study, lubiprostone-treated ischemic ileal tissues had significantly greater villous lengths compared with other ischemic tissues. It is unclear whether this response was due to lubiprostone' s ability to inhibit villous contraction or stimulate villous lengthening during repair. Lubiprostone could have had a direct action on the principal contractile cells within the villus: myofibroblasts. These cells are arranged in linked chains of cells adjacent to the central lacteal and subjacent to the epithelial basement membrane. More studies, including a detailed time course of lubiprostone's effects on villous architecture during recovery of ischemic injury, are required to determine this and lubiprostone's overall relevance to intestinal barrier repair. In contrast to lubiprostone, PEG 3350-treated ileal tissues had significantly shorter villi compared with ischemic controls measured 120 min post-ischemic injury. This may represent ongoing injury in these tissues supported by increased paracellular permeability induced by PEG 3350.

Overall, this study demonstrates that lubiprostone stimulates recovery of mucosal barrier function in ischemic intestinal tissues, whereas the PEG laxative enhanced intestinal permeability. These results suggest that, unlike osmotic laxatives, lubiprostone stimulates repair of the injured intestinal barrier.

COMMENTS

Background

A number of important intestinal diseases, including ischemia/reperfusion injury, are characterized by damage to the epithelium lining the gut. Mechanisms are in place to rapidly repair epithelial defects, including epithelial migration (restitution). More recently, studies have shown the importance of the interepithelial tight junctions in recovery of the epithelial barrier. Studies have shown that prostaglandins and prostones increase the rate of epithelial recovery *via* re-assembly of tight junctions.

Research frontiers

The prostone lubiprostone, a new medication on the market indicated for treatment of chronic constipation and irritable bowel syndrome has its effect on chloride channels (ClC-2) within tight junctions. These channels are involved in secretion of chloride in the intestine, but to a far lesser extent than the chloride channel that is genetically disrupted in patients with cystic fibrosis (CFTR). Recent studies have shown that ClC-2, when activated by prostaglandins or prostones such as lubiprostone, also play an important role in re-assembly of tight junctions, resulting in increases in the speed of epithelial repair.

Innovations and breakthroughs

Stimulation of one of the minor chloride channels, ClC-2, is an innovative way to induce low level secretion into the gut, thereby serving as a laxative. This is in contrast to traditional laxatives such as Polyethylene Glycol 3350 (PEG), which result in increased fluid in the intestinal lumen because of its osmotic properties. Other effects of ClC-2 activation by lubiprostone have recently been discovered, particularly the ability to increase mucosal repair. A comparison of lubiprostone and PEG showed that only lubiprostone facilitated the repair of the mucosa. This increased repair could not be seen at the histological level. This is consistent with prior studies showing that enhanced repair is noted at the level of tight junctions.

Applications

When treating constipation, the choice of medication includes laxatives such as PEG and the ClC-2 activator lubiprostone. The present study suggests that use of lubiprostone will also hasten the recovery of injured gut mucosa in patients with more severe intestinal disease. Further basic science research followed by clinical trials will be needed to determine the validity of these findings.

Terminology

The term ClC-2 is used to describe a chloride channel in the gut epithelium that is localized to interepithelial tight junctions. The term prostone refers to a new group of compounds which are distinct from prostaglandins and specifically activate ClC-2.

Peer review

The present study was performed using porcine tissues; it demonstrates that lubiprostone stimulates recovery of barrier function in ischemic intestinal tissues whereas the PEG laxative had deleterious effects on mucosal repair. This is an interesting study and well written manuscript.

REFERENCES

1 **Deitch EA**, Rutan R, Waymack JP. Trauma, shock, and gut translocation. *New Horiz* 1996; **4**: 289-299
2 **American Gastroenterological Association Medical Position Statement: guidelines on intestinal ischemia**. *Gastroenterology* 2000; **118**: 951-953
3 **Boros M**. Microcirculatory dysfunction during intestinal ischemia-reperfusion. *Acta Physiol Hung* 2003; **90**: 263-279
4 **Blikslager AT**, Roberts MC, Argenzio RA. Prostaglandin-induced recovery of barrier function in porcine ileum is triggered by chloride secretion. *Am J Physiol* 1999; **276**: G28-G36
5 **Blikslager AT**, Roberts MC, Young KM, Rhoads JM, Argenzio RA. Genistein augments prostaglandin-induced recovery of barrier function in ischemia-injured porcine ileum. *Am J Physiol Gastrointest Liver Physiol* 2000; **278**: G207-G216
6 **Doig CJ**, Sutherland LR, Sandham JD, Fick GH, Verhoef M, Meddings JB. Increased intestinal permeability is associated with the development of multiple organ dysfunction syndrome in critically ill ICU patients. *Am J Respir Crit Care Med* 1998; **158**: 444-451
7 **Faries PL**, Simon RJ, Martella AT, Lee MJ, Machiedo GW. Intestinal permeability correlates with severity of injury in trauma patients. *J Trauma* 1998; **44**: 1031-1035; discussion 1035-1036
8 **Stechmiller JK**, Treloar D, Allen N. Gut dysfunction in critically ill patients: a review of the literature. *Am J Crit Care* 1997; **6**: 204-209
9 **Blikslager AT**. Treatment of gastrointestinal ischemic

injury. *Vet Clin North Am Equine Pract* 2003; **19**: 715-727
10 **Moeser AJ**, Nighot PK, Engelke KJ, Ueno R, Blikslager AT. Recovery of mucosal barrier function in ischemic porcine ileum and colon is stimulated by a novel agonist of the ClC-2 chloride channel, lubiprostone. *Am J Physiol Gastrointest Liver Physiol* 2007; **292**: G647-G656
11 **Baker DE**. Lubiprostone: a new drug for the treatment of chronic idiopathic constipation. *Rev Gastroenterol Disord* 2007; **7**: 214-222
12 **Lubiprostone: RU 0211, SPI 0211**. *Drugs R D* 2005; **6**: 245-248
13 **Cuppoletti J**, Malinowska DH, Tewari KP, Li QJ, Sherry AM, Patchen ML, Ueno R. SPI-0211 activates T84 cell chloride transport and recombinant human ClC-2 chloride currents. *Am J Physiol Cell Physiol* 2004; **287**: C1173-C1183
14 **Lubiprostone (Amitiza) for irritable bowel syndrome with constipation**. *Med Lett Drugs Ther* 2008; **50**: 53-54
15 **Dorn SD**, Ringel Y. Lubiprostone: easing the strain of constipation? *Gastroenterology* 2008; **134**: 355-357
16 **Gyomorey K**, Yeger H, Ackerley C, Garami E, Bear CE. Expression of the chloride channel ClC-2 in the murine small intestine epithelium. *Am J Physiol Cell Physiol* 2000; **279**: C1787-C1794
17 **Moeser AJ**, Haskell MM, Shifflett DE, Little D, Schultz BD, Blikslager AT. ClC-2 chloride secretion mediates prostaglandin-induced recovery of barrier function in ischemia-injured porcine ileum. *Gastroenterology* 2004; **127**: 802-815
18 **Videla S**, Lugea A, Vilaseca J, Guarner F, Treserra F, Salas A, Crespo E, Medina C, Malagelada JR. Polyethylene glycol enhances colonic barrier function and ameliorates experimental colitis in rats. *Int J Colorectal Dis* 2007; **22**: 571-580
19 **Wu L**, Zaborina O, Zaborin A, Chang EB, Musch M, Holbrook C, Shapiro J, Turner JR, Wu G, Lee KY, Alverdy JC. High-molecular-weight polyethylene glycol prevents lethal sepsis due to intestinal Pseudomonas aeruginosa. *Gastroenterology* 2004; **126**: 488-498
20 **Chang HY**, Kelly EC, Lembo AJ. Current gut-directed therapies for irritable bowel syndrome. *Curr Treat Options Gastroenterol* 2006; **9**: 314-323
21 **Lacy BE**, Levy LC. Lubiprostone: a chloride channel activator. *J Clin Gastroenterol* 2007; **41**: 345-351
22 **Madara JL**. Increases in guinea pig small intestinal transepithelial resistance induced by osmotic loads are accompanied by rapid alterations in absorptive-cell tight-junction structure. *J Cell Biol* 1983; **97**: 125-136
23 **Blikslager AT**, Moeser AJ, Gookin JL, Jones SL, Odle J. Restoration of barrier function in injured intestinal mucosa. *Physiol Rev* 2007; **87**: 545-564
24 **Moore R**, Carlson S, Madara JL. Villus contraction aids repair of intestinal epithelium after injury. *Am J Physiol* 1989; **257**: G274-G283
25 **Erickson RA**, Tarnawski A, Dines G, Stachura J. 16,16-Dimethyl prostaglandin E2 induces villus contraction in rats without affecting intestinal restitution. *Gastroenterology* 1990; **99**: 708-716
26 **Blikslager AT**, Roberts MC. Mechanisms of intestinal mucosal repair. *J Am Vet Med Assoc* 1997; **211**: 1437-1441

S- Editor Li DL **L- Editor** Negro F **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6018

World J Gastroenterol 2008 October 21; 14(39): 6018-6023
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Management of recurrent rectal cancer: A population based study in greater Amsterdam

Roel Bakx, Otto Visser, Judith Josso, Sybren Meijer, J Frederik M Slors, J Jan B van Lanschot

Roel Bakx, J Frederik M Slors, J Jan B van Lanschot, Department of Surgery, Academic Medical Centre, Amsterdam AZ 1105, Netherlands
Otto Visser, Judith Josso, Comprehensive Cancer Centre Amsterdam, Amsterdam CX 1066, Netherlands
Sybren Meijer, Department of Surgery, Free University Medical Centre, Amsterdam HV 1081, Netherlands
J Jan B van Lanschot, Presently at the Erasmus Medical Center, Rotterdam CE 3015, Netherlands
Author contributions: Bakx R and Visser O contributed equally to this work, wrote the manuscript, analyzed the data and designed the research project; Josso J was responsible for the data-collection; Meijer S and van Lanschot JJB designed the research project and corrected the manuscript.
Correspondence to: Dr. Roel Bakx, Academic Medical Center, Department of Surgery, Meibergdreef 9, G4-137, Amsterdam AZ 1105, Netherlands. r.bakx@amc.uva.nl
Telephone: +31-20-5669111 Fax: +31-20-6914858
Received: March 6, 2008 Revised: September 23, 2008
Accepted: September 30, 2008
Published online: October 21, 2008

Abstract

AIM: To analyze, retrospectively in a population-based study, the management and survival of patients with recurrent rectal cancer initially treated with a macroscopically radical resection obtained with total mesorectal excision (TME).

METHODS: All rectal carcinomas diagnosed during 1998 to 2000 and initially treated with a macroscopically radical resection (632 patients) were selected from the Amsterdam Cancer Registry. For patients with recurrent disease, information on treatment of the recurrence was collected from the medical records.

RESULTS: Local recurrence with or without clinically apparent distant dissemination occurred in 62 patients (10%). Thirty-two patients had an isolated local recurrence. Ten of these 32 patients (31%) underwent radical re-resection and experienced the highest survival (three quarters survived for at least 3 years). Eight patients (25%) underwent non-radical surgery (median survival 24 mo), seven patients (22%) were treated with radio- and/or chemotherapy without surgery (median survival 15 mo) and seven patients (22%) only received best supportive care (median survival 5 mo). Distant dissemination occurred in 124 patients (20%) of whom 30 patients also had a local recurrence. The majority (54%) of these patients were treated with radio- and/or chemotherapy without surgery (median survival 15 mo). Twenty-seven percent of these patients only received best supportive care (median survival 6 mo), while 16% underwent surgery for their recurrence. Survival was best in the latter group (median survival 32 mo).

CONCLUSION: Although treatment options and survival are limited in case of recurrent rectal cancer after radical local resection obtained with TME, patients can benefit from additional treatment, especially if a radical resection is feasible.

Key words: Rectal cancer; Total mesorectal excision; Local recurrence; Relative survival

Peer reviewer: Bernardino Rampone, PhD, Department of General Surgery and Surgical Oncology, University of Siena, viale Bracci, Siena 53100, Italy

Bakx R, Visser O, Josso J, Meijer S, Slors JFM, van Lanschot JJB. Management of recurrent rectal cancer: A population based study in greater Amsterdam. *World J Gastroenterol* 2008; 14(39): 6018-6023 Available from: URL: http://www.wjgnet.com/1007-9327/14/6018.asp DOI: http://dx.doi.org/10.3748/wjg.14.6018

INTRODUCTION

Colorectal cancer is the second most common cancer in the Western world and approximately one third of these tumours are located in the rectum or rectosigmoid[1]. Annually, over 3000 patients are registered with a newly diagnosed rectal or rectosigmoid carcinoma in the Netherlands[2,3]. In these patients, locally recurrent disease is a major concern and is often accompanied with intractable pain and severely disabling complications which are difficult to treat[4-6]. It has a tremendous impact on quality of life[7] and frequently induces an awful last period of a patient's life. Therefore, the focus in rectal cancer research has been on the prevention of locally recurrent disease, which resulted in the introduction of preoperative radiotherapy and total mesorectal excision (TME)[8-12].

There are many reports on the treatment of

recurrent rectal cancer[4,6,13-16]. However, these reports present mainly results from randomised clinical trials or specialised institutes, which are known to be biased[17]. There are only a few population-based reports on the treatment of locally recurrent rectal cancer disease[13,18], although they are probably the best reflection of daily practice.

In 1996, TME was introduced in Greater Amsterdam, the region of the Comprehensive Cancer Centre Amsterdam (CCCA). Its introduction was facilitated by the CCCA. Surgeons were supervised by teacher-surgeons in order to qualify as TME-surgeon and a documentation project was started to investigate the influence of TME-surgery on the incidence of local recurrences and survival[19]. From 1998 on, all patients in Greater Amsterdam are treated with TME in case of rectal resection.

The aim of the present study was to analyze, retrospectively in a population-based setting, the management and survival of patients with recurrent rectal cancer, initially treated with macroscopically radical local resection obtained with TME.

MATERIALS AND METHODS

Cancer registry data

All primary rectal carcinomas (rectosigmoid excluded) diagnosed in patients with residence in Greater Amsterdam, the region of the CCCA, between January 1, 1998 and December 31, 2000, and who underwent a macroscopically radical resection obtained with TME in the absence of distant dissemination, were selected from the Amsterdam Cancer Registry of the CCCA. The Amsterdam Cancer Registry is a regional, population-based cancer registry with complete regional coverage. Non-epithelial cancers, carcinoids and cases with preceding invasive cancers were excluded. The population of the region amounted to 2.8 million inhabitants on December 31, 2000, approximately 17% of the total population of the Netherlands.

The information for the cancer registry is routinely extracted from detailed hospital and outpatient clinic records by registration clerks. Apart from demographic data, data are collected on morphological classification, stage of the tumour and primary treatment of the patients. The TNM system for classification of malignant tumours is prospectively registered to classify all rectal carcinomas. Stage grouping in this study was performed according to the 6th edition of the TNM-classification[20], based on the available information after surgery (pTNM).

Of the selected cases, a supplementary data set was extracted from the medical records. This data set included the occurrence and the date of local recurrence or distant dissemination. Local recurrence was defined as cancer recurrence within the lower pelvis. Additional treatment of recurrence, the presence of microscopic or macroscopic residual disease after salvage surgery for recurrent disease, the date of salvage surgery and the cause of death were also collected. Cases were generally followed for five, but at least three years after the date of initial surgery.

Table 1 Initially applied radiotherapy in surgically treated, primary rectal carcinoma patients according to pTNM-stage in Greater Amsterdam, the Netherlands, 1998 to 2000

Stage of disease	Number of cases (% of total)	Radiotherapy, number of patients (%)		
		No RT	Postoperative RT	Preoperative RT
I	209 (33)	115 (55)	1 (0)	93 (45)
II A	180 (28)	72 (40)	26 (14)	82 (46)
II B	20 (3)	2 (10)	6 (30)	12 (60)
III A	32 (5)	8 (25)	11 (34)	13 (41)
III B	113 (18)	26 (23)	32 (28)	55 (49)
III C	72 (11)	13 (18)	23 (32)	36 (50)
Unknown	6 (1)	2 (33)	-	4 (67)
Total	632	238 (38)	99 (16)	295 (47)

RT: Radiotherapy.

Vital status

The vital status was updated by active follow-up in the hospitals, by linking files with deceased persons to the cancer registry and by linkage to the electronic death registry of the Central Office for Genealogy in September, 2003 and February, 2005, as described earlier[21]. Completeness of follow-up of the vital status is estimated to be over 99.5%.

Statistical analysis

$P < 0.05$ was considered statistically significant. All statistical analyses were performed using a two-sided 5% level of significance.

Survival probabilities were estimated using the Kaplan-Meier method[22]. Multivariate analyses using the Cox proportional-hazard method were performed to calculate the hazard ratio (HR) for death after recurrent disease[23]. Cox regression and Kaplan-Meier survival curves were calculated with STATA (Stata Corporation, College Station, TX, USA).

RESULTS

Initial treatment and incidence of local recurrence

A total of 632 patients diagnosed with primary rectal carcinoma in the absence of clinically manifest distant dissemination between 1998 and 2000 underwent a macroscopically radical local resection obtained with TME. Characteristics of the initial treatment of the primary tumour in these patients are given in Table 1. Local recurrence within five years after diagnosis occurred in 62 patients (10%), including 30 cases with distant dissemination (6%). Of these 30 patients, 24 patients had synchronous local and distant recurrence, while 6 patients developed distant dissemination after the local recurrence.

Treatment of local recurrence

There were 32 out of 62 patients (52%) without signs

Table 2 Secondary treatment of local recurrence (in the absence of distant dissemination) according to treatment with radiotherapy and stage at initial diagnosis (after a macroscopically radical resection obtained with TME)

Secondary treatment	Stage and treatment with radiotherapy at initial diagnosis									Total *n* (%)
	Stage I			Stage II			Stage III			
	No RT	Post RT	Pre RT	No RT	Post RT	Pre RT	No RT	Post RT	Pre RT	
Radical surgery[1] (± radiotherapy and/or chemotherapy)	3	-	1	3	-	1	1	-	1	10 (3)
Non-radical surgery[1] (± radiotherapy and/or chemotherapy)	-	-	-	2	1	2	-	3	-	8 (2)
Radiotherapy and/orchemotherapy without surgery	2	-	-	1	1	-	1	2	-	7 (2)
Best supportive care	1	-	-	-	1	3	1	-	1	7 (2)
Total	6	-	1	6	3	6	3	5	2	32

[1]Radical surgery was defined as surgery without microscopically residual disease; all other surgery cases were classified as non-radical. No RT: No radiotherapy; Post RT: Postoperative radiotherapy; Pre RT: Preoperative radiotherapy.

Table 3 Multivariate analysis of potentially prognostic factors for improved survival after treatment of patients with a locally recurrent rectal carcinoma in Greater Amsterdam (cases with distant dissemination and/or macroscopic residual disease at time of initial treatment are excluded)

Parameter	Number of cases	HR (95% CI)
Sex		
Male (reference) *vs* female	33/29	1.9 (0.9-3.7)
Radiotherapy at initial treatment		
No radiotherapy	23	1.0
Preoperative radiotherapy	23	1.2 (0.6-2.5)
Postoperative radiotherapy	16	0.9 (0.4-1.8)
Distant dissemination at time of local recurrence		
Absent (reference) *vs* present	32/30	0.8 (0.4-1.6)
Surgical treatment of locally recurrent disease		
No surgery	42	1.0
Radical surgery	10	0.1 (0.0-0.3)
Non-radical surgery	10	0.5 (0.2-1.3)

HR > 1: Worse prognosis; HR < 1: Better prognosis.

of distant dissemination at the time of diagnosis of recurrent disease. Median survival after recurrence in the absence of distant dissemination was 25 mo. Ten of these 32 patients underwent a microscopically radical resection of their recurrence (Table 2). As is depicted in Figure 1, radical surgery resulted in a significantly better survival than non-radical surgery, radio- and/or chemotherapy without surgery or best supportive care (log-rank test radical surgery *vs* other treatments: $P < 0.001$). About three quarters of the patients who underwent a radical resection survived for at least three years. Median survival after non-radical surgery (8 patients) was 24 mo, 7 mo after radio- and/or chemotherapy without surgery (7 patients) and was 5 mo in case of best supportive care only (7 patients).

In 30 patients (48%), distant dissemination was present at the time of diagnosis of local recurrent disease. Median survival after local recurrence in the presence of distant dissemination was 10 mo. None of these patients underwent curative surgery, two patients underwent non-radical surgical resection, 14 patients were treated with radio- and/or chemotherapy without surgery (median survival 14 mo) and 14 patients received best supportive care only (median survival 9 mo).

Table 4 Treatment of distant dissemination in patients initially treated with a macroscopically radical local resection obtained with TME in the absence of distant metastasis

Treatment	No local recurrence		Local recurrence[1]		Total	
	n	%	*n*	%	*n*	%
Surgery[1] (± radiotherapy and/or chemotherapy)	18	19	2	7	20	16
Radiotherapy and/or chemotherapy	54	57	13	43	67	54
Other	1	1	2	7	3	2
Best supportive care	21	22	13	43	34	27
Total	94		30		124	

[1]Synchronous with distant metastasis or prior to distant metastasis.

Prognostic factors for survival after recurrence

Several factors were analysed to identify prognostic factors for improved survival after local recurrence. The results of the multivariate analysis are shown in Table 3. Surgery for recurrent disease (radical and non-radical) was a prognostic factor for improved survival, while radiotherapy applied during the initial treatment did not influence survival after local recurrence (Figure 2).

Distant dissemination

Distant dissemination within five years after diagnosis occurred in 124 patients (20%). The majority of patients (54%) with distant dissemination were treated with radio- and/or chemotherapy (Table 4). The median survival after distant dissemination was 15 mo.

Twenty patients (16%) underwent surgery for their recurrence, including liver resections in eight patients, lung resections in five patients, and other surgical procedures in seven patients. Median survival after surgery was 32 mo, while median survival after radiotherapy and/or chemotherapy without surgery was 15 mo and 6 mo if best supportive care was applied (Figure 3). Patients with distant dissemination who were treated surgically experienced the highest survival (log-rank test surgery *vs* other treatments: $P < 0.001$).

DISCUSSION

This is the first population-based study concerning

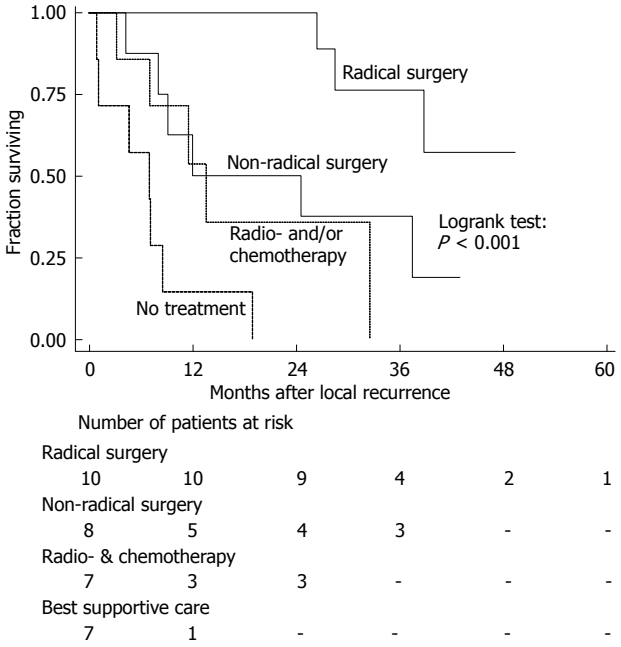


Figure 1 Crude survival after isolated local recurrence in rectal cancer patients initially treated with a macroscopically radical local resection obtained with TME in Greater Amsterdam according to treatment for recurrence. Radical surgery is defined as surgery without macroscopically or microscopically residual disease. Non-radical surgery is defined as surgery with macroscopically or microscopically residual disease.

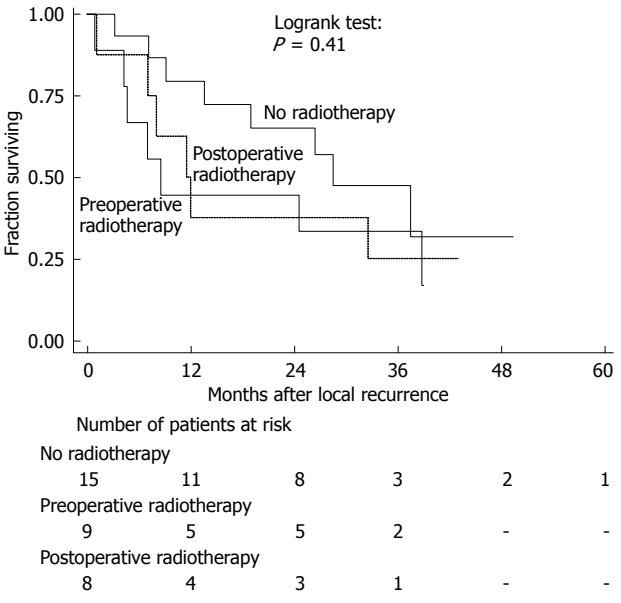


Figure 2 Crude survival after isolated local recurrence in rectal cancer patients initially treated with a macroscopically radical local resection obtained with TME in Greater Amsterdam according to radiotherapeutic treatment of the primary tumour.

recurrent rectal cancer treatment after the introduction of TME. All patients in this study were initially diagnosed between 1998 and 2000 in Greater Amsterdam and treated by macroscopically radical local resection obtained with TME. A local recurrence occurred in 62 of the 632 patients (10%), while distant dissemination was found in 124 patients (20%).

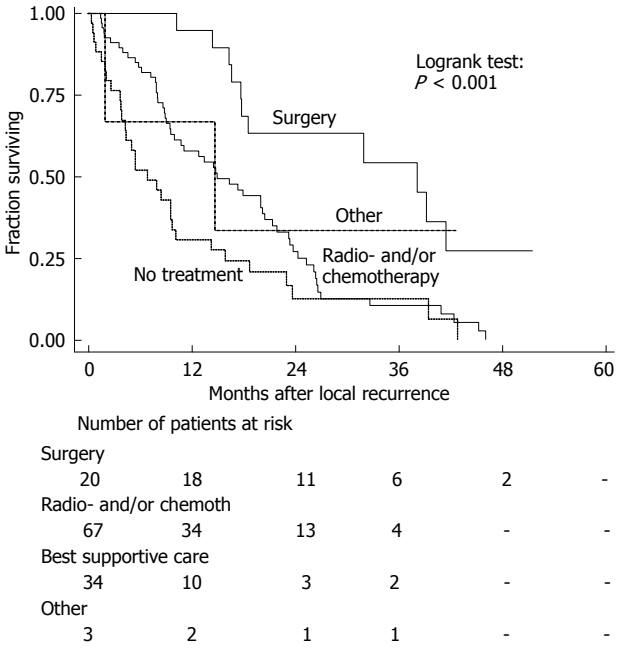


Figure 3 Crude survival after distant dissemination in rectal cancer patients initially treated with a macroscopically radical local resection obtained with TME in Greater Amsterdam according to treatment for distant dissemination.

Treatment of patients with isolated recurrent disease

Of the 32 patients with an isolated local recurrence, 31% were treated by a radical resection. These patients experienced a significantly better survival compared to patients who underwent a non-radical resection for their recurrence. As has been shown previously, radical resection of locally recurrent disease can achieve long-term survival[4,13-15,24] and should, therefore, be aimed at, even if extended resection (*e.g.* abdominosacral resection or exenteration)[16,25,26] or flap-reconstruction[27] is required.

Survival in patients treated with non-radical surgery and patients treated with radiotherapy and/or chemotherapy without surgery was comparable, but was significantly worse in patients not treated with surgery, radiotherapy or chemotherapy. Although no information concerning the extent of recurrent disease was available in this study, treatment has probably been more aggressive in case of limited disease and, therefore, selection bias may have played an important role in the outcome of the various treatment modalities.

Treatment of patients with distant dissemination

The median survival after distant dissemination was 15 mo for patients diagnosed between 1998 to 2000. In a previous study, we have described that patients diagnosed in 1988 between 1991 in Greater Amsterdam only survived 9 mo after distant dissemination (log-rank test: $P = 0.004$)[19]. The majority of patients diagnosed between 1998-2000 (54%) with distant dissemination were treated with radiotherapy and/or chemotherapy without surgery, while 16% were treated with surgical resection and 27% received only best supportive care. Survival was significantly better in the group of patients

treated with surgery compared to other groups. This is probably due to the limited spread of disease in these patients (selection bias). As no treatment data were available for the patients diagnosed between 1988 to 1991 in Greater Amsterdam, it is unclear which treatment modality has contributed to the increase in the median survival.

CONCLUSION

In this population-based study, treatment options and survival were limited in patients with recurrent rectal cancer after macroscopically radical local resection obtained with TME. Approximately one third of the patients only received best supportive care with a subsequent poor survival. On the other hand, in one third of the patients with an isolated local recurrence, radical resection was feasible with a favourable survival. We conclude that a locally recurrent rectal cancer without distant dissemination does not automatically lead to a hopeless situation[28]. However, survival after local recurrence in combination with distant dissemination remains extremely poor.

ACKNOWLEDGMENTS

The surgeons in the 20 participating hospitals (Academic Medical Centre, Free University Medical Centre, Sint Lucas Andreas Hospital, Netherlands Cancer Institute, BovenIJ Hospital, Onze Lieve Vrouwe Hospital, Slotervaart Hospital, Waterland Hospital, Amstelland Hospital, Zaans Medical Centre, Flevo Hospital, IJsselmeer Hospitals, Gooi-Noord Hospital, Hilversum Hospital, Kennemer Hospital, Red Cross Hospital, Spaarne Hospital, Gemini Hospital, Westfries Hospital, Medical Centre Alkmaar) are greatly acknowledged.

COMMENTS

Background

Colorectal cancer is a common cancer in the Western world and rectal cancer has been subject to significant treatment changes over the last decade. Despite this change, recurrent rectal cancer treatment remains a frustrating and ongoing process.

Research frontiers

In the recent decade, the main changes in rectal cancer treatment have been the application of preoperative radiotherapy and the introduction of total mesorectal excision (TME) which led to a significant decrease in local recurrence percentages. In the current article, the influence the introduction of TME on recurrent rectal cancer treatment is evaluated in a population based cohort.

Applications

The current results show that recurrent rectal cancer treatment is in some cases worthwhile, especially if a radical resection is feasible. However, avoiding a local recurrence remains an important aspect in future rectal cancer treatment.

Terminology

TME is a surgical technique initiated by B. Heald during which the entire mesorectum is excised using the so-called "holy" plane. This provides the opportunity to remove the entire rectum with possibly infiltrated lymph nodes.

Peer review

This is an interesting study which was well organized. It demonstrated that although treatment options and survival are limited in case of recurrent rectal cancer after radical local resection obtained with TME, patients can benefit from additional treatment, especially if a radical resection is feasible.

REFERENCES

1 **Landis SH**, Murray T, Bolden S, Wingo PA. Cancer statistics, 1999. *CA Cancer J Clin* 1999; **49**: 8-31, 1
2 **Visser O**, Siesling S, Dijck JAAM. Incidence of Cancer in the Netherlands 1999/2000. 11th ed, Utrecht: Association of Comprehensive Cancer Centres, 2003: 22-23
3 **Ferlay J**, Bray F, Pisani P, Parkin DM. GLOBOCAN 2000; cancer incidence, mortality and prevalence worldwide. Version 1.0. IARC CancerBase No. 5. Lyon; IARC Press, 2001
4 **Sagar PM**, Pemberton JH. Surgical management of locally recurrent rectal cancer. *Br J Surg* 1996; **83**: 293-304
5 **Frykholm GJ**, Pahlman L, Glimelius B. Treatment of local recurrences of rectal carcinoma. *Radiother Oncol* 1995; **34**: 185-194
6 **van den Brink M**, Stiggelbout AM, van den Hout WB, Kievit J, Klein Kranenbarg E, Marijnen CA, Nagtegaal ID, Rutten HJ, Wiggers T, van de Velde CJ. Clinical nature and prognosis of locally recurrent rectal cancer after total mesorectal excision with or without preoperative radiotherapy. *J Clin Oncol* 2004; **22**: 3958-3964
7 **Camilleri-Brennan J**, Steele RJ. The impact of recurrent rectal cancer on quality of life. *Eur J Surg Oncol* 2001; **27**: 349-353
8 **Heald RJ**, Ryall RD. Recurrence and survival after total mesorectal excision for rectal cancer. *Lancet* 1986; **1**: 1479-1482
9 **MacFarlane JK**, Ryall RD, Heald RJ. Mesorectal excision for rectal cancer. *Lancet* 1993; **341**: 457-460
10 **Kapiteijn E**, Marijnen CA, Nagtegaal ID, Putter H, Steup WH, Wiggers T, Rutten HJ, Pahlman L, Glimelius B, van Krieken JH, Leer JW, van de Velde CJ. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. *N Engl J Med* 2001; **345**: 638-646
11 **Glimelius B**, Gronberg H, Jarhult J, Wallgren A, Cavallin-Stahl E. A systematic overview of radiation therapy effects in rectal cancer. *Acta Oncol* 2003; **42**: 476-492
12 **Improved survival with preoperative radiotherapy in resectable rectal cancer**. Swedish Rectal Cancer Trial. *N Engl J Med* 1997; **336**: 980-987
13 **Manfredi S**, Benhamiche AM, Meny B, Cheynel N, Rat P, Faivre J. Population-based study of factors influencing occurrence and prognosis of local recurrence after surgery for rectal cancer. *Br J Surg* 2001; **88**: 1221-1227
14 **Garcia-Aguilar J**, Cromwell JW, Marra C, Lee SH, Madoff RD, Rothenberger DA. Treatment of locally recurrent rectal cancer. *Dis Colon Rectum* 2001; **44**: 1743-1748
15 **Bakx R**, van Tinteren H, van Lanschot JJ, Zoetmulder FA. Surgical treatment of locally recurrent rectal cancer. *Eur J Surg Oncol* 2004; **30**: 857-863
16 **Yamada K**, Ishizawa T, Niwa K, Chuman Y, Aikou T. Pelvic exenteration and sacral resection for locally advanced primary and recurrent rectal cancer. *Dis Colon Rectum* 2002; **45**: 1078-1084
17 **Pahlman L**. Population based study of factors influencing occurrence and prognosis of local recurrence after surgery for rectal cancer. *Tech Coloproctol* 2003; **7**: 120-121
18 **Guyot F**, Faivre J, Manfredi S, Meny B, Bonithon-Kopp C, Bouvier AM. Time trends in the treatment and survival of recurrences from colorectal cancer. *Ann Oncol* 2005; **16**: 756-761
19 **Visser O**, Bakx R, Zoetmulder FA, Levering CC, Meijer S, Slors JF, van Lanschot JJ. The influence of total mesorectal excision on local recurrence and survival in rectal cancer patients: a population-based study in Greater Amsterdam. *J Surg Oncol* 2007; **95**: 447-454
20 **Sobin LH**, Wittekind CH. TNM Classification of Malignant Tumours. International Union Against Cancer (UICC). 6th ed. New York: John Willey & Sons, 2002
21 **Visser O**, van Leeuwen FE. Stage-specific survival of epithelial cancers in North-Holland/Flevoland, The Netherlands. *Eur J Cancer* 2005; **41**: 2321-2330
22 **Kaplan EL**, Meier P. Nonparametric estimation from

incomplete observations. *J Am Stat Assoc* 1958; **53**: 457-481
23 **Cox DR**. Regression models and life-tables. *J R Stat Soc* 1972; **34**: 187-220
24 **Caricato M**, Borzomati D, Ausania F, Valeri S, Rosignoli A, Coppola R. Prognostic factors after surgery for locally recurrent rectal cancer: an overview. *Eur J Surg Oncol* 2006; **32**: 126-132
25 **Temple WJ**, Ketcham AS. Sacral resection for control of pelvic tumors. *Am J Surg* 1992; **163**: 370-374
26 **Bakx R**, van Lanschot JJ, Zoetmulder FA. Sacral resection in cancer surgery: surgical technique and experience in 26 procedures. *J Am Coll Surg* 2004; **198**: 846-851
27 **Bakx R**, van Lanschot JJ, Zoetmulder FA. Inferiorly based rectus abdominis myocutaneous flaps in surgical oncology: Indications, technique, and experience in 37 patients. *J Surg Oncol* 2004; **85**: 93-97
28 **Daland EM**, Welch CE, Nathanson I. One hundred untreated cancers of the rectum. *N Engl J Med* 1936; **214**: 451-458

S- Editor Li DL **L- Editor** Rippe RA **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6024

World J Gastroenterol 2008 October 21; 14(39): 6024-6029
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Prospective evaluation of laparoscopic Roux en Y gastric bypass in patients with clinically severe obesity

Konstantinos Vagenas, Spyros Panagiotopoulos, Ioannis Kehagias, Stavros Nikolaos Karamanakos, Nancy Mead, Fotis Kalfarentzos

Kostantinos Vagenas, Spyros Panagiotopoulos, Ioannis Kehagias, Stavros Nikolaos Karamanakos, Fotis Kalfarentzos, Department of Surgery, University of Patras School of Medicine, University Hospital, Rion 26504, Greece
Nancy Mead, Fotis Kalfarentzos, Nutrition Support and Morbid Obesity Clinic, University of Patras School of Medicine, University Hospital, Rion 26504, Greece
Author contributions: Vagenas K and Kalfarentzos F designed the study; Kehagias I and Karamanakos SN performed the research; and Panagiotopoulos S and Mead N wrote the paper.
Correspondence to: Fotis Kalfarentzos, MD, FACS, Professor and Chairman, Department of Surgery, School of Medicine, University of Patras, Platia Voriou Ipirou 5, Patras 26441, Greece. fkalfar@med.upatras.gr
Telephone: +30-26-10999299 Fax: +30-26-10993984
Received: September 1, 2008 Revised: September 20, 2008
Accepted: September 27, 2008
Published online: October 21, 2008

Abstract

AIM: To evaluate and present our experience with laparoscopic Roux en Y gastric bypass (RYGB) in a selected patient population.

METHODS: A cohort of 130 patients with a body mass index (BMI) between 35 and 50 kg/m^2 were evaluated in relation to postoperative morbidity, weight loss and resolution of co-morbidities for a period of 4 years following laparoscopic RYGB.

RESULTS: Early morbidity was 10.0%, including 1 patient with peritonitis who was admitted to Intensive Care Unit (ICU) and 1 conversion to open RYGB early in the series. There was no early or late mortality. Maximum weight loss was achieved at 12 mo postoperatively, with mean BMI 30 kg/m^2, mean percentage of excess weight loss (EWL%) 66.4% and mean percentage of initial weight loss (IWL%) 34.3% throughout the follow-up period. The majority of preexisting comorbidities were resolved after weight loss and no major metabolic disturbances or nutritional deficiencies were observed.

CONCLUSION: Laparoscopic RYGB appears to be a safe and effective procedure for patients with BMI 35-50 kg/m^2 with results that are comparable to previously published data mostly from the USA but from Europe as well.

Key words: Laparoscopic gastric bypass; Evaluation; Obesity

Peer reviewer: Leonidas G Koniaris, Professor, Alan Livingstone Chair in Surgical Oncology, 3550 Sylvester Comprehensive Cancer Center (310T), 1475 NW 12th Ave, Miami, FL 33136, United States

Vagenas K, Panagiotopoulos S, Kehagias I, Karamanakos SN, Mead N, Kalfarentzos F. Prospective evaluation of laparoscopic Roux en Y gastric bypass in patients with clinically severe obesity. *World J Gastroenterol* 2008; 14(39): 6024-6029 Available from: URL: http://www.wjgnet.com/1007-9327/14/6024.asp DOI: http://dx.doi.org/10.3748/wjg.14.6024

INTRODUCTION

Obesity has reached epidemic proportions in society today[1] and it is associated with high rates of morbidity and mortality due to the presence of severe comorbidities, many of which show complete resolution after weight loss[2]. According to published data, surgery is the only reliable way of achieving both adequate and long-term weight loss in the morbidly obese population[3]. Roux en Y gastric bypass (RYGB), which was first described by Mason and Ito at the University of Iowa in 1967, is now considered the gold standard of bariatric procedures[4]. In 1994, Wittgrove *et al*[5] first introduced a totally laparoscopic approach to this procedure, which is now a routine practice for the majority of bariatric centers in United States and this trend seems to be spreading throughout Europe as well. However, there are few published studies to date regarding the implementation of this procedure in Europe[6].

The aim of this study, therefore, is to report the experience of a single centre in Greece with the laparoscopic RYGB.

MATERIALS AND METHODS

Since 1994, 1095 surgical procedures for clinically severe obesity have been performed at our institution. The type of procedure performed is based on specific selection criteria according to an algorithm developed

Table 1 Patients' preoperative characteristics

Period of study	September 2003 to June 2008
Number of patients	130
Male:Female	21:109
Age (yr)	37 ± 9.4 (18-55)
Weight (kg)	125 ± 13.4 (95-170)
Excess weight (kg)	65 ± 9.7 (39-103)
% EBW	208 ± 14.5 (160.9-253.7)
BMI (kg/m^2)	46 ± 3.0 (37.5-50)

Values are mean ± SD (range).

Table 2 Preoperative comorbidities

Comorbidities	No. of patients (%)
Hypertension	21 (16.3)
COPD	22 (17.0)
Sleep apnea	13 (10.0)
Osteoarthritis	21 (16.3)
Depression	11 (8.5)
Diabetes Mellitus	24 (18.6)
OGTT	8 (6.2)
Hypercholesterolemia	64 (49.6)
Hypertriglyceridemia	36 (27.9)

COPD: Chronic obstructive pulmonary disease.

by our center, whereby patients with body mass index (BMI) over 50 kg/m^2 undergo biliopancreatic diversion with RYGB (BPD-RYGB), as modified in our center[7], while patients with BMI < 50 kg/m^2 undergo RYGB with long limb (RYGB-LL) or sleeve gastrectomy (SG) depending on comorbidities and eating habits. Sweet consumers and patients with diabetes mellitus undergo RYGB-LL whereas volume consumers may undergo SG. Since October 2003 in patients with BMI < 50 kg/m^2 all procedures have been performed laparoscopically, and the present study refers to 130 of these patients who underwent RYGB-LL as their primary procedure.

All patients participated in a multistage educational and informational program to accomplish full disclosure preoperatively and to engage them in compliance with bariatric management postoperatively.

A multidisciplinary team including the surgeon, an endocrinologist, a cardiologist, a pneumonologist, a psychiatrist and a nutritionist-dietitian evaluate all patients preoperatively and postoperatively to assess and optimize their physical condition. Table 1 shows the patients' preoperative characteristics and Table 2 shows the patients' preoperative comorbidities.

At the operating room, during induction in anesthesia, a single dose of antibiotics (cephalosporin second generation 1.5 g and metronidazole 500 mg) was given and continued for 48 h postoperatively. Thromboembolic prophylaxis using low molecular weight heparin at a dose adapted to patients' weight was started the day before operation and continued till the discharge. Complementary measures consisting of air compression boots and specially designed mattress were used.

Surgical technique

The main characteristics of the RYGB-LL are a gastric pouch of 15 ± 5 mL, a biliopancreatic limb of 50 cm, an alimentary limb of 150 cm and the remainder of small intestine as the common limb. We prefer to pass the alimentary limb antecolic and antegastric and to create a gastrojejunostomy using a 25 mm circular stapler performing a circular end to end anastomosis (CEEA) by passing the anvil transorally. The gastrojejunostomy is reinforced with interrupted non-absorbable sutures. The proximal end of Roux limb is closed with application of linear stapler. A side to side jejunojejunostomy is performed using a linear stapler and the mesenteric defects are closed with non-absorbable running sutures.

Cholecystectomy is always added to the main procedure. Furthermore, because fatty liver and non-alcoholic steatohepatitis are common, liver biopsy is routinely performed to assess preoperatively liver histopathology and will be used as a baseline for comparison if a problem in hepatic function arises in the future.

Postoperative management

Patients were mobilized in the evening of surgery. Pain management consisted of parecoxib 40 mg intravenously every 12 h and meperidine 50 mg intramuscularly as needed for 3 d.

On the fourth postoperative day, after an uneventful upper gastrointestinal evaluation, a clear liquid diet was begun with gradual progression to full liquids and blenderized food. High protein supplements were administered to all patients until 4 wk postoperatively when per os feeding was advanced by the nutritionist.

All patients received a daily multivitamin and mineral supplement and 1000 mg of calcium. An iron supplement was also prescribed for premenopausal women at a dose of 80 mg/d. After the 6th postoperative month, vitamin B_{12} supplementation was given intramuscularly at a dose of 1000-3000 μg depending upon the measured values.

Follow-up visits were scheduled at 1, 3, 6 and 12 mo postoperatively and annually thereafter. Each follow-up visit included complete medical and nutritional evaluation, with laboratory workup and evaluation by other specialists as needed.

Statistical analysis

The data presented are expressed as mean ± SD, unless otherwise stated. During the study comparisons of the observed values at various time periods were performed using ANOVA (one way analysis of variance). If there were any statistic significant differences, the Tukey post-test was used to determine the time points that contribute to this significance.

RESULTS

From October 2003 to June 2008, 130 patients with BMI 35-50 kg/m^2 underwent laparoscopic RYGB-LL as their primary bariatric operation. In addition,

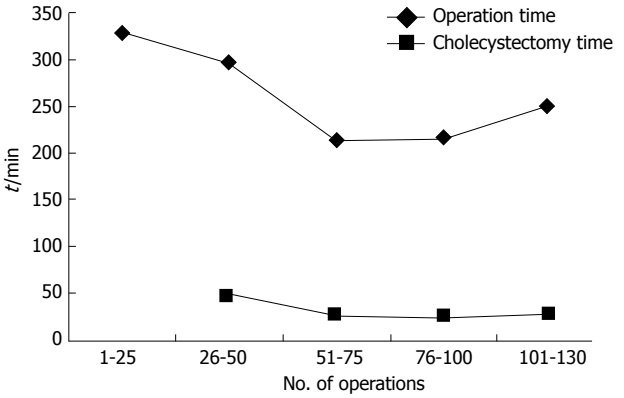


Figure 1 Operation time for the first 130 patients. Values in graph lines are mean values.

cholecystectomy was performed in all but nine patients who had previously undergone this procedure elsewhere and other abdominal procedures were performed in five patients. The mean total operative time was 260 ± 87.4 min (range 120-270) and the mean operative time for cholecystectomy was 18 ± 17.2 min (range 15-70). The operative time decreased significantly over time, especially after the first 50 cases ($P < 0.05$) (Figure 1). Conversion to open RYGB was necessary in one patient (0.8%) early in the series.

The mean length of hospital stay was 9 d (range 8-29) primarily due to standard hospital protocol in Greece. The mean follow-up time was 24.3 mo (range 1-48). The number of patients who successfully completed follow-up was 110 of 112 patients (98.2%) at 12 mo, 85 of 90 patients (94.4%) at 24 mo, 51 of 55 patients (92.7%) at 36 mo and 16 of 18 patients (88.9%) at 48 mo. Four patients were lost to follow-up. Thus far there has been no need for revision surgery.

Mortality

There was no early or late mortality.

Complications

Intraoperative period: One conversion to open RYGB was necessary early in the series, due to inability to construct the jejuno-jejuno anastomosis.

Early postoperative period (< 30 d): In the early postoperative period, as shown in Table 3, there were three gastrointestinal haemorrhages, one of which was intraluminal, probably from the jejunojejunal anastomosis, presenting as small obstruction and requiring reoperation, while the others were intrabdominal and treated conservatively. In addition, there were two enterocutaneous fistulas, caused by attachment of small intestine loop to mesh after simultaneous repair of incisional hernias, one episode of ileus due to volvulus and one case of peritonitis, all of which required surgical intervention. The last patient required admission to the Intensive Care Unit (ICU) after reoperation for peritonitis caused by leakage of the alimentary limb stump on the 6th postoperative day. The latter was caused by herniation of a small bowel limb to port site. The patient was finally discharged 168 d after surgery following a complicated and difficult postoperative course, including acute compartment syndrome requiring reoperation and development of acute renal insufficiency requiring dialysis. There was also one episode of portal vein thrombosis treated with anticoagulant medication, one wound infection of port site treated with antibiotics, one lung atelectasis, two orthopaedic injuries due to inappropriate patient positioning and securing intraoperatively. Finally, there was one anastomotic leakage from gastrojejunal anastomosis, which presented on the 6th postoperative day with severe sepsis without hemodynamic instability and was treated successfully conservatively with drainage under computer tomography (CT) guidance and total parenteral nutrition. The total early postoperative morbidity was 10.0% (13 patients).

Table 3 Incidence of postoperative complications and management

Time	Complications	Treatment	% of patients
Early < 30 d			
	3 gastrointestinal hemorrhages	Conservative (2) Operation (1)	2.4
	2 enterocutaneous fistulas	Operation	1.6
	1 peritonitis	Operation, ICU admission	0.8
	1 ileus due to volvulus	Operation	0.8
	1 portal vein thrombosis	Anticoagulants	0.8
	1 wound infection of port site	Antibiotics	0.8
	2 orthopedic injuries	Immobilization	1.6
	1 atelectasis	Antibiotics peros	0.8
	No incisional hernia		0.0
	1 anastomotic leak	Conservative	0.8
Intermediate 30-90 d			
	1 incisional hernia	No operation	0.8
Late > 90 d			
	5 incisional hernias	Operation (4)	3.9
	4 episodes of ileus	Operation (1) Conservative (3)	3.1
	1 suicide attempt		0.8
	1 gastrojejunal anastomosis stenosis	Endoscopic dilatation	0.8

Intermediate postoperative period (30-90 d): During the intermediate postoperative period there was one incisional hernia with no other major complications.

Late postoperative period (> 90 d): During the late postoperative period there were 5 incisional hernias, 4 of which were treated surgically, 4 episodes of small bowel obstruction, one of which was due to adhesions requiring surgical intervention, one episode of gastrojejunal anastomosal stenosis, which was treated with endoscopic dilation and one suicide attempt 5 mo after surgery due to undetected severe pre-existing depression.

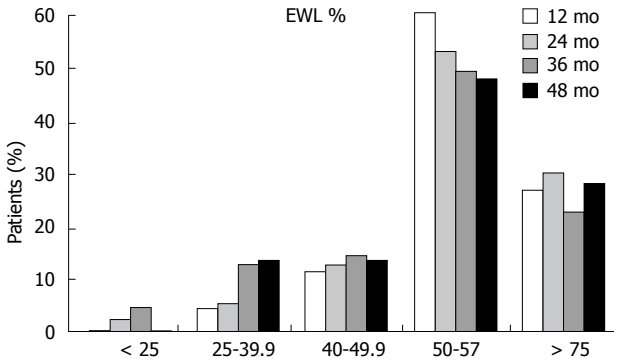


Figure 2 Percentage of EWL during follow-up.

Weight loss

The results of weight loss expressed as actual weight, BMI, percentage of excess weight loss (EWL%) and percentage of initial weight loss (IWL%) at 12, 24, 36 and 48 mo, respectively, are shown in Table 4. Maximum weight loss was observed at 12 mo, with mean BMI 30 kg/m^2, mean EWL 66.4% and mean IWL 34.3% which remained stable throughout the follow-up period. Ninety-two (83.6%) of 110 patients achieved an EWL of at least 50% at 12 mo which was maintained throughout the follow-up period (Figure 2). An EWL of 50% is considered successful.

Comorbidities

Comorbidities were present in 117 (90.7%) patients preoperatively with an average of 3 ± 2.6 (range 0-10) per patient. The majority of these showed significant improvement during the 48 mo follow-up. Table 5 presents the postoperative results of relevant comorbidities.

Hypertension was present in 21 patients (16.3%) preoperatively, 16 of whom (76.2%) were on medication. After the 2nd postoperative year, only one patient (4.8%) remained on antihypertensive medication.

Preoperatively 24 patients had diabetes mellitus (blood glucose > 125 mg/dL), 14 of whom were on oral hypoglycemic agents while 2 were being treated with insulin. An additional 8 patients had impaired oral glucose tolerance test (OGTT) preoperatively. Postoperatively, diabetes mellitus was completely resolved in 22 patients (91.7%) by the 12 mo follow-up. Only one patient remained on insulin therapy throughout the study period.

Hypercholesterolemia (blood cholesterol > 200 mg/dL) was present in 64 patients preoperatively. Mean cholesterol levels were 230 ± 28.8 mg/dL preoperatively and had decreased to 179 ± 42 mg/dL by the 12 mo follow-up and remained at these levels for the rest of the follow-up period. Among the 52 of 64 patients who completed the 12 mo follow-up, 14 (26.9%) still had elevated blood cholesterol levels > 200 mg/dL. By the 36th month following surgery, only 6 of the 24 patients (25.0%) still had cholesterol levels > 200 mg/dL.

There were 34 patients with hypertriglyceridemia (TGs > 160 mg/dL) preoperatively, with mean levels 225 ± 64.8 mg/dL. Postoperatively, mean TGs levels dropped to 96 ± 33.2 mg/dL and 96 ± 37.7 mg/dL at 12 and 24 mo respectively and none of the patients had hypertriglyceridemia.

Table 4 Weight loss during follow-up

	0 mo	12 mo	24 mo	36 mo	48 mo
Patients	130	110	85	51	16
Weight (kg)	125	81.6	81.4	84.1	79.2
BMI	46	30	31	32	31
IWL %		34.2	34.3	32.2	31.4
EWL %		66.2	65.9	61.5	61.1
Pts > 50% EWL		92	68	35	12

Values for weight, BMI, EWL, IWL are mean values.

Table 5 Postoperative resolution of comorbidities

Comorbidities	No. of patients	Follow-up period (mo)	Resolved (%)	Improved (%)	Without change (%)
Hypertension	21	24	84		16
COPD	22	12		100	
Sleep apnea	13	12		100	
Osteoarthritis	21	12		100	
Depression	11	24		89	11
Diabetes Mellitus	24	12	91.7		8.3
OGTT	8	12	100		
Hypercholesterolemia	64	36	94.4		5.6
Hypertriglyceridemia	36	12	100		

Metabolic complications

Anaemia: Anaemia, as defined by haemoglobin level < 13.5 mg/dL in men and < 12.5 mg/dL in women, was observed at mean rate of 20.1%. More specifically, a low haemoglobin level was seen in 14.8%, 20.2%, 22% and 26.7% of patients at 12, 24, 36 and 48 mo, respectively. Regarding other parameters associated with anaemia including iron (< 35 μg/dL), vitamin B12 (< 200 pg/mL) and ferritin (< 9 ng/mL), the mean rates of postoperative deficiency were 6.1%, 18.7% and 10.0%, respectively. No deficiency of folic acid was observed following surgery and none of the above deficiencies were clinically severe. All patients were treated successfully with oral supplementation.

Hypoalbuminemia: Hypoalbuminemia did not occur at any time during the postoperative follow-up. The mean levels of albumin were > 4 g/dL at all follow-up evaluation and no patient presented with albumin levels below the minimum normal level.

DISCUSSION

In this study, 130 patients underwent laparoscopic RYGB-LL with an acceptable total early complication rate (minor and major) 10.0%, a low conversion rate (0.8%) and no mortality.

The mean hospital stay was quite high (9 d) but this is due to the existing health care system in Greece, which

lacks the services of early postoperative care for patients from distant places.

Maximum weight loss in our series was achieved at 12 mo, with a mean EWL of 66.4%, which was maintained through out the study period. However, 14% of patients did not achieve adequate weight loss (EWL < 50%) and the reason for these failures has not been clarified yet causing problems for the surgeons and the patients.

In addition to adequate weight loss, improvement of pre-existing comorbidities is one of the most important criteria for evaluating success of a bariatric procedure. In our study, as in others[1,2,6,8-10], that was achieved, as shown in Table 2. Diabetes mellitus was completely resolved in the majority of the patients, finding supported by other reports, which also proposed mechanisms that this can be achieved[11,12]. Hypercholesterolemia was resolved in the majority of patients and hypertriglyceridemia in all patients, results similar to those described for malabsorptive procedures[13,14]. Regarding metabolic complications the most significant were vitamin B_{12} and iron deficiency. However there was no clinical impact and all the patients were treated conservatively with intramuscular injections of vitamin B_{12} and oral iron supplements as necessary.

The surgical complications rate in our series was comparable to that described by others[6,15-20] and included primarily gastrointestinal haemorrhages, enterocutaneous fistulas and obstructive ileus. We had no internal hernias, which was probably due to the antecolic position of the Roux limb[21]. Furthermore, the low incidence of anastomotic gastrointestinal stricture (0.8%) may be attributed to the use of 25 mm circular stapler. The prevalence of wound infection and anastomotic leakage was similar to that reported by others[15,17,18].

The laparoscopic approach to bariatric surgery is technically challenging, but with experience it can be mastered. The learning curve is steep and long operating times are required[22]. As shown in Figure 2, our operating time decreased significantly as our experience accumulated, which was supported by Wittgrove *et al*[5]. Since practice makes perfection, we could also manage to improve our port-site closure technique and after the first 5 unfortunate incisional hernias there was no new case recorded. Furthermore, our 2 cases of enterocutaneous fistulas were attributed to simultaneous mesh placement and eventually this approach was abandoned.

Our results suggest that laparoscopic RYGB-LL as performed at our institution provides adequate weight loss and resolution or improvement of comorbidities up to 4 years. Recently, it has been increasingly recognised that RYGB may have an effect on appetite and eating behaviour, which, in turn, may improve long-term results. These effects may be related to changes in the circulating levels of the gastrointestinal hormones known to influence appetite including PYY[7,23-28], and this may play a role in the overall success of the procedure.

CONCLUSION

In our experience laparoscopic Roux-en-Y gastric bypass appears to be a safe and effective surgical procedure for patients with BMI 35-50 kg/m^2 with results which are comparable to previously published data primarily from the USA, but some from Europe as well. It is associated with minor postoperative pain, early patient mobilization, low incidence of perioperative complications, improvement or complete resolution of preexisting comorbidities and weight loss results that are comparable to those seen following the open procedure. For all these reasons we consider laparoscopic RYGB-LL to be a procedure of choice for the treatment of clinically severe obesity in properly selected patients.

COMMENTS

Background

Obesity, has reached epidemic proportions in society today and it is associated with high rates of morbidity and mortality due to the presence of severe comorbidities. Surgery is the only reliable way of achieving adequate and long-term weight loss in the morbidly obese population. Nowadays, Roux en Y gastric bypass (RYGB) is considered as the gold standard of bariatric procedures. In the present study, the experience of a single centre in Greece with the laparoscopic RYGB is reported.

Research frontiers

Laparoscopic RYGB-LL as performed at authors' institution provides adequate weight loss and resolution or improvement of comorbidities up to 4 years. Apparently, RYGB seems to have an effect on appetite and eating behaviour, which, in turn, may improve long term results. These effects are related to changes in the circulating levels of the gastrointestinal hormones known to influence appetite including ghrelin, PYY and GLP-1. Further in depth research upon this topic is required in order to elucidate the precise mechanisms involved in weight loss.

Innovations and breakthroughs

According to authors' experience, laparoscopic Roux-en-Y gastric bypass appears to be a safe and effective surgical procedure for patients with BMI 35-50 kg/m^2. The vast majority of their complications occurred early at the learning curve. The authors were able to minimize their complication rate and further improve operation and shorten hospitalization.

Applications

Laparoscopic RYGB-LL is a safe and efficious bariatric procedure as it is associated with minor postoperative pain, early patient mobilization, low incidence of perioperative complications, improvement or complete resolution of preexisting comorbidities and durable weight loss. For these reasons, it should be a procedure of choice for the treatment of clinically severe obesity in properly selected patients.

Peer review

A single institutional series of 130 patients with laparoscopic gastric bypass is presented. Although not a huge series the results are excellent and the series is from a European country where lap gastric bypass is less common. The manuscript is likely of interest.

REFERENCES

1 **Elder KA**, Wolfe BM. Bariatric surgery: a review of procedures and outcomes. *Gastroenterology* 2007; **132**: 2253-2271
2 **Deitel M**. How much weight loss is sufficient to overcome major co-morbidities? *Obes Surg* 2001; **11**: 659
3 **Sugerman HJ**. Treatment of obesity. *J Gastrointest Surg* 2003; **7**: 476-477
4 **DeMaria EJ**. Bariatric surgery for morbid obesity. *N Engl J Med* 2007; **356**: 2176-2183
5 **Wittgrove AC**, Clark GW, Tremblay LJ. Laparoscopic Gastric Bypass, Roux-en-Y: Preliminary Report of Five Cases. *Obes Surg* 1994; **4**: 353-357
6 **Suter M**, Paroz A, Calmes JM, Giusti V. European experience with laparoscopic Roux-en-Y gastric bypass in

466 obese patients. *Br J Surg* 2006; **93**: 726-732
7 **Kalfarentzos F**, Papadoulas S, Skroubis G, Kehagias I, Loukidi A, Mead N. Prospective evaluation of biliopancreatic diversion with Roux-en-Y gastric bypass in the super obese. *J Gastrointest Surg* 2004; **8**: 479-488
8 **O'Brien PE**, McPhail T, Chaston TB, Dixon JB. Systematic review of medium-term weight loss after bariatric operations. *Obes Surg* 2006; **16**: 1032-1040
9 **Lujan JA**, Frutos MD, Hernandez Q, Liron R, Cuenca JR, Valero G, Parrilla P. Laparoscopic versus open gastric bypass in the treatment of morbid obesity: a randomized prospective study. *Ann Surg* 2004; **239**: 433-437
10 **Puzziferri N**, Austrheim-Smith IT, Wolfe BM, Wilson SE, Nguyen NT. Three-year follow-up of a prospective randomized trial comparing laparoscopic versus open gastric bypass. *Ann Surg* 2006; **243**: 181-188
11 **Polyzogopoulou EV**, Kalfarentzos F, Vagenakis AG, Alexandrides TK. Restoration of euglycemia and normal acute insulin response to glucose in obese subjects with type 2 diabetes following bariatric surgery. *Diabetes* 2003; **52**: 1098-1103
12 **Pories WJ**, Swanson MS, MacDonald KG, Long SB, Morris PG, Brown BM, Barakat HA, deRamon RA, Israel G, Dolezal JM. Who would have thought it? An operation proves to be the most effective therapy for adult-onset diabetes mellitus. *Ann Surg* 1995; **222**: 339-350; discussion 350-352
13 **Buchwald H**, Avidor Y, Braunwald E, Jensen MD, Pories W, Fahrbach K, Schoelles K. Bariatric surgery: a systematic review and meta-analysis. *JAMA* 2004; **292**: 1724-1737
14 **Nguyen NT**, Varela E, Sabio A, Tran CL, Stamos M, Wilson SE. Resolution of hyperlipidemia after laparoscopic Roux-en-Y gastric bypass. *J Am Coll Surg* 2006; **203**: 24-29
15 **Nguyen NT**, Goldman C, Rosenquist CJ, Arango A, Cole CJ, Lee SJ, Wolfe BM. Laparoscopic versus open gastric bypass: a randomized study of outcomes, quality of life, and costs. *Ann Surg* 2001; **234**: 279-289; discussion 289-291
16 **Schauer PR**, Ikramuddin S, Gourash W, Ramanathan R, Luketich J. Outcomes after laparoscopic Roux-en-Y gastric bypass for morbid obesity. *Ann Surg* 2000; **232**: 515-529
17 **Westling A**, Gustavsson S. Laparoscopic vs open Roux-en-Y gastric bypass: a prospective, randomized trial. *Obes Surg* 2001; **11**: 284-292
18 **Jones DB**, Provost DA, DeMaria EJ, Smith CD, Morgenstern L, Schirmer B. Optimal management of the morbidly obese patient. SAGES appropriateness conference statement. *Surg Endosc* 2004; **18**: 1029-1037
19 **Brolin RE**, Kenler HA, Gorman JH, Cody RP. Long-limb gastric bypass in the superobese. A prospective randomized study. *Ann Surg* 1992; **215**: 387-395
20 **Nguyen NT**, Longoria M, Chalifoux S, Wilson SE. Gastrointestinal hemorrhage after laparoscopic gastric bypass. *Obes Surg* 2004; **14**: 1308-1312
21 **Iannelli A**, Facchiano E, Gugenheim J. Internal hernia after laparoscopic Roux-en-Y gastric bypass for morbid obesity. *Obes Surg* 2006; **16**: 1265-1271
22 **Schauer P**, Ikramuddin S, Hamad G, Gourash W. The learning curve for laparoscopic Roux-en-Y gastric bypass is 100 cases. *Surg Endosc* 2003; **17**: 212-215
23 **le Roux CW**, Aylwin SJ, Batterham RL, Borg CM, Coyle F, Prasad V, Shurey S, Ghatei MA, Patel AG, Bloom SR. Gut hormone profiles following bariatric surgery favor an anorectic state, facilitate weight loss, and improve metabolic parameters. *Ann Surg* 2006; **243**: 108-114
24 **Batterham RL**, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, Wren AM, Brynes AE, Low MJ, Ghatei MA, Cone RD, Bloom SR. Gut hormone PYY(3-36) physiologically inhibits food intake. *Nature* 2002; **418**: 650-654
25 **Batterham RL**, Cohen MA, Ellis SM, Le Roux CW, Withers DJ, Frost GS, Ghatei MA, Bloom SR. Inhibition of food intake in obese subjects by peptide YY3-36. *N Engl J Med* 2003; **349**: 941-948
26 **Stock S**, Leichner P, Wong AC, Ghatei MA, Kieffer TJ, Bloom SR, Chanoine JP. Ghrelin, peptide YY, glucose-dependent insulinotropic polypeptide, and hunger responses to a mixed meal in anorexic, obese, and control female adolescents. *J Clin Endocrinol Metab* 2005; **90**: 2161-2168
27 **le Roux CW**, Batterham RL, Aylwin SJ, Patterson M, Borg CM, Wynne KJ, Kent A, Vincent RP, Gardiner J, Ghatei MA, Bloom SR. Attenuated peptide YY release in obese subjects is associated with reduced satiety. *Endocrinology* 2006; **147**: 3-8
28 **Karamanakos SN**, Vagenas K, Kalfarentzos F, Alexandrides TK. Weight loss, appetite suppression, and changes in fasting and postprandial ghrelin and peptide-YY levels after Roux-en-Y gastric bypass and sleeve gastrectomy: a prospective, double blind study. *Ann Surg* 2008; **247**: 401-407

S- Editor Li DL **L- Editor** Ma JY **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6030

World J Gastroenterol 2008 October 21; 14(39): 6030-6035
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Is ineffective esophageal motility associated with gastropharyngeal reflux disease?

Kyung Yup Kim, Gwang Ha Kim, Dong Uk Kim, Soo Geun Wang, Byung Joo Lee, Jin Choon Lee, Do Youn Park, Geun Am Song

Kyung Yup Kim, Gwang Ha Kim, Dong Uk Kim, Geun Am Song, Department of Internal Medicine, Pusan National University School of Medicine, Busan, South Korea
Soo Geun Wang, Byung Joo Lee, Jin Choon Lee, Department of Otolaryngology, Pusan National University School of Medicine, Busan, South Korea
Do Youn Park, Department of Pathology, Pusan National University School of Medicine, Busan, South Korea
Author contributions: Kim KY and Kim GH contributed equally to this work; Kim GH, Park DY and Song GA designed the research; Kim KY, Kim GH, Kim DU, Wang SG, Lee BJ and Lee JC performed the research; Kim GH and Park DY analyzed the data; and Kim KY and Kim GH wrote the paper.
Supported by A grant of The Korean Institute of Medicine
Correspondence to: Gwang Ha Kim, Department of Internal Medicine, Pusan National University School of Medicine and Medical Research Institute, Pusan National University Hospital, 1-10 Ami-dong, Seo-Gu, Busan 602-739, South Korea. doc0224@pusan.ac.kr
Telephone: +82-51-2407869 Fax: +82-51-2448180
Received: September 3, 2008 Revised: September 27, 2008
Accepted: October 4, 2008
Published online: October 21, 2008

Abstract

AIM: To evaluate the association between ineffective esophageal motility (IEM) and gastropharyngeal reflux disease (GPRD) in patients who underwent ambulatory 24-h dual-probe pH monitoring for the evaluation of supraesophageal symptoms.

METHODS: A total of 632 patients who underwent endoscopy, esophageal manometry and ambulatory 24-h dual-pH monitoring due to supraesophageal symptoms (*e.g.* globus, hoarseness, or cough) were enrolled. Of them, we selected the patients who had normal esophageal motility and IEM. The endoscopy and ambulatory pH monitoring findings were compared between the two groups.

RESULTS: A total of 264 patients with normal esophageal motility and 195 patients with the diagnosis of IEM were included in this study. There was no difference in the frequency of reflux esophagitis and hiatal hernia between the two groups. All the variables showing gastroesophageal reflux and gastropharyngeal reflux were not different between the two groups. The frequency of GERD and GPRD, as defined by ambulatory pH monitoring, was not different between the two groups.

CONCLUSION: There was no association between IEM and GPRD as well as between IEM and GERD. IEM alone cannot be considered as a definitive marker for reflux disease.

Key words: Ineffective esophageal motility; Gastroesophageal reflux disease; Gastropharyngeal reflux disease

Peer reviewers: Diego Garcia-Compean, MD, Professor, Faculty of Medicine, University Hospital, Department of Gastroenterology, Autonomous University of Nuevo Leon, Ave Madero y Gonzalitos, 64700 Monterrey, N.L.Mexico; Fabio Pace, Professor, Division of Gastroenterology, "L. Sacco" University Hospital, University of Milan, Via G. B. Grassi, 74, Milano 20157, Italy

Kim KY, Kim GH, Kim DU, Wang SG, Lee BJ, Lee JC, Park DY, Song GA. Is ineffective esophageal motility associated with gastropharyngeal reflux disease? *World J Gastroenterol* 2008; 14(39): 6030-6035 Available from: URL: http://www.wjgnet.com/1007-9327/14/6030.asp DOI: http://dx.doi.org/10.3748/wjg.14.6030

INTRODUCTION

Gastroesophageal reflux disease (GERD) is characterized by increased exposure of the esophageal mucosa to the gastric contents. This is mainly due to a various combinations of an increased number of gastroesophageal reflux episodes and abnormally prolonged clearance of the refluxed material[1,2]. The mechanisms for efficient clearance are effective peristalsis, the volume of saliva and gravity.

Ineffective esophageal motility (IEM) is the most recently described esophageal motility abnormality. IEM is defined as contractions with an amplitude of less than 30 mmHg and/or with a rate of nontransmission to the distal esophagus in number of 30% or more of water swallows[3,4]. IEM is associated with an increased acid clearance times in the distal esophagus[3]. Increased acid exposure in these patients is associated with the development of erosive esophagitis and GERD-

associated respiratory symptoms[5,6].

Gastropharyngeal reflux, also called laryngopharyngeal reflux, is a term used to describe esophageal acid reflux into the laryngeal and pharyngeal areas. It causes supraesophageal manifestations (*e.g.* globus, chronic cough, hoarseness, asthma, chronic sinusitis, or other pulmonary or otorhinolaryngologic diseases). Currently, the best way to demonstrate gastropharyngeal reflux is ambulatory 24-h dual probe pH monitoring[7].

It might be hypothesized that patients with IEM would be unable to clear refluxed acid; this would lead to a prolonged esophageal dwell time of the refluxed acid and then the refluxed acid would reach to a higher level. As a result, it would be presumed that patients with IEM have more gastropharyngeal reflux than those patients with normal esophageal motility.

Therefore, the aim of this study was to evaluate the association between IEM and gastropharyngeal reflux in a large series of patients who underwent ambulatory 24-h dual-probe pH monitoring for the evaluation of supraesophageal symptoms.

MATERIALS AND METHODS

Study population

We retrospectively analyzed the medical records and the findings from endoscopy, esophageal manometry and ambulatory 24-h pH monitoring of an unselected group of consecutive patients who were referred to our motility laboratory from July, 2003 to December, 2006. A total of 632 patients received all three examinations due to supraesophageal symptoms (*e.g.* globus, hoarseness or cough). Of them, we selected the patients who had normal esophageal motility and a diagnosis of IEM. We did not enroll those patients who had a history of gastric surgery, a diagnosis of scleroderma or those who were on anti-reflux medications at the time of the study.

This study was reviewed and approved by the Institutional Review Board of Pusan National University Hospital.

Assessment by endoscopy

The presence or absence of reflux esophagitis, hiatal hernia and endoscopically suspected esophageal metaplasia (ESEM) were determined by two endoscopists (G.H. Kim, G.A. Song).

Reflux esophagitis: If esophagitis was present, it was graded according to the Los Angeles classification[8].

Hiatal hernia: Hiatal hernia was defined as a circular extension of the gastric mucosa above the diaphragmatic hiatus greater than 2 cm in the axial length.

Endoscopically suspected esophageal metaplasia: The presence or absence of endoscopically suspected esophageal metaplasia (ESEM) was examined in the lower portion of the esophagus, including the esophagogastric junction, during inflation of the esophagus before inserting the endoscope into the stomach. The esophagogastric junction was defined as the oral side end of the fold, which exists continuously from the gastric lumen[9], as well as the end of the anal side of the fine longitudinal vessel, because the veins in the lower part of the esophagus were distributed uniformly, running parallel and longitudinally in the lamina propria[10,11]. The squamo-columnar junction was defined by a clear change in the color of the mucosa. ESEM was defined as the area between the squamo-columnar junction and the esophagogastric junction.

Esophageal manometry

All antisecretory and prokinetic medications were discontinued at least 7 d before testing. Esophageal manometry was performed, after an overnight fast, with using an eight-lumen catheter (Synetics Medical Co., Stockholm, Sweden) with side holes 3 cm, 4 cm, 5 cm, 6 cm, 8 cm, 13 cm, 18 cm, and 23 cm from the catheter tip and a water-perfused, low-compliance perfusion system (Synetics Medical Co., Stockholm, Sweden), according to a standard protocol. Briefly, the manometry protocol included the following: First, a station pull-through was performed through the lower esophageal sphincter (LES) to determine the end-expiratory resting pressure, the LES length and the location relative to the nares. The catheter was then positioned with the most distal side-hole 2 cm below the upper margin of the LES. Ten 5-mL water swallows were given to evaluate peristalsis; only the esophageal body contractions, measured at 3 cm, 8 cm and 13 cm above the LES, were recorded for data analysis. The catheter was then pulled through the upper esophageal sphincter (UES) in the same manner (station pull-through) to determine the resting UES pressure, the length and the location relative to the nares. Patients were identified as having IEM when the total sum of the low amplitude peristaltic contractions (the distal amplitude measured at 3 or 8 cm above the LES was < 30 mmHg) and the nontransmitted peristaltic contractions (dropouts at either 3 cm or 8 cm above the LES) was equal or greater than 30% of the total number of swallows used for the esophageal body study[4].

Ambulatory 24-h dual-probe pH monitoring

Ambulatory 24-h dual-probe pH monitoring was performed immediately after esophageal manometry with using a single-use monocrystalline antimony dual-site pH probe (Zinetics 24, Medtronic Inc., Minneapolis, USA) with the electrodes placed at the tip and 15 cm proximal to the tip. A cutaneous reference electrode placed on the upper chest was also used. All the electrodes were calibrated in buffer solutions of pH 7 initially and then pH 1. The pH catheter was introduced transnasally into the stomach and it was withdrawn back into the esophagus until the electrodes were 5 cm above the proximal margin of the LES. The subjects were encouraged to eat regular meals with restriction for the intake of drink or food with a pH below 4. All the subjects recorded their meal times (start and end), body position (supine and upright) and any symptoms

in a diary. The data were collected using a portable data logger (Digitrapper Mark Ⅲ, Synetics Medical Co., Stockholm, Sweden) with a sampling rate of 4 seconds, and the data was then transferred to a computer for analysis using "Polygram for Windows®" (Release 2.04, Synetics Medical Co., Stockholm, Sweden). For both sites, a decrease in pH below 4, which was not induced by eating or drinking, was considered the beginning of a reflux episode, and the following rise to pH above 4 was considered the end of such an episode. To be accepted as a gastropharyngeal reflux event, the decrease at the proximal probe had to be abrupt and simultaneous with the decrease at the distal probe, or it was preceded by a decrease in pH of a similar or larger magnitude at the distal probe. Thus, acid episodes induced by oral intake, aero-digestive tract residue and secretions, proximal probe movement or loss of mucosal contact in which the proximal pH decline may precede the esophageal pH drop were not included as gastropharyngeal reflux episodes.

The variables assessed for gastroesophageal reflux at the distal probe were the total percentage of time the pH was < 4, the percentage of time the pH was < 4 in the supine and upright positions, the number of episodes the pH was < 4, the number of episodes the pH was < 4 for ⩾ 5 min, the duration of the longest episode the pH was < 4 and the DeMeester composite score[12].

The variables assessed for gastropharyngeal reflux at the proximal probe were the total percentage of time the pH was < 4, the percentage of time the pH was < 4 in the supine and upright positions, and the number of episodes the pH was < 4.

For the diagnosis of GERD at the distal probe, two different aspects were analyzed[13,14]; (1) the total reflux time: the total proportion of the recorded time with pH < 4; a value of > 4% was considered abnormal; (2) the number of reflux episodes: the total number of pH episodes with pH< 4 during the recording; a value of > 35 episodes was considered abnormal.

For the diagnosis of gastropharyngeal reflux disease (GPRD) at the proximal probe, we considered more than 0.1% for the total time, 0.2% for the upright time and 0% for the supine time of pH < 4 to be pathological. For the number of reflux episodes, more than 4 reflux episodes were considered pathological[15,16].

Statistical analysis

The data are expressed as mean ± SE unless otherwise noted. The student *t*-test was used to assess the statistical significance of age, the body mass index, the pressure and length of the LES and the parameters of ambulatory pH monitoring between the two groups. The differences in gender, alcohol intake, smoking, typical reflux symptoms, indications for pH monitoring, reflux esophagitis, hiatal hernia, ESEM, GERD and GPRD, as defined by the ambulatory pH monitoring between the two groups were assessed using the χ^2 test. A $P < 0.05$ was considered statistically significant. Statistical calculations were performed using the SPSS version 12.0 for Windows software (SPSS Inc., Chicago, IL, USA).

Table 1 Patient profiles and the endoscopic findings in the patients with normal esophageal motility and ineffective esophageal motility *n* (%)

	Normal (*n* = 264)	IEM (*n* = 195)	*P* value
Age (yr, mean ± SD)	50.8 ± 11.1	51.1 ± 12.0	0.782
Gender (men/women)	99/165	87/108	0.125
BMI (kg/m^2, mean ± SD)	23.6 ± 2.7	23.2 ± 2.7	0.393
Alcohol intake	58 (22.0)	31 (15.9)	0.104
Smoking	43 (16.3)	19 (9.7)	0.043
Heartburn/acid regurgitation[1]	128 (48.5)	104 (53.3)	0.304
Indication for pH monitoring			0.542
Globus	118 (44.7)	88 (45.1)	
Hoarseness	63 (23.9)	35 (17.9)	
Cough	27 (10.2)	24 (12.3)	
Sore throat	30 (11.4)	28 (14.4)	
Others[2]	26 (9.8)	20 (10.3)	
Reflux esophagitis[3]	30 (11.4)	30 (15.4)	0.206
A	22	16	
B	7	11	
C	1	2	
D	0	1	
Hiatal hernia	17 (6.4)	10 (5.1)	0.555
Endoscopically suspected esophageal metaplasia	23 (8.7)	11 (5.6)	0.214

[1]More than 2 d per wk; [2]Other indications were halitosis, throat clearing and laryngeal pathology such as vocal polyp; [3]Los Angeles classification grade. IEM: Ineffective esophageal motility.

RESULTS

A total of 264 patients with normal esophageal motility and 195 patients with the diagnosis of IEM were included in this study. Age, gender, the body mass index, typical reflux symptoms and indications for pH monitoring were not different between the two groups. There was no difference in the frequency of reflux esophagitis and hiatal hernia between the two groups (Table1).

The LES pressure was lower in the patients with IEM than in those patients with normal esophageal motility. All the variables showing gastroesophageal reflux at the distal probe were not different between the two groups. There was no difference in all the variables showing gastropharyngeal reflux at the proximal probe between the two groups (Table 2).

The frequency of GERD and GPRD, as defined by ambulatory pH monitoring was not different between the two groups (Table 3, Figure 1).

DISCUSSION

Esophageal acid clearance consists of two processes, first is rapid removal of most of the intraluminal refluxate, which is achieved by gravity and primary or secondary peristalsis (volume clearance), and this is followed by a slow neutralization of the acidified mucosa by the swallowed saliva (chemical clearance). Previous analysis of the relationship between peristaltic dysfunction and the efficacy of esophageal emptying, with using concurrent manometry and fluoroscopy, illustrated that absent or incomplete peristaltic contractions invariably

Table 2 Results of the lower esophageal sphincter and ambulatory 24-h dual probe pH monitoring in the patients with normal esophageal motility and ineffective esophageal motility

	Normal (n = 264)	IEM (n = 195)	P value
Lower esophageal sphincter			
Pressure	21.4 ± 0.5	18.6 ± 0.5	< 0.001
Length	3.3 ± 0.1	3.3 ± 0.1	0.921
Proximal probe			
Time pH < 4 (total) (%)	0.4 ± 0.1	0.7 ± 0.2	0.225
Time pH < 4 (upright) (%)	0.7 ± 0.1	1.0 ± 0.2	0.230
Time pH < 4 (supine) (%)	0.1 ± 0.0	0.6 ± 0.4	0.214
No. of reflux episodes	8.7 ± 1.0	12.2 ± 2.6	0.219
Distal probe			
Time pH < 4 (total) (%)	2.9 ± 0.2	3.5 ± 0.3	0.185
Time pH < 4 (upright) (%)	4.8 ± 0.7	5.0 ± 0.5	0.827
Time pH < 4 (supine) (%)	1.6 ± 0.3	1.9 ± 0.4	0.564
No. of reflux episodes	44.1 ± 2.5	47.8 ± 4.0	0.430
No. of reflux episodes ⩾ 5 min	1.4 ± 0.2	1.9 ± 0.3	0.116
Longest reflux episode (min)	8.6 ± 0.8	10.2 ± 1.0	0.199
DeMeester composite score	12.1 ± 0.9	14.1 ± 1.3	0.217

IEM: Ineffective esophageal motility.

Table 3 Distribution of the patients with gastroesophageal reflux disease and/or gastropharyngeal reflux disease, as defined by ambulatory pH monitoring, in the patients with normal esophageal motility and ineffective esophageal motility n (%)

	Normal (n = 264)	IEM (n = 195)
GERD and GPRD	108 (40.9)	74 (37.9)
GPRD only	56 (21.2)	33 (16.9)
GERD only	19 (7.2)	18 (9.2)
Normal	81 (30.7)	70 (35.9)

GERD: Gastroesophageal reflux disease; GPRD: **G**astropharyngeal reflux disease; IEM: Ineffective esophageal motility.

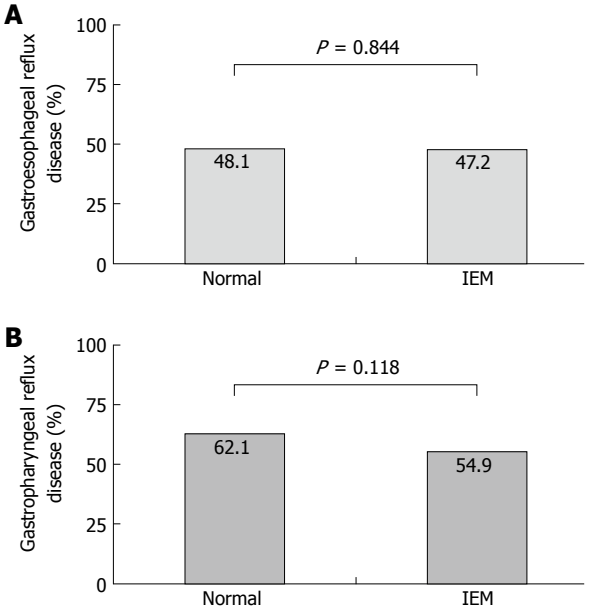


Figure 1 Frequency of gastroesophageal reflux disease (**A**) and gastropharyngeal reflux disease (**B**), as defined by ambulatory pH monitoring, in the patients with normal esophageal motility and ineffective esophageal motility (IEM).

resulted in little or no volume clearance and ineffective esophageal propulsion of a bolus occurs when the amplitude of the peristaltic waves is below 30 mmHg[17]. Thus, peristaltic dysfunction could potentially prolong esophageal acid clearance by delaying the first phase, that of esophageal emptying.

GERD motility abnormalities are part of the nonspecific motor disorders that have been described many years ago[18], and IEM has been found in 20%-50% of the patients with GERD[19]. In addition, there have been some studies suggesting a link between IEM and delayed esophageal acid clearance[3,5,20]. When GERD patients underwent pH monitoring, there were significantly more recumbent and upright reflux episodes and delayed acid clearance in the patients with IEM than in those patients without IEM[3,20]. A greater frequency of IEM was found in patients with respiratory presentations of GERD (chronic cough, asthma and laryngitis) and identification of IEM was particularly useful for patients with supraesophageal GERD[5].

In present study, we selected the patients who had normal esophageal motility and IEM among the patients who received the endoscopy, esophageal manometry and ambulatory pH monitoring due to supraesophageal symptoms. We then analyzed the degree of gastroesophageal and gastropharyngeal reflux in both group. Our results indicated that IEM was not associated with GPRD as well as GERD, as defined by ambulatory pH monitoring. In addition, all the variables for gastropharyngeal reflux and gastroesophageal reflux were not higher in the patients with IEM than those with normal esophageal motility. These findings are consistent with the previous studies[21,22] showing that there was no association between esophageal dysmotility and abnormal acid reflux in patients with supraesophageal GERD symptoms. We also examined the degree of gastroesophageal and gastropharyngeal reflux according to the severity of IEM, but there was no association (data not shown), which was similar to the previous report[23] showing that the severity of IEM was not different in erosive and in nonerosive GERD patients. These results suggest that IEM alone is unlikely to be the major determinant of abnormal esophageal acid exposure.

Although many studies have assessed the link between IEM and esophagitis, this issue remains controversial. Most of the previous studies restricted the enrolled subjects to the GERD patients. IEM was associated with reflux esophagitis in some studies of patients with confirmed GERD[6,24]. However, other studies showed that the presence of reflux esophagitis was similar between the patients with IEM and those patients with normal esophageal peristalsis[20] and there was no difference in the severity of IEM when comparing the erosive and non-erosive GERD patients[23]. In our present study, we included the patients who had normal esophageal motility and IEM over a defined period, providing that the ambulatory study had been done in the absence of anti-secretory therapy, thereby insuring the presence of a control group with normal esophageal acid exposure. Our result showed that reflux esophagitis was not associated with IEM.

There were some merits of this study when

comparing it with the previous studies. First, in contrast to previous reports[5,21,22] that focused on an association between IEM and supraesophageal reflux disease, our study limited the enrolled subjects to patients with normal esophageal motility and those with IEM to maximize the effect of IEM on GPRD. Second, in the current analysis, we defined GERD and GPRD according to the strict criteria of ambulatory 24-h dual-probe pH monitoring, which is the best available test for diagnosing GPRD, as well as GERD[7]. Third, because all the patients in the current study underwent upper endoscopy, we were able to classify them according to the presence or absence of esophagitis and hiatal hernia.

There were some limitations in this study. First, the ambulatory pH monitoring is not 100% accurate and it has a sensitivity as low as 70% in patients with esophagitis, and the sensitivity is substantially lower in patients with nonerosive disease[25], so that some of our patients may have been misclassified. Yet, we included a large number of cases (459 cases), so this limitation was probably lessened. Second, a great deal of controversy exists about the location of the proximal probe. Recording the pH in the hypopharynx is technically difficult. Acid exposure in the hypopharynx can easily be missed because of the relatively large space within the hypopharynx[15]. On the contrary, placement of the proximal probe in or below the upper esophageal sphincter allows for more permanent contact with the mucosa during the 24-h period and this results in fewer artifacts[15,16]. We used a dual-site pH probe with electrodes placed at the tip and 15 cm proximal to the tip, and we could not choose the exact location of the proximal probe. Yet in most cases (75.4%, 346/459), the proximal probe was located in the UES. So, for the diagnosis of GPRD, we used the criteria proposed by Smit *et al*[15,16].

Why is IEM not associated with GPRD as well as GERD? Conventional manometry may be unable to evaluate the "true effectiveness" of esophageal peristalsis[26,27]. In addition, the refluxed acid is neutralized by both the esophageal submucosal secretions and the swallowed salivary secretions, so it becomes non-acid reflux material. Therefore, even though this non-acid refluxate in the upper level actually increased in the patients with IEM, the proximal pH probe cannot detect it. To solve this problem, a prospective study using a combined multichannel intraluminal impedance and pH measurement, which are able to detect both acid and non-acid reflux, as well as the proximal extent of the refluxate, will be needed.

In conclusion, by analyzing a large cohort of patients who had normal esophageal motility and IEM, we demonstrated that there was no correlation between IEM and GPRD, as well as between IEM and GERD, as defined by ambulatory pH monitoring. Although we do not completely exclude that such an association may be possible, IEM alone cannot be considered a definitive marker for reflux (gastroesophageal or gastropharyngeal).

ACNOWLEDGEMENTS

We wish to thank Jin Seon Kim (Gastrointestinal Motility Laboratory, Pusan National University Hospital) for her assistance with this study.

COMMENTS

Background

Ineffective esophageal motility (IEM) is associated with an increased acid clearance times in the distal esophagus. Gastropharyngeal reflux causes supraesophageal manifestations such as globus, chronic cough, hoarseness, asthma, chronic sinusitis, or other otorhinolaryngologic diseases. It might be hypothesized that patients with IEM would be unable to clear refluxed acid; this would lead to a prolonged esophageal dwell time of the refluxed acid and then the refluxed acid would reach to a higher level. As a result, it would be presumed that patients with IEM have more gastropharyngeal reflux than those patients with normal esophageal motility.

Research frontiers

The research front in this area is focused on evaluating the association of IEM and gastropharyngeal reflux disease (GPRD), as well as gastroesophageal reflux disease (GERD). Although many studies have assessed the link between IEM and esophagitis, this issue remains controversial. Most of the previous studies restricted the enrolled subjects to GERD patients. IEM was associated with reflux esophagitis in some studies of patients with confirmed GERD. However, other studies showed that the presence of reflux esophagitis was similar between the patients with IEM and those patients with normal esophageal peristalsis. This study showed no association between IEM and GPRD, as well as between IEM and GERD in a large series of patients who underwent ambulatory 24-hour dual-probe pH monitoring, for the evaluation of supraesophageal symptoms.

Innovations and breakthroughs

There are few reports on the association between IEM and GPRD. Most previous studies are symptom-based and lack objective tests such as ambulatory 24-h dual-probe pH monitoring. This study is the largest study to evaluate the association of IEM and GPRD in patients who underwent ambulatory 24-h dual-probe pH monitoring for the evaluation of supraesophageal symptoms.

Applications

IEM is not associated with GPRD, as well as GERD. Further studies using a combined multichannel intraluminal impedance and pH measurement, which are able to detect both acid and non-acid reflux, as well as the proximal extent of the refluxate, will be needed.

Terminology

Ineffective esophageal motility (IEM) is defined as contractions with an amplitude of less than 30 mmHg and/or with a rate of nontransmission to the distal esophagus in number of 30% or more of water swallows. Esophageal acid reflux into the laryngeal and pharyngeal areas causes extraesophageal manifestations such as chronic cough, hoarseness, asthma, globus sensation, chronic sinusitis, or other otorhinolaryngologic diseases. This condition is called as gastropharyngeal reflux disease (GPRD).

Peer review

This is an interesting study since physicians who perform esophageal manometry frequently find IEM. This study is well structured and definitions of esophagitis, GERD and GPRD are adequate since they were based on endoscopy and 24-h dual esophageal pH monitoring.

REFERENCES

1 **Dodds WJ**, Dent J, Hogan WJ, Helm JF, Hauser R, Patel GK, Egide MS. Mechanisms of gastroesophageal reflux in patients with reflux esophagitis. *N Engl J Med* 1982; **307**: 1547-1552

2 **Orlando RC**. Overview of the mechanisms of gastro-esophageal reflux. *Am J Med* 2001; **111** Suppl 8A: 174S-177S

3 **Leite LP**, Johnston BT, Barrett J, Castell JA, Castell DO. Ineffective esophageal motility (IEM): the primary finding in patients with nonspecific esophageal motility disorder. *Dig Dis Sci* 1997; **42**: 1859-1865

4 **Spechler SJ**, Castell DO. Classification of oesophageal motility abnormalities. *Gut* 2001; **49**: 145-151
5 **Fouad YM**, Katz PO, Hatlebakk JG, Castell DO. Ineffective esophageal motility: the most common motility abnormality in patients with GERD-associated respiratory symptoms. *Am J Gastroenterol* 1999; **94**: 1464-1467
6 **Diener U**, Patti MG, Molena D, Fisichella PM, Way LW. Esophageal dysmotility and gastroesophageal reflux disease. *J Gastrointest Surg* 2001; **5**: 260-265
7 **Dobhan R**, Castell DO. Normal and abnormal proximal esophageal acid exposure: results of ambulatory dual-probe pH monitoring. *Am J Gastroenterol* 1993; **88**: 25-29
8 **Lundell LR**, Dent J, Bennett JR, Blum AL, Armstrong D, Galmiche JP, Johnson F, Hongo M, Richter JE, Spechler SJ, Tytgat GN, Wallin L. Endoscopic assessment of oesophagitis: clinical and functional correlates and further validation of the Los Angeles classification. *Gut* 1999; **45**: 172-180
9 **Nandurkar S**, Talley NJ. Barrett's esophagus: the long and the short of it. *Am J Gastroenterol* 1999; **94**: 30-40
10 **Vianna A**, Hayes PC, Moscoso G, Driver M, Portmann B, Westaby D, Williams R. Normal venous circulation of the gastroesophageal junction. A route to understanding varices. *Gastroenterology* 1987; **93**: 876-889
11 **Noda T**. Angioarchitectural study of esophageal varices. With special reference to variceal rupture. *Virchows Arch A Pathol Anat Histopathol* 1984; **404**: 381-392
12 **Johnson LF**, DeMeester TR. Development of the 24-hour intraesophageal pH monitoring composite scoring system. *J Clin Gastroenterol* 1986; **8** Suppl 1: 52-58
13 **Johnsson F**, Joelsson B, Isberg PE. Ambulatory 24 hour intraesophageal pH-monitoring in the diagnosis of gastroesophageal reflux disease. *Gut* 1987; **28**: 1145-1150
14 **Weusten BLAM**, Smout AJPM. Ambulatory monitoring of esophageal pH and pressure In: Castell DO, Richter JE eds. The Esophagus. 4th ed. Philadelphia: Lippincott Williams & Wilkins, 2004: 135-150
15 **Smit CF**, Mathus-Vliegen LM, Devriese PP, Schouwenburg PF, Kupperman D. Diagnosis and consequences of gastropharyngeal reflux. *Clin Otolaryngol Allied Sci* 2000; **25**: 440-455
16 **Smit CF**, Tan J, Devriese PP, Mathus-Vliegen LM, Brandsen M, Schouwenburg PF. Ambulatory pH measurements at the upper esophageal sphincter. *Laryngoscope* 1998; **108**: 299-302
17 **Kahrilas PJ**, Dodds WJ, Hogan WJ. Effect of peristaltic dysfunction on esophageal volume clearance. *Gastroenterology* 1988; **94**: 73-80
18 **Richter JE**, Wu WC, Johns DN, Blackwell JN, Nelson JL 3rd, Castell JA, Castell DO. Esophageal manometry in 95 healthy adult volunteers. Variability of pressures with age and frequency of "abnormal" contractions. *Dig Dis Sci* 1987; **32**: 583-592
19 **Kahrilas PJ**, Pandolfino JE. Ineffective esophageal motility does not equate to GERD. *Am J Gastroenterol* 2003; **98**: 715-717
20 **Ho SC**, Chang CS, Wu CY, Chen GH. Ineffective esophageal motility is a primary motility disorder in gastroesophageal reflux disease. *Dig Dis Sci* 2002; **47**: 652-656
21 **Vinjirayer E**, Gonzalez B, Brensinger C, Bracy N, Obelmejias R, Katzka DA, Metz DC. Ineffective motility is not a marker for gastroesophageal reflux disease. *Am J Gastroenterol* 2003; **98**: 771-776
22 **Knight RE**, Wells JR, Parrish RS. Esophageal dysmotility as an important co-factor in extraesophageal manifestations of gastroesophageal reflux. *Laryngoscope* 2000; **110**: 1462-1466
23 **Lemme EM**, Abrahao-Junior LJ, Manhaes Y, Shechter R, Carvalho BB, Alvariz A. Ineffective esophageal motility in gastroesophageal erosive reflux disease and in nonerosive reflux disease: are they different? *J Clin Gastroenterol* 2005; **39**: 224-227
24 **Fornari F**, Callegari-Jacques SM, Scussel PJ, Madalosso LF, Barros EF, Barros SG. Is ineffective oesophageal motility associated with reflux oesophagitis? *Eur J Gastroenterol Hepatol* 2007; **19**: 783-787
25 **Kahrilas PJ**, Quigley EM. Clinical esophageal pH recording: a technical review for practice guideline development. *Gastroenterology* 1996; **110**: 1982-1996
26 **Simren M**, Silny J, Holloway R, Tack J, Janssens J, Sifrim D. Relevance of ineffective oesophageal motility during oesophageal acid clearance. *Gut* 2003; **52**: 784-790
27 **Tutuian R**, Castell DO. Esophageal function testing: role of combined multichannel intraluminal impedance and manometry. *Gastrointest Endosc Clin N Am* 2005; **15**: 265-275

S- Editor Xiao LL **L- Editor** Rippe RA **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6036

World J Gastroenterol 2008 October 21; 14(39): 6036-6043
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Decreased phagocytic activity of Kupffer cells in a rat nonalcoholic steatohepatitis model

Tatsuhiro Tsujimoto, Hideto Kawaratani, Toshiyuki Kitazawa, Toshiko Hirai, Hajime Ohishi, Mitsuteru Kitade, Hitoshi Yoshiji, Masahito Uemura, Hiroshi Fukui

Tatsuhiro Tsujimoto, Hideto Kawaratani, Toshiyuki Kitazawa, Mitsuteru Kitade, Hitoshi Yoshiji, Masahito Uemura, Hiroshi Fukui, Third Department of Internal Medicine, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan
Toshiko Hirai, Hajime Ohishi, Department of Endoscopy and Ultrasound, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan
Author contributions: Tsujimoto T and Fukui H designed the research project; Tsujimoto T, Kawaratani H, Kitazawa T and Kitade M contributed to the immunohistochemical staining; Tsujimoto T, Hirai T and Ohishi H contributed to contrast enhanced ultrasonography (CEUS) examination; Tsujimoto T, Kawaratani H, Yoshiji H and Uemura M analysed the data; and Tsujimoto T wrote the paper.
Supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, No. 19590784
Correspondence to: Tatsuhiro Tsujimoto, MD, PhD, Third Department of Internal Medicine, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan. tat-tyan@xa2.so-net.ne.jp
Telephone: +81-744-223051 Fax: +81-744-247122
Received: June 20, 2008 Revised: September 16, 2008
Accepted: September 23, 2008
Published online: October 21, 2008

Abstract

AIM: To investigate Kupffer cell dynamics and phagocytic activity, using a rat nonalcoholic steatohepatitis (NASH) model.

METHODS: Male F344 rats were fed either a control diet or a choline-deficient L-amino acid-defined (CDAA) diet, followed by contrast enhanced ultrasonography (CEUS) using Levovist®. The uptake of latex beads by the Kupffer cells was determined by fluorescent microscopy. The status of the Kupffer cells was compared between the two groups, using the immunohistochemical staining technique.

RESULTS: After 4 or more wk of the CDAA diet, CEUS examination revealed a decrease in the signal intensity, 20 min after intravenous Levovist®. Fluorescent microscopic examination showed that the uptake of latex beads by the Kupffer cells was reduced at week 1 and 2 in the study group, compared with the controls, with no further reduction after 3 wk. Immunohistochemical staining revealed no significant difference in the Kupffer cell counts between the control group and the CDAA group.

CONCLUSION: CEUS examination using Levovist® demonstrated reduced contrast effect and phagocytic activity in the liver parenchymal phase, although the Kupffer cell numbers were unchanged, indicating reduced phagocytic function of the Kupffer cells in the rat NASH model. We believe that CEUS examination using Levovist® is a useful screening modality, which can detect NASH in fatty liver patients.

Key words: Nonalcoholic steatohepatitis; Kupffer cells; Contrast enhanced ultrasonography; Levovist; Ultrasound contrast agent; Phagocytic activity

Peer reviewers: Natalia A Osna, Liver Study Unit, Research Service (151), VA Medical Center, 4101 Woolworth Avenue, Omaha NE 68105, United States; Ursula M Gehling, PhD, Department of Hepatobiliary Surgery and Visc, University Hospital Hamburg-Eppendorf, Martinistrasse 52, Hamburg 20246, Germany

Tsujimoto T, Kawaratani H, Kitazawa T, Hirai T, Ohishi H, Kitade M, Yoshiji H, Uemura M, Fukui H. Decreased phagocytic activity of Kupffer cells in a rat nonalcoholic steatohepatitis model. *World J Gastroenterol* 2008; 14(39): 6036-6043 Available from: URL: http://www.wjgnet.com/1007-9327/14/6036.asp DOI: http://dx.doi.org/10.3748/wjg.14.6036

INTRODUCTION

Nonalcoholic steatohepatitis (NASH) is characterized by hepatic steatosis, inflammation and fibrosis, with increased risk of developing cirrhosis and hepatocellular carcinoma (HCC)[1-3]. The progression from simple steatosis to cirrhosis has been attributed to inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), oxidative stress and endotoxins, in combination with fatty degeneration due to insulin resistance[4]. At present, histopathological examination of liver biopsy tissue is the only way to definitively diagnose NASH[5-8].

The diagnosis of NASH is important in clinical practice, since this condition can progress to cirrhosis and HCC. When patients with NASH undergo contrast enhanced ultrasonography (CEUS) using Levovist

® (galactose-palmitic acid ultrasound contrast agent), a reduced contrast effect is seen in the liver parenchymal phase[9]. The activity of Kupffer cells and hepatic sinusoids can be evaluated using the contrast effect in the liver parenchymal phase during CEUS examination with Levovist®. The findings strongly implicate Kupffer cells in the pathogenesis of NASH. Reduced function or uneven distribution of Kupffer cells in the liver may play a part in the development of NASH, although this hypothesis remains conjectural at the present time.

In this study, we investigated the contrast effect in the liver parenchymal phase of CEUS using Levovist®, and assessed the Kupffer cell dynamics and phagocytic activity.

MATERIALS AND METHODS

Animals, NASH model induction

Six-week-old male F344 rats weighing 180-200 g were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). The animals were housed in stainless steel mesh cages under controlled conditions of temperature (23 ± 3℃) and relative humidity (50% ± 20%), with 10 to 15 air changes per hour, and light illumination for 12 h a day. The animals were allowed access to tap water ad libitum throughout the duration of the study. A choline-deficient L-amino acid-defined (CDAA), and a choline-supplemented L-amino acid-defined (CSAA) diet were purchased from CLEA Japan Inc. (Tokyo, Japan). The details of both diets are described elsewhere[10]. The study group was fed a CDAA diet for 8 wk in order to produce NASH. All procedures were approved by the institutional animal care committee and conducted in accordance with Nara Medical University Guidelines for the Care and Use of Laboratory Animals.

Serum alanine aminotransferase (ALT)

Serum samples from CSAA-fed rats and CDAA-fed rats, killed at 1, 4 and 8 wk were used to measure the serum ALT levels. The levels of serum ALT were determined using a 7170 Clinical Analyzer (Hitachi High-Technologies, Tokyo, Japan).

Histological examination

The liver tissues were fixed in 10% formalin, and the first section was stained with hematoxylin and eosin for histological examination. Another section was stained with Azan and Sirius red to detect fibrosis. Histological grading and staging were performed using a modified scoring system based on the classification of either Matteoni *et al*[5] or Brunt *et al*[6]. Matteoni *et al*[5] proposed NAFLD types 1-4 based on long-term outcome studies; Brunt *et al*[6] proposed a system of grading and staging for NASH that follows methods of separate assessment of necroinflammatory lesions (grading) and fibrosis (staging), accepted in other forms of non-biliary chronic liver diseases.

Levovist® CEUS studies

We compared the contrast effects in the CEUS liver parenchymal phase before administering the CDAA diet (control group, n = 5), and after 1, 4, and 8 wk of the CDAA diet (1-, 4-, and 8-wk groups, n = 5 for each group). The studies were performed with the Logiq 7 ultrasonographic system (GE Healthcare, Tokyo, Japan), using a 7L probe (3-7 MHz), and employing the Coded Harmonic Angio mode. Levovist® (Schering AG, Berlin, Germany) was diluted to 300 mg/mL, and injected into the animal's tail vein at a dose of 0.1 mL/100 g body weight. Following confirmation that the contrast had entered the right kidney, we scanned the liver, using 1-s intermittent transmission scans at 5, 10, 15, and 20 min, with a different section for each scan. The fluorescent intensity in the region of interest (ROI) in each image was calculated by the equipment software using the time intensity curve (TIC).

Observation of FITC-latex beads phagocytosis by Kupffer cells in vivo

An injection of 2×10^{10}/kg 1 μm fluorescent latex beads (Polyscience, Warrington, PA, USA) was given into the animal's tail vein before CDAA (control group, n = 5), and after 1, 2, 3, 4, and 8 wk of CDAA (1-, 2-, 3-, 4- and 8-wk groups, n = 5 for each group). Two hours after the injection, the animals were killed and 4% paraformaldehyde and 0.5% glutaraldehyde in phosphate buffer saline (PBS) was perfused into the portal vein. After fixation with the same fixative for 60 min, the liver specimens were sliced into 5-μm-thick sections with a cryostat microtome CM 1510 (Leica Microsystems, Wetzlar, Germany). The uptake of latex beads by the Kupffer cells was determined using fluorescent microscopy Leica DM IRB (Leica Microsystems), and the fluorescent intensities were analyzed and compared using IP LabTM software (BD Biosciences, Rockville, MD, USA).

Isolation and culture of Kupffer cells

The Kupffer cells were harvested from the liver using the isolation buffers described by Seglen[11]. The liver was perfused in situ with Ca^{2+}-free minimum essential medium (Sigma, St. Louis, MI, USA) followed by 0.3% pronase (Roche Diagnostics Corp., Indianapolis, IN, USA), and 0.05% type IV collagenase (Sigma) in Dulbecco's modified eagle's medium/F-12 (Sigma) at a rate of 10 mL/min through the portal vein. The liver was carefully removed and minced with scissors. The minced liver was incubated in a shaker water bath with 0.035% pronase and 62.5 U/mL DNase (Sigma) in Dulbecco's modified eagle's medium/F-12 at 37℃ for 20 min, and was then filtered through gauze; the parenchymal cells were removed by low-speed centrifugation. The resultant supernatant was laid on top of 4 separate densities (1.035, 1.045, 1.058, and 1.085) of arabinogalactan solution (Sigma) in one test tube and centrifuged at 400 r/min for 45 min at 37℃ using a Beckman SW41-Ti rotor (Beckman Instruments, Fullerton, CA, USA). The third and fourth layers from the top were recovered and washed twice with Ca^{2+}-free Hanks balanced salt solution (Sigma). The final cell pallet was subsequently suspended in RPMI 1640 medium,

and cultured in a culture flask at 37℃ in humidified atmosphere containing 50 mL/L CO_2 and air for 2 h.

The purity of the isolated Kupffer cells was over 98%, as determined by the uptake of 1 μm latex beads[12], and the viability was over 95% in the trypan-blue-dye exclusion test.

The Kupffer cells were seeded in 12-well plastic plates and incubated in RPMI 1640 medium at a concentration of 5×10^5 cells/mL. The dishes were washed with Hanks balanced salt solution to remove the unattached cells. The Kupffer cells were cultured in RPMI 1640 supplemented with 50 μg/mL streptomycin and 50 μg/mL ampicillin (Nakalai tesque, Kyoto, Japan).

Observation of FITC-latex beads phagocytosis by Kupffer cells in vitro

Fluorescent latex beads (1×10^7 1 μm) were placed in each well, and culture was performed at 37℃ for 2 h under 50 mL/L CO_2 on plastic dishes that were washed three times with Hanks balanced salt solution to remove un-phagocytosed latex beads. The uptake of latex beads by the Kupffer cells was determined using fluorescent microscopy, and the fluorescent intensities were analyzed and compared using IP Lab™ software.

Kupffer cells immunohistochemical staining

The Kupffer cell dynamics were analyzed by comparing the cell counts of each group, using immuno-histochemical staining with anti-rat macrophage/dendritic cell monoclonal antibody (RM-4: Trans Genic Inc., Kobe, Japan), a Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA, USA), and DAB peroxidase substrate solution (Vector Laboratories), with counterstaining by Hematoxylin Mayer. The stained areas were analyzed and compared using NIH-image software (Version 1.61; U. S. National Institute of Health, Bethesda, MD, USA).

Statistical analysis

P-values were calculated, assuming equal sample variance, using the paired *t*-test, and Statview software (version 5.0; SAS Institute, Cary, NC, USA), considering $P < 0.05$ as statistically significant. The values are mentioned as mean ± SD.

RESULTS

Serum ALT

The mean serum ALT level in the control rats was 41.7 ± 7.4 IU/L, compared to 524.6 ± 101.7 IU/L, 267.9 ± 47.5 IU/L and 251.4 ± 81.6 IU/L in the 1-, 4-, and 8-wk, respectively in the CDAA-fed rats. All NASH groups showed statistically significant elevations, compared with the control animals ($P < 0.01$) (Figure 1).

Changes in liver histology

Histological examination of the liver of 1-wk CDAA-fed rats revealed inflammation and fat deposits, but no fibrosis, and was considered as Matteoni's type 2.

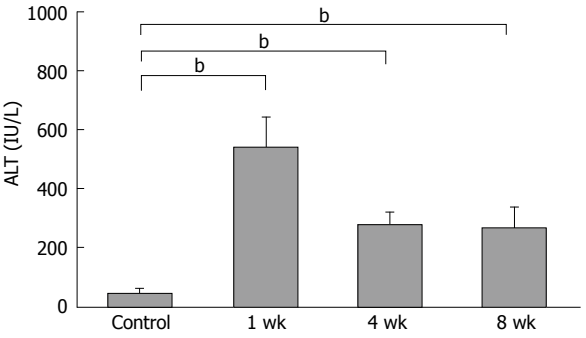


Figure 1 Serum ALT levels were elevated significantly in 1-wk CDAA-fed rats, and decreased gradually in the 4 and 8-wk CDAA-fed rats, although, both were elevated significantly compared with the control animals ($n = 5$), $^bP < 0.01$.

The 4-wk CDAA-fed rats had more inflammation, fat deposits, and fibrosis, which was equivalent to Matteoni's type 3 and Brunt's NASH classification of grade 2/stage 2. The histological findings in the 8-wk CDAA-fed rats were equivalent to Matteoni's type 4 and Brunt's grade 2/stage 3. In the 4- and 8-wk groups, Sirius red staining revealed abundant collagen (Figure 2).

Levovist® CEUS examination

Assessment of changes in fluorescent intensity up to 20 min (-25.5 ± 6.4 dB, -37.5 ± 7.5 dB, -55.2 ± 3.9 dB, -59.3 ± 5.6 dB in the control, 1-, 4-, and 8-wk CDAA-fed rats) after administration of Levovist®, with the values at 5 min (-30.2 ± 4.4 dB, -31.8 ± 1.8 dB, -38.8 ± 4.1 dB, -39.7 ± 6.2 dB in the control, 1-, 4-, and 8-wk CDAA-fed rats, respectively) considered as the standard, showed that the fluorescent intensity in the control group tended to rise from 10 min onwards, and remained elevated. In the 1-wk group, the contrast effect remained fairly constant from 5 min to 20 min. In the 4- and 8-wk groups, the contrast effect was decreased significantly at 20 min (Figure 3A). When the fluorescent intensity was quantified using TIC, the fluorescent intensity at 20 min tended to be lower in the 1-wk group and significantly lower in the 4- and 8-wk groups compared with the control group (Figure 3B).

Phagocytosis of FITC-latex beads by Kupffer cells in vivo

Fluorescent microscopic examination showed that the latex bead uptake per Kupffer cell *in vivo* was lower in the 1-wk group compared to the controls, with no further reduction after 4 and 8 wk (Figure 4A and B). To avoid the influence of Kupffer cell cross-sections, and to count the Kupffer cells more accurately, we observed the images again at lower magnification. In comparison with the control group, the uptake was reduced to approximately 50% in the 1-wk group, and to 30% in the 2-wk group, with no further decrease after 3 wk (Figure 4C).

Phagocytosis of FITC-latex beads by Kupffer cells in vitro

Similar to the *in vivo* findings, the fluorescent microscopic examination showed that the *in vitro* latex bead uptake

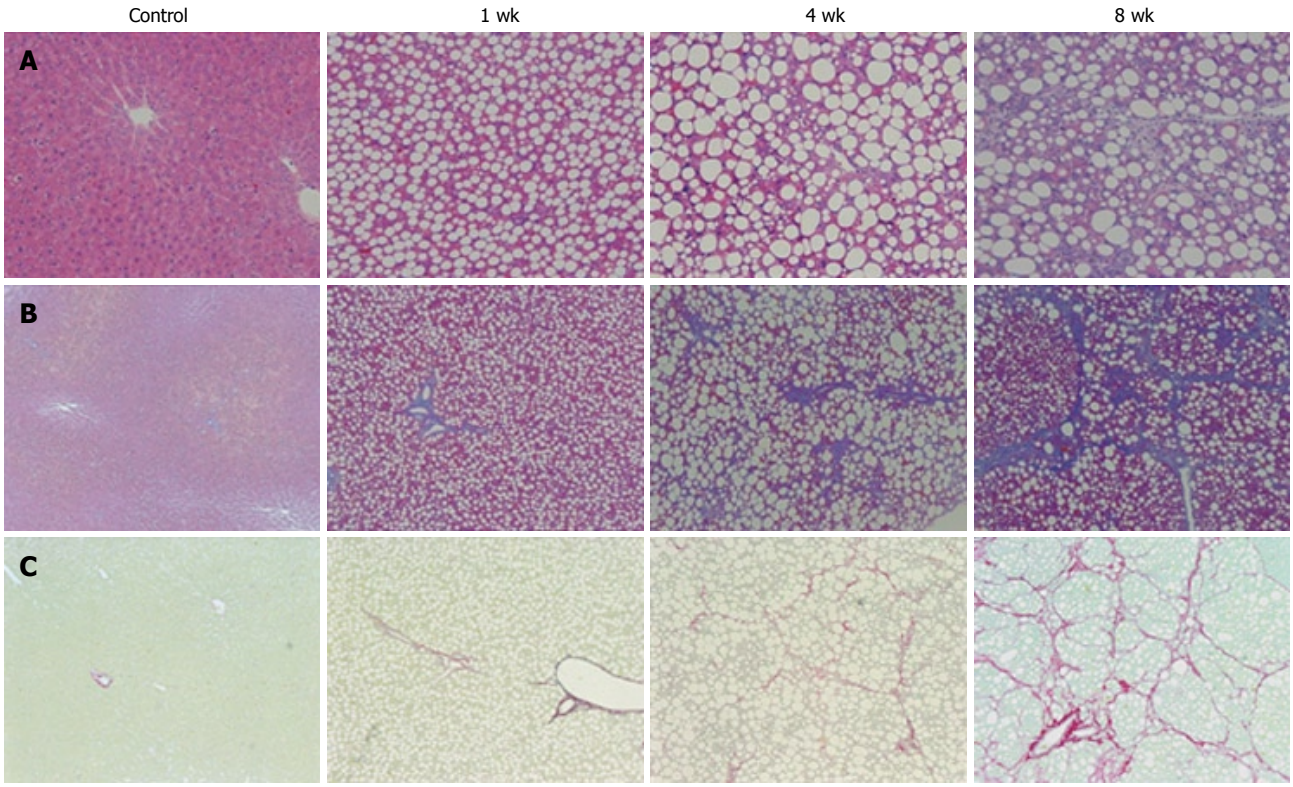


Figure 2 Histological analysis of the liver sections. **A**: Hematoxylin and eosin stain (x 200); **B**: Azan stain (x 100); **C**: Sirius red stain (x 100). Histological examination of the liver tissue of 1-wk CDAA-fed rats showed inflammation and fat deposits, but no fibrosis, corresponding to Matteoni's type 2. The 4-wk CDAA-fed rats had more inflammation, fat deposits, and fibrosis, which was equivalent to Matteoni's type 3 and grade 2/stage 2 of Brunt's NASH classification. The histological findings in the 8-wk CDAA-fed rats were equivalent to Matteoni's type 4 and Brunt's grade 2/stage 3. In the 4 and 8-wk groups, Sirius red staining revealed abundant collagen (n = 5).

per Kupffer cell was lower in the 1-wk group than in the controls, with no further reduction after 4 and 8 wk. To avoid the influence of Kupffer cell cross-sections, and to count the Kupffer cells more accurately, we observed the images again at lower magnification. In comparison with the control group, the uptakes were reduced to approximately 60% in the 1-wk group, and to 30% in the 2-wk group, with no further decrease after 3 wk (Figure 5).

Kupffer cells immunohistochemical staining

Immunohistochemical staining showed no significant difference in the number of stained cells per field between the control group and any of the CDAA groups. Quantitative analysis using NIH Image™ also showed no significant difference between the groups (Figure 6).

Relationship of Kupffer cell phagocytic activity with Kupffer cell count and liver histology

There was no correlation between the changes in the contrast effect, and the Kupffer cell count or the degree of fat deposition.

DISCUSSION

Ultrasonographic examinations have been performed to assist in the diagnosis of abdominal diseases since the 1970s, and continue to be widely used in clinical practice because of the ease of use and low level of invasiveness[13]. Until recently, ultrasonography enhanced by CO_2 microbubbles, delivered through an intra-arterial catheter (developed in the 1980s), was the only CEUS technique available[14,15]. The intravenous ultrasonographic contrast agent Levovist®, available for clinical use in Japan since September 1999, facilitates the hepatic blood flow imaging with an inherently low level of invasiveness[16-19]. Levovist® CEUS provides information on both the blood flow and the parenchyma through the characteristics of the contrast agent[20,21]. Microbubbles injected *via* the intravenous route travel through the blood vessels, producing vascular images, and the gas is finally eliminated through the lungs[22]. However, some kinds of microbubbles accumulate in the organs such as the liver[23] and the spleen[24], which allows delayed phase imaging[25]. Recently, Levovist® CEUS has been reported to be a useful screening modality for NASH[9]. The reduced contrast effect in the liver parenchyma has been attributed to sinusoidal or Kupffer cell dysfunction, although this finding remains to be established[26].

In the present study, we performed Levovist® CEUS examination using a rat NASH model induced by the CDAA diet. We also assessed Kupffer cell dynamics and phagocytic activity, and confirmed that the contrast effect is reduced in the hepatic parenchymal phase. The liver histology progressed from steatosis and inflammation to marked fibrosis during 8 wk of CDAA

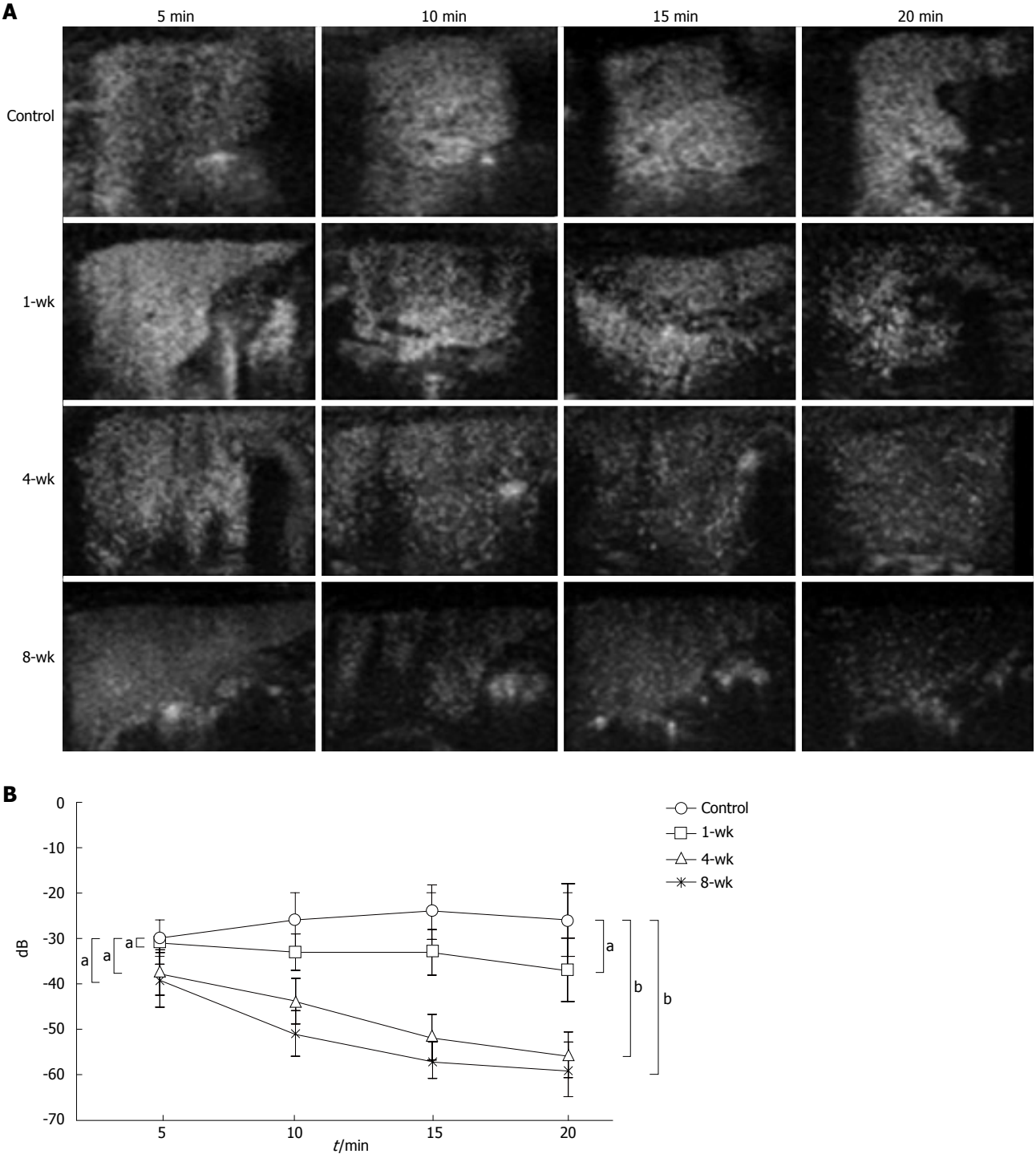


Figure 3 Results of Levovist® CEUS in each group. **A**: Examination of changes in the fluorescent intensity up to 20 min after administration of Levovist®, with the values at 5 min considered as the standard, showed that the fluorescent intensity in the control group tended to rise from 10 min onwards, and remained elevated. In the 1-wk group, the contrast effect remained fairly constant from 5 min to 20 min. After 4 or more wk of the CDAA diet, CEUS examination revealed a decrease in the signal intensity, 20 min after intravenous Levovist®; **B**: Changes in the fluorescent intensity in Levovist® contrast enhanced ultrasonograms in each group. In the control group, the fluorescent intensity increased significantly at 20 min compared with the findings at 5 min, whereas a significant chronological decrease was seen in the NASH groups ($n = 5$). $^{a}P > 0.05$, $^{b}P < 0.01$.

feeding. The changes in fluorescent intensity up to 20 min after the administration of Levovist® revealed distinct differences depending on the duration of the CDAA diet. In the early phase, the effect of Levovist® in the hepatic sinusoids and the blood stream was strong, whereas at 20 min the effect of the contrast taken up by the Kupffer cells was apparent. When the values obtained at 5 min were taken as the standard, the contrast effect at 20 min was low even in the 1-wk CDAA-fed group. The contrast effect at 20 min was significantly lower in the 4-wk and 8-wk CDAA-fed groups compared with the findings in the control group. The decrease in the phagocytic activity of Kupffer cells in the early phase of rat steatohepatitis was further

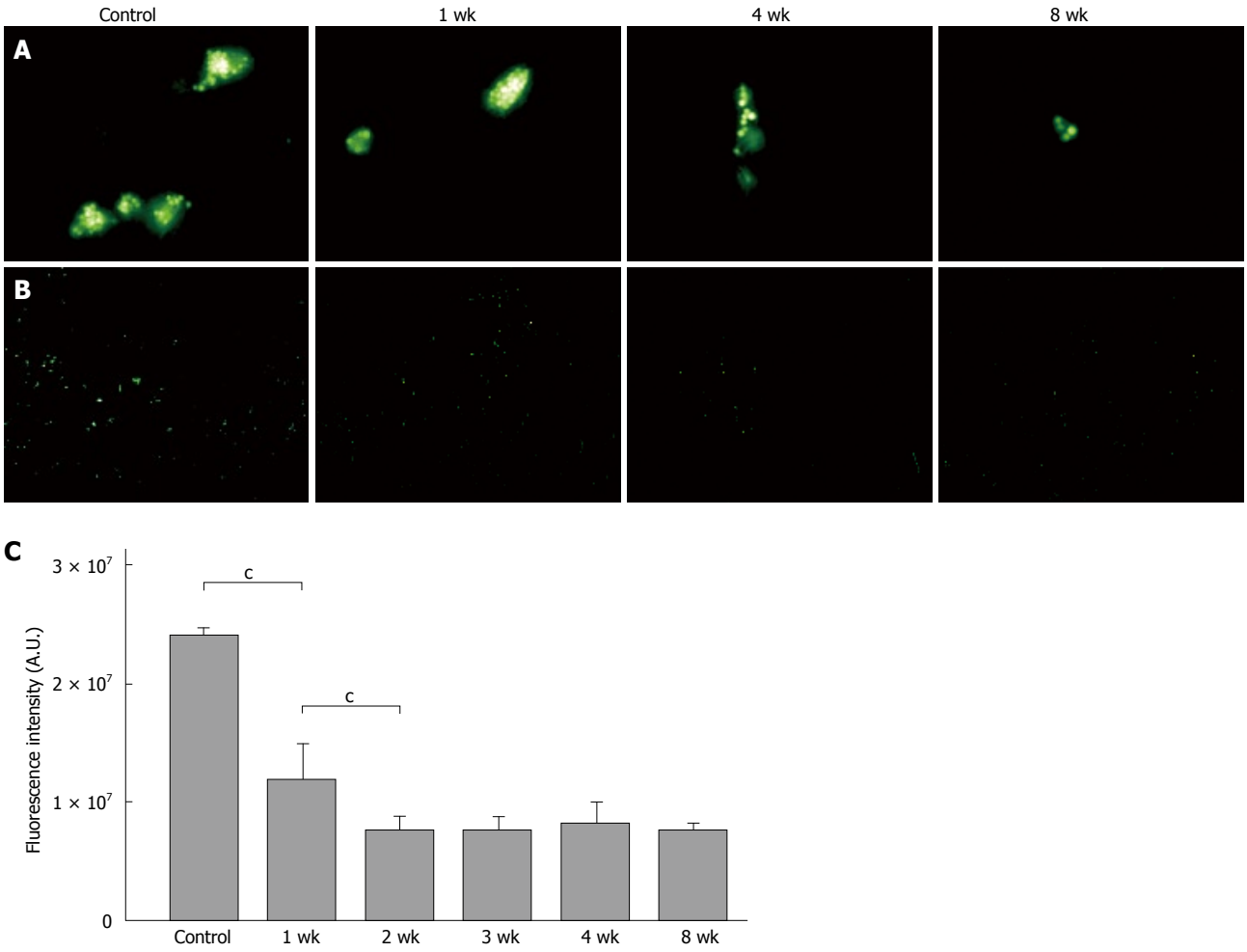


Figure 4 **A**: Fluorescence micrographs (x 1000). In the control group, there was no uptake of multiple latex beads (phagocytic activity) by the triangular shaped Kupffer cells, whereas the latex bead uptake was reduced in the NASH groups; **B**: Phagocytosis of FITC-latex beads by Kupffer cells *in vivo* (x 100). Decreased latex bead uptake was seen in the NASH groups compared with the control group; **C**: When compared with the control group, fluorescence was reduced to approximately 50% in the 1-wk group, and to 30% in the 2-wk group, but there was no further decrease after 3 wk (n = 5). $^{c}P < 0.001$.

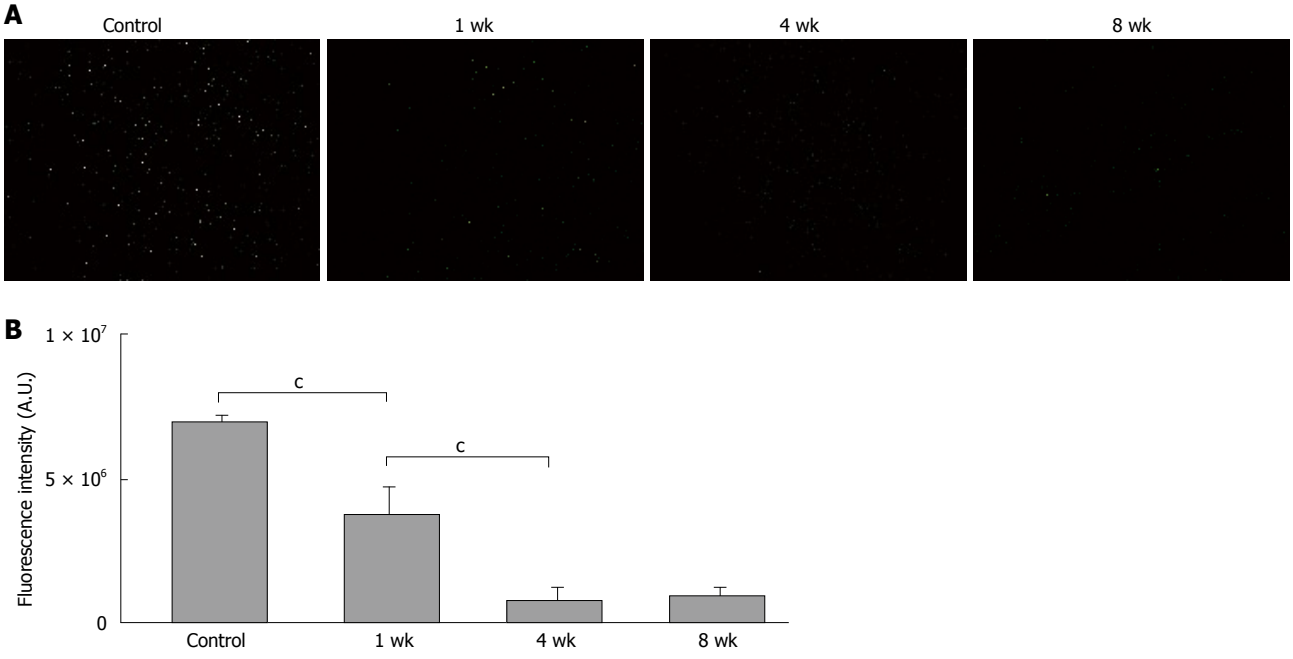


Figure 5 **A**: Phagocytosis of FITC-latex beads by the Kupffer cells *in vitro* (x 100). There was reduced latex bead uptake in the NASH groups compared with the control group; **B**: When compared with the control group, fluorescence was reduced to approximately 60% in the 1-wk group, and to 30% in the 2-wk group, but no further decrease was observed after 4 wk (n = 5). $^{c}P < 0.001$.

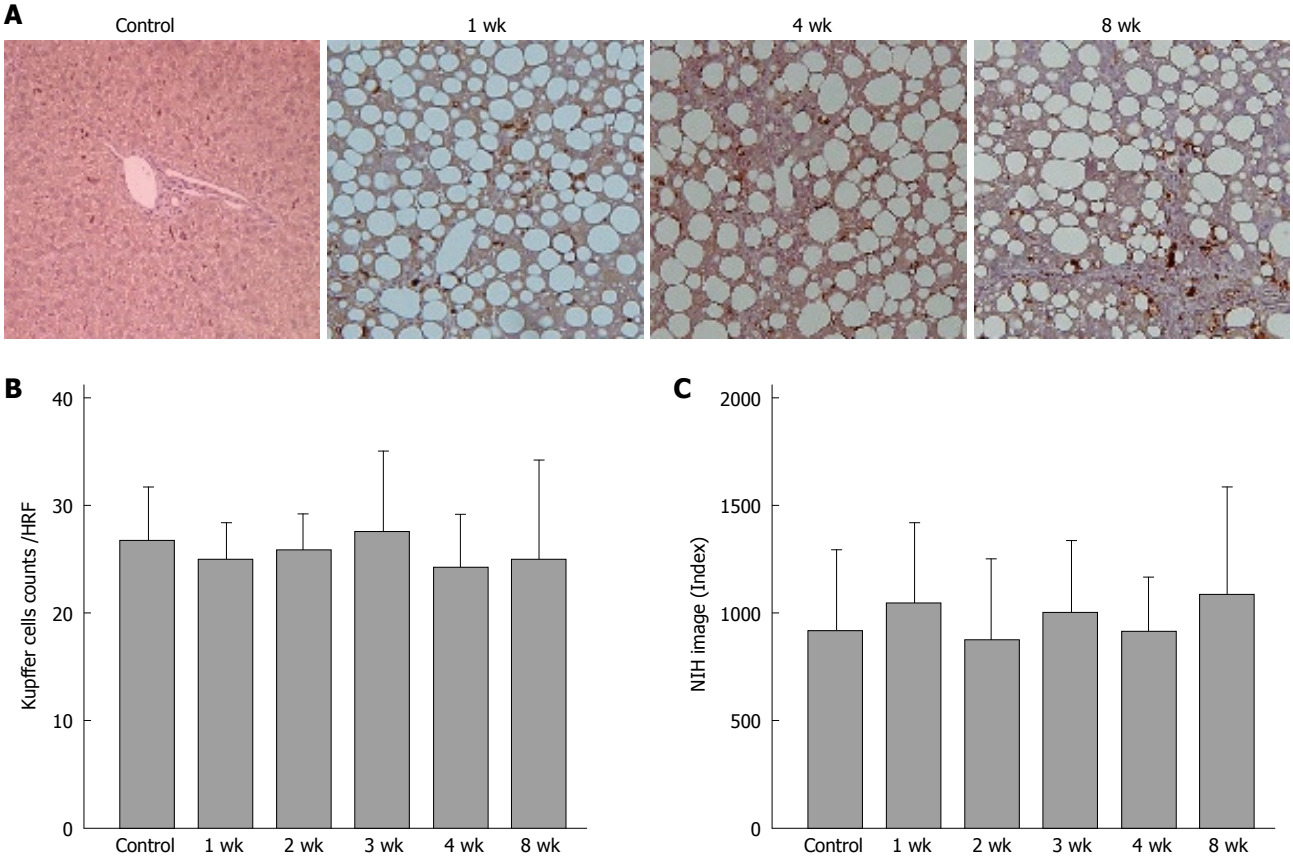


Figure 6 **A**: Kupffer cell immunohistochemical staining (x 200). Brown-stained cells are positive; **B**: There were no significant differences between the different groups in the number of stained cell per field; **C**: No significant differences were found between the groups in the quantitative analyses (n = 5).

confirmed by the latex bead uptake test. In the present *in vitro* study, we demonstrated that isolated Kupffer cells from CDAA-fed rats had reduced phagocytic activity. The marked decrease in the phagocytic activity in the presence of normal Kupffer cell counts suggests the presence of Kupffer cell functional abnormalities in our NASH model. We propose that persistent inflammation leads to reduced phagocytic activity of the Kupffer cells. Although the cause of the marked decrease in the phagocytic activity is unclear[27], it may be attributable to intestinal bacterial endotoxins, which are believed to play a key role in the choline deficiency model of liver injury[28]. Further studies are necessary to exclude the possibility of hypoperfusion of the sinusoids, and to confirm Kupffer cell abnormality in steatohepatitis.

In the present study, we used the CDAA model, because the changes induced in the liver are reproducible and similar to those observed in NASH. The development of NASH in patients receiving total parenteral nutrition has been attributed to choline deficiency[29]. Furthermore, alcoholic liver disease may be associated with hepatic choline deficiency and hepatic steatosis, abnormalities that are also observed in rats fed a CDAA diet[27]. Although, our steatohepatitis model lacks obesity and insulin resistance, two major characteristics of human NASH, a possible association between Kupffer cells, with inflammation and fibrosis may resemble the findings in humans. Further studies on cytokine production by the Kupffer cells in the present NASH model may reveal Kupffer cell dysfunction and add new insight to the hepatic consequences of human NASH.

In conclusion, ultrasound examination by Levovist® confirmed the presence of a reduced contrast effect in the liver parenchymal phase in the rat NASH model. We believe that CEUS examination using Levovist® is a useful screening modality which can detect NASH in patients with fatty liver.

COMMENTS

Background

The diagnosis of nonalcoholic steatohepatitis (NASH) is important in clinical practice since this condition can progress to hepatic cirrhosis and hepatocellular carcinoma (HCC). At present, histopathological examination of liver biopsy tissue is the only way to definitively diagnose NASH. When NASH patients undergo contrast enhanced ultrasonography (CEUS) using Levovist®, reduced contrast effect is seen in the liver parenchymal phase.

Research frontiers

Levovist® CEUS provides useful information on both the blood flow and the liver parenchyma, based on the characteristics of the contrast agent. Recently, Levovist® CEUS is being increasingly used as a screening modality for NASH. The reduced contrast effect in the liver parenchyma has been attributed to sinusoidal or Kupffer cell dysfunction, but this finding remains to be established. Therefore, the present workers performed Levovist® CEUS in a rat NASH model, induced by a choline-deficient L-amino acid-defined (CDAA) diet, and examined the Kupffer cell dynamics and phagocytic activity. The results obtained confirmed that the contrast effect is reduced in the liver parenchymal phase in the rat NASH model.

Innovations and breakthroughs

The present study demonstrated the contrast effect in the liver parenchymal

phase of CEUS using Levovist®. In addition, Kupffer cell dynamics and phagocytic activity were assessed in the rat NASH model. The results confirmed the effectiveness of CEUS in diagnosing NASH.

Applications

The authors believe that the CEUS with Levovist® is a useful screening modality, which can detect NASH in patients with fatty liver.

Terminology

Levovist (Schering AG, Berlin, Germany) is a galactose-palmitic acid ultrasound contrast agent, currently in use in European and Asian countries.

Peer review

The manuscript includes well designed figures, and the results clearly show a reduction in KC function, which was not attributed to changes in KC number, during the course of CDAA feeding. The hypothesis is simple and clear.

REFERENCES

1 **Zen Y**, Katayanagi K, Tsuneyama K, Harada K, Araki I, Nakanuma Y. Hepatocellular carcinoma arising in non-alcoholic steatohepatitis. *Pathol Int* 2001; **51**: 127-131

2 **Shimada M**, Hashimoto E, Taniai M, Hasegawa K, Okuda H, Hayashi N, Takasaki K, Ludwig J. Hepatocellular carcinoma in patients with non-alcoholic steatohepatitis. *J Hepatol* 2002; **37**: 154-160

3 **Bugianesi E**, Leone N, Vanni E, Marchesini G, Brunello F, Carucci P, Musso A, De Paolis P, Capussotti L, Salizzoni M, Rizzetto M. Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. *Gastroenterology* 2002; **123**: 134-140

4 **Day CP**, James OF. Steatohepatitis: a tale of two "hits"? *Gastroenterology* 1998; **114**: 842-845

5 **Matteoni CA**, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology* 1999; **116**: 1413-1419

6 **Brunt EM**, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol* 1999; **94**: 2467-2474

7 **Brunt EM**. Nonalcoholic steatohepatitis: definition and pathology. *Semin Liver Dis* 2001; **21**: 3-16

8 **Brunt EM**. Nonalcoholic steatohepatitis. *Semin Liver Dis* 2004; **24**: 3-20

9 **Moriyasu F**, Iijima H, Tsuchiya K, Miyata Y, Furusaka A, Miyahara T. Diagnosis of NASH using delayed parenchymal imaging of contrast ultrasound. *Hepatol Res* 2005; **33**: 97-99

10 **Nakae D**, Mizumoto Y, Yoshiji H, Andoh N, Horiguchi K, Shiraiwa K, Kobayashi E, Endoh T, Shimoji N, Tamura K. Different roles of 8-hydroxyguanine formation and 2-thiobarbituric acid-reacting substance generation in the early phase of liver carcinogenesis induced by a choline-deficient, L-amino acid-defined diet in rats. *Jpn J Cancer Res* 1994; **85**: 499-505

11 **Seglen PO**. Preparation of rat liver cells. 3. Enzymatic requirements for tissue dispersion. *Exp Cell Res* 1973; **82**: 391-398

12 **Kubo S**, Rodriguez T Jr, Roh MS, Oyedeji C, Romsdahl MM, Nishioka K. Stimulation of phagocytic activity of murine Kupffer cells by tuftsin. *Hepatology* 1994; **19**: 1044-1049

13 **Tsujimoto T**, Kuriyama S, Yoshiji H, Fujimoto M, Kojima H, Yoshikawa M, Fukui H. Ultrasonographic findings of amebic colitis. *J Gastroenterol* 2003; **38**: 82-86

14 **Matsuda Y**, Yabuuchi I. Hepatic tumors: US contrast enhancement with CO2 microbubbles. *Radiology* 1986; **161**: 701-705

15 **Kudo M**, Tomita S, Tochio H, Kashida H, Hirasa M, Todo A. Hepatic focal nodular hyperplasia: specific findings at dynamic contrast-enhanced US with carbon dioxide microbubbles. *Radiology* 1991; **179**: 377-382

16 **Harvey CJ**, Blomley MJ, Eckersley RJ, Heckemann RA, Butler-Barnes J, Cosgrove DO. Pulse-inversion mode imaging of liver specific microbubbles: improved detection of subcentimetre metastases. *Lancet* 2000; **355**: 807-808

17 **Vallone P**, Gallipoli A, Izzo F, Fiore F, Delrio P. Local ablation procedures in primary liver tumors: Levovist US versus spiral CTto evaluate therapeutic results. *Anticancer Res* 2003; **23**: 5075-5079

18 **Kim CK**, Choi D, Lim HK, Kim SH, Lee WJ, Kim MJ, Lee JY, Jeon YH, Lee J, Lee SJ, Lim JH. Therapeutic response assessment of percutaneous radiofrequency ablation for hepatocellular carcinoma: utility of contrast-enhanced agent detection imaging. *Eur J Radiol* 2005; **56**: 66-73

19 **Maruyama H**, Matsutani S, Kondo F, Yoshizumi H, Kobayashi S, Okugawa H, Ebara M, Saisho H. Ring-shaped appearance in liver-specific image with Levovist: a characteristic enhancement pattern for hypervascular benign nodule in the liver of heavy drinkers. *Liver Int* 2006; **26**: 688-694

20 **Iijima H**, Moriyasu F, Miyahara T, Yanagisawa K. Ultrasound contrast agent, Levovist microbubbles are phagocytosed by Kupffer cells-In vitro and in vivo studies. *Hepatol Res* 2006; **35**: 235-237

21 **Yanagisawa K**, Moriyasu F, Miyahara T, Yuki M, Iijima H. Phagocytosis of ultrasound contrast agent microbubbles by Kupffer cells. *Ultrasound Med Biol* 2007; **33**: 318-325

22 **Toft KG**, Hustvedt SO, Hals PA, Oulie I, Uran S, Landmark K, Normann PT, Skotland T. Disposition of perfluorobutane in rats after intravenous injection of Sonazoid. *Ultrasound Med Biol* 2006; **32**: 107-114

23 **Quaia E**, Blomley MJ, Patel S, Harvey CJ, Padhani A, Price P, Cosgrove DO. Initial observations on the effect of irradiation on the liver-specific uptake of Levovist. *Eur J Radiol* 2002; **41**: 192-199

24 **Lim AK**, Patel N, Eckersley RJ, Goldin RD, Thomas HC, Cosgrove DO, Taylor-Robinson SD, Blomley MJ. Hepatic vein transit time of SonoVue: a comparative study with Levovist. *Radiology* 2006; **240**: 130-135

25 **Blomley MJ**, Albrecht T, Cosgrove DO, Eckersley RJ, Butler-Barnes J, Jayaram V, Patel N, Heckemann RA, Bauer A, Schlief R. Stimulated acoustic emission to image a late liver and spleen-specific phase of Levovist in normal volunteers and patients with and without liver disease. *Ultrasound Med Biol* 1999; **25**: 1341-1352

26 **Iijima H**, Moriyasu F, Tsuchiya K, Suzuki S, Yoshida M, Shimizu M, Sasaki S, Nishiguchi S, Maeyama S. Decrease in accumulation of ultrasound contrast microbubbles in non-alcoholic steatohepatitis. *Hepatol Res* 2007; **37**: 722-730

27 **Tsujimoto T**, Kuriyama S, Yamazaki M, Nakatani Y, Okuda H, Yoshiji H, Fukui H. Augmented hepatocellular carcinoma progression and depressed Kupffer cell activity in rat cirrhotic livers. *Int J Oncol* 2001; **18**: 41-47

28 **Eastin CE**, McClain CJ, Lee EY, Bagby GJ, Chawla RK. Choline deficiency augments and antibody to tumor necrosis factor-alpha attenuates endotoxin-induced hepatic injury. *Alcohol Clin Exp Res* 1997; **21**: 1037-1041

29 **Fong DG**, Nehra V, Lindor KD, Buchman AL. Metabolic and nutritional considerations in nonalcoholic fatty liver. *Hepatology* 2000; **32**: 3-10

S- Editor Li DL **L- Editor** Anand BS **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6044

World J Gastroenterol 2008 October 21; 14(39): 6044-6051
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Torque teno virus: Its prevalence and isotypes in North India

Mohammad Irshad, Shiwani Singh, Khushboo Irshad, Sanjay Kumar Agarwal, Yogendra Kumar Joshi

Mohammad Irshad, Shiwani Singh, Clinical Biochemistry Division, Department of Laboratory Medicine, All India Institute of Medical Sciences, New Delhi-110029, India
Khushboo Irshad, Department of Biochemistry, All India Institute of Medical Sciences, New Delhi-110029, India
Sanjay Kumar Agarwal, Department of Nephrology, All India Institute of Medical Sciences, New Delhi-110029, India
Yogendra Kumar Joshi, Department of Gastroenterology and Human Nutrition, All India Institute of Medical Sciences, New Delhi-110029, India
Supported by Indian Council of Medical Research (ICMR), New Delhi-110049 for financial grant
Author contributions: Irshad M planned the study; Singh S and Irshad K performed the experiments; Agarwal SK and Joshi YK examined the patients and provided patients' material.
Correspondence to: Dr. Mohammad Irshad, Professor, Clinical Biochemistry Division, Department of Laboratory Medicine, PO Box-4938, A.I.I.M.S., New Delhi-110029, India. drirshad54@yahoo.com
Telephone: +11-91-26594981 Fax: +11-91-26588641
Received: May 30, 2008 Revised: August 11, 2008
Accepted: August 18, 2008
Published online: October 21, 2008

Abstract

AIM: To investigate the prevalence and genotype distribution of Torque teno virus (TTV) in patients with different liver diseases and chronic renal failure treated at a referral hospital in North India.

METHODS: Whereas prevalence of TTV was based on amplification of conserved region of ORF2 of TTV genome, the genotyping of TTV was carried out using restriction fragment length polymorphism (RFLP) procedure on the N22 region of ORF1.

RESULTS: TTV-DNA was detected in 137 of 513 (26.7%) patients with liver diseases and 38 of 65 (58.5%) patients with chronic renal failure. TTV was also detected in 27% of healthy controls. The sequence analysis of the PCR product from 10 randomly selected cases failed to show a significant sequence divergence when compared with that of the TRM1 isolate of TTV genotype 1. The results of genotyping in 55 randomly selected patients showed the presence of genotype 1 (G1) in 53 (96.4%) and genotype 2 (G2) in 2 cases (3.6%), respectively. Other genotypes were not identified in this patient subgroup, suggesting that G1 is predominant in this area. The results of genotyping by RFLP were also supported by phylogenetic tree analysis, where G1 was found to be the major genotype.

CONCLUSION: These results indicate that TTV is moderately present in Indian patients, with G1 to be the major genotype in North India. The pathogenicity and etiological role of TTV in different diseases is still a question mark and warrant further studies.

Key words: Torque teno virus; Genotype; Restriction fragment length polymorphism; Torque teno; Hepatocellular carcinoma

Peer reviewer: Dr. Adam G Testro, Department of Gastroenterology and Liver Transplantation, Austin Health Institution, Heidelberg 3032, Australia

Irshad M, Singh S, Irshad K, Agarwal SK, Joshi YK. Torque teno virus: Its prevalence and isotypes in North India. *World J Gastroenterol* 2008; 14(39): 6044-6051 Available from: URL: http://www.wjgnet.com/1007-9327/14/6044.asp DOI: http://dx.doi.org/10.3748/wjg.14.6044

INTRODUCTION

Torque teno virus (TTV), formerly known as transfusion-transmitted virus, is a small, non-enveloped, icosahedral, single-stranded, circular-DNA virus[1] approximately 30 nm in diameter. TTV contains a 3.6-3.9 kb DNA genome of minus polarity[2-4] and belongs to a novel virus family, *Circoviridae*[3]. At least 40 TTV genotypes from 5 major phylogenetic groups have been identified[5]. They display over 30% nucleotide diversity[6]. TTVs are ubiquitous in nature and have been demonstrated in high proportion of serum samples from healthy individuals where they persist overtime[3,7-9]. A tissue culture system that supports efficient replication of TTV is not available[10], and this has delayed the study of both TTV genome replication and TTV gene expression. Antibodies reacting with two proteins encoded by the 2.8-kb mRNA have been detected in TTV-infected individuals[11,12]. The first is the large protein encoded by TTV ORF1 (the ORF1 protein), which is predicted to be 736 amino acids in length and initiates from a methionine at nucleotide (nt) 581 (O1AUG). The second protein is encoded by the 2.8-kb mRNA in ORF2, initiating at a methionine in the position of nt 354 (O2AUG) and extending for 117 amino acids[13].

TTV belongs to the group of *Anelloviruses* that are widely diverse. Indeed, based on their heterogeneity,

Anelloviruses are currently classified into two species, each subdivided into numerous genotypes. Thus, TTV, the first anellovirus species identified[1], is currently subdivided into approximately 40 genotypes, which cluster in five clearly distinct phylogenetic groups designated from 1 to 5[1,6,10,14]. TTV is transmitted parenterally through transfusion with blood or blood products, but the natural route of its transmission is still unknown[15,16].

TTV is found in the plasma of > 80% of the human population worldwide. Co-infection of single individuals with multiple TTV isolates is frequent[17]. The epidemiology and pathogenic potential of TTV is poorly understood. In several studies, however, the viral genome has been detected at comparable prevalence rates in the blood of healthy persons and patients and this led to the hypothesis that TTV might be essentially non-pathogenic in nature[18].

TTV can be transmitted by parenteral route, although its role in causing post-transfusion hepatitis has not been established. The majority of individuals who become TTV-DNA-positive after blood transfusion usually have normal ALT and do not develop chronic hepatitis, although TTV viremia frequently persists for several years. Patients who develop chronic hepatitis are invariably coinfected with HBV or HCV and chronic hepatitis is closely correlated with HBV or HCV infection. This raises the possibility that TTV is merely an innocent bystander rather than a primary hepatitis virus[19].

Although TTV appears to be widespread in the general population of several geographical regions, its prevalence in many areas is still unknown. The reports on status of TTV available from India are very preliminary and therefore there is a need of extensive studies to understand the endemicity, epidemiology and etiological potential of TTV infection in various diseases. Also, very little is known about the genotyping of TTV strains circulating in this country. Thus, the present study was undertaken to elucidate the prevalence and detect genotypes distribution of TTV in patients with liver and renal diseases in North India.

MATERIALS AND METHODS

Patients and blood samples

Five hundred and seventy eight adult patients of both sexes were included. There were 126 patients with acute viral hepatitis (AVH, age range: 21-48 years), 111 patients with chronic viral hepatitis (CVH, age range: 19-48 years), 132 patients with liver cirrhosis (CIR, age range: 34-57 years), 51 patients with fulminant hepatic failure (FHF, age range: 28-46 years), 93 patients with hepatocellular carcinoma (HCC, age range: 24-71 years) and 65 patients with chronic renal failure (CRF, age range: 20-74 years)[19]. All these patients attended either the Outpatient Department or were admitted to the Liver and Renal Units of All India Institute of Medical Sciences, New Delhi, from June 2001 to March 2008. They were evaluated clinically and biochemically and their sera were tested for various markers and parameters. The diagnosis of different types of diseases was based on accepted clinical, biochemical and histological criteria as outlined elsewhere[20].

AVH was diagnosed when patients exhibited overt jaundice and/or increased alanine aminotransferase levels (at least 3 times above the normal value) documented at least twice at a 1-wk interval without any history of pre-existing liver disease. None of the patients had a past history of alcohol intake or were using any drug or had clinical or serological evidence of autoimmune diseases or biliary infection. The patients with CVH and liver cirrhosis were diagnosed based on histopathological criteria established by the International Study Group on Chronic Hepatitis[21]: All of them had persistent elevation of transaminases (at least twice the upper limit of the normal range) for more than six months and histologic evidence of chronic hepatitis on liver biopsy at the beginning of follow-up. FHF was diagnosed if the patients developed hepatic encephalopathy within 4 wk from the onset of acute hepatitis, as outlined elsewhere[20]. The diagnosis of HCC was based on histological criteria. CRF was diagnosed using criteria as detailed elsewhere[22]. One hundred age- and sex-matched healthy subjects were used as controls.

From each of the above patients, 6-10 mL of venous blood was drawn and aliquoted in plain tubes without anticoagulant. Serum was separated after centrifugation and then stored at -70℃ until further analysis. Repeated freezing and thawing of serum was avoided as far as possible. These serum samples were used to analyze liver function tests and routine hematogram.

Detection of TTV-DNA by PCR

Viral DNA was extracted from 200 μL of sera stored at -20℃ using QiAmp Mini Elute viral spin kit (Qiagen, Germany) and following the manufacturer's instructions. The DNA was eluted in 50 μL of elution buffer supplied with the kit. TTV-DNA (conserved region of ORF2) was detected by nested PCR using primers NS1 (sense) 5'-GGGTGCCGAAGGTGAGTTTAC-3' (175-195), NS2 (anti-sense) 5'-GCGGGGCACGAA-GCACAGAAG-3' (474-494), NS3 (sense) 5'-AGTTTA-CACACCGAAGTCAAG-3' (189-209) and NS4 (anti-sense) 5'-AGCACAGAAGCAAGATGATTA-3' (463-483) as described by Biagini *et al*[23], 1999 (Accession No. AB008394). Briefly, 10 μL of DNA was used for the amplification in a 50 μL reaction mixture containing 10 × PCR buffer, 25 pmol/μL of each primer, 10 mmol/L of each dNTPs, and 1.5 U Taq polymerase (Qiagen). Each of the 35 cycles of the 1st round and 25 cycles for the 2nd round of amplification consisted in an initial denaturation step at 95℃ for 5 min and a cycling denaturation at 94℃ for 30 s, annealing at 60℃ for 45 s, extension at 74℃ for 45 s, with a final extension for 3 min at 74℃.

Genotyping of TTV was done by amplifying the N22 region using specific primers as described by Okamoto *et al*[4], 1999. Briefly, a first round of amplification was performed with sense primer NG059 5'-ACAGAC-

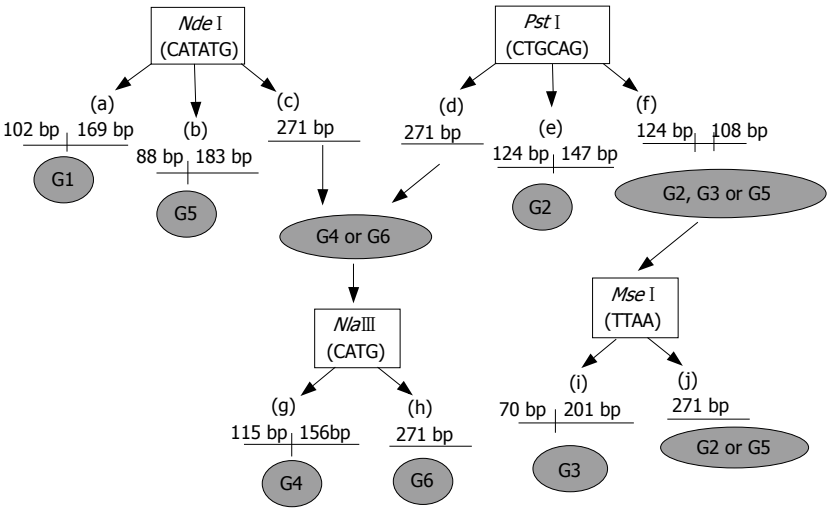


Figure 1 Strategy for RFLP analysis (Tanaka *et al*[24], 1998).

AGAGGAGAAGGCAACATG-3' and anti-sense primer NG063 5'-CTGGCATTTTACCATTTCCAAAGTT-3' for 10 min at 95℃ (initial denaturation) followed by 35 cycles of denaturation at 95℃ for 30 s, annealing at 55℃ for 1 min, and extension at 74℃ for 1 min with a final extension at 74℃ for 5 min. The second round of PCR was performed using sense primer NG061 5'-GGCAACATGYTRTGGATAGACTGG-3' and anti-sense primer NG063 5'-CTGGCATTTT-ACCATTTCCAAAGTT-3' following same conditions as used for first round amplification. Three microliters of the PCR product were electrophoresed on 2% agarose gel and stained by ethidium bromide to observe as 295 bp product of ORF2 region and 271 bp product of N22 region.

Sequence analysis

PCR products of ORF2 were recovered from 2% agarose gels after staining with ethidium bromide and visualized under UV and were purified with QIAquick Gel Extraction Kit, Qiagen, Germany. They were sequenced in both directions using an automated DNA sequencer at M/s Lab India. The same set of primers was used for both sequencing and amplification by PCR.

Restriction fragment length polymorphism (RFLP)

Restriction digestion was carried out overnight using 10 μL of the second round PCR product of the N22 region and 10 × enzyme buffer according to the manufacturer's instructions. Reactions were carried out with 20 U each of *Nde* I , *Pst* I (New England Biolabs, MA, USA), *Nla* Ⅲ (MBI Fermantas, Canada) at 37℃. Similarly, 20 U of *Mse* I (MBI Fermantas, Canada) were used for digestion at 65℃. The digested PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide. The RFLP pattern was then evaluated under UV light[24]. The procedure using restriction digestion enzymes for genotyping by RFLP is shown schematically in Figure 1. Briefly, restriction digestion pattern with *Nde* I producing two fragments of 102 bp and 169 bp shows the presence of genotype 1 (G1). Production of fragments of 88 bp and 183 bp with *Nde* I shows the presence of genotype 5 (G5). Similarly, a digestion pattern with *Pst* I producing two fragments of 124 bp and 147 bp size shows the presence of genotype 2 (G2). Restriction digestion with *Nla* Ⅲ produces two fragments of 115 bp and 156 bp if genotype 4 (G4) is present. Digestion pattern of *Mse* I , producing two fragments of 70 bp and 201 bp length, shows the presence of genotype 3 (G3). Genotype 6 (G6) does not contain site of any of the above restriction enzymes.

Phylogenetic analysis

DNA sequences derived from TTV ORF2 positive samples were compared to an online database for the best possible match using the BLAST (Basic Local Alignment Search Tool) program of National Center for Biotechnology Information (www.ncbi.nlm.nic.gov) and CLUSTALX program[25]. Phylogenetic tree was constructed using the neighbor-joining method in CLUSTALX program and PHYLIP version 3.5[26]. The data set was bootstrap re-sampled 1000 times to ascertain support for major branches of the tree.

RESULTS

Presence of TTV in sera samples from healthy persons and patients with different liver and renal diseases was detected using PCR amplifying the conserved region of ORF2 (107 to 715 nt) (Accession No. AB008394)[23]. TTV-DNA was detected in 175 of 578 patients, giving an overall prevalence of 30.3% (Table 1). Similarly, it was detected in 27 of 100 healthy persons (prevalence of 27%). Typical TTV amplicons of 295 bp are shown in Figure 2. The break-up of TTV prevalence in different disease groups shown its presence in 29 of the 126 (23.0%) patients with AVH, 26 of 111 (23.4%) with CVH, 46 of 132 (34.8%) with CIR, 15 of 51 (29.4%) with FHF, 21 of 93 (22.6%) with HCC and 38 of 65

Table 1 Prevalence of TTV-DNA in different liver & renal disease groups

Disease group	Number of samples	TTV-DNA positivity *n* (%)
Acute viral hepatitis (AVH)	126	29 (23.0)
Chronic viral hepatitis (CVH)	111	26 (23.4)
Cirrhosis (CIR)	132	46 (34.8)
Fulminant hepatic failure (FHF)	51	15 (29.4)
Hepatocellular carcinoma (HCC)	93	21 (22.6)
Chronic renal failure (CRF)	65	38 (58.5)
Healthy controls	100	27 (27.0)

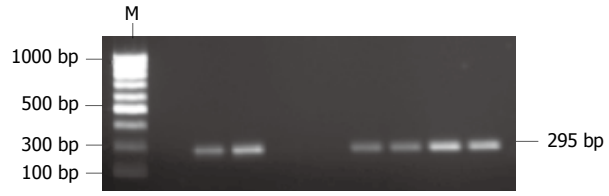


Figure 2 TTV detection by PCR (ORF2: nt 107-712). Amplifiction of ORF-2 region of TTV-DNA using NS1, NS2, NS3 and NS4 primers. (Biagini *et al*[23], 1999. Accession No.: AB008394)

Table 2 Relation between TTV infection & ALT level in different liver disease groups *n* (%)

Diseases group	TTV-DNA+ samples	ALT level (IU/L)			
		50-200	201-400	401-600	> 600
AVH	29	17 (59)	8 (28)	0	4 (14)
CVH	26	18 (69)	6 (23)	0	2 (8)
CIR	46	35 (76)	19 (41)	0	2 (4)
FHF	15	2 (13)	6 (40)	1 (7)	6 (40)
HCC	21	8 (38)	5 (24)	3 (14)	5 (24)

Percent value was computed in comparison to total number positive for TTV-DNA.

Table 3 TTV genotype analysis by RFLP *n* (%)

Restriction enzymes used	Number of cases treated	Digestion noticed	Possible TTV genotypes	Genotypes prevalence
Nde Ⅰ	55	53	G1	53 (96.4)
Pst Ⅰ	55	2	G2	2 (3.6)
Mse Ⅰ	55	0	-	0
Nla Ⅲ	55	0	-	0

Each sample was treated with four restriction enzymes (*Nde* Ⅰ, *Pst* Ⅰ, *Mse* Ⅰ, *Nla* Ⅲ) to determine genotyping of TTV following criteria mentioned in material and methods
The DNA remaining undigested with REs indicated absence of corresponding genotypes
There was no effect of *Mse* Ⅰ and *Nla* Ⅲ on TTV-DNA on digestion with these enzymes

(58.5%) with CRF.

The relation of TTV-DNA with ALT level in sera in these disease groups is shown in Table 2. The majority of cases with liver diseases had low levels of ALT, suggesting a very benign role of TTV in causing liver cell necrosis. The raised level of ALT up to the level of more than 600 IU was detected only in FHF cases, where TTV infection may not be the major cause.

The PCR products from 10 sera were processed for DNA sequencing. The sequence of TTV-DNA from each case was compared to the corresponding region of ORF2 from the TRM1 isolate of genotype 1 (Accession No. AB026345), gathered from the published literature. The full length sequence of amplicons compared to the published sequence of this same region is shown in Figure 3. The results of sequencing indicate the substitution of C by T or Y at nt position 632, A by C at 640 position, substitution of A by G at position 643, T by C at position 696 and at 770 C is being substituted by T or Y. Significant sequence divergence involving more than 2 bp was not observed. Sequencing of additional cases is underway.

Characterization of the TTV genotypes prevalent in the current study population was conducted in 55 cases by RFLP analysis of N22 nucleotide sequence belonging to ORF1. Digestion of the N22 amplicon with *Nde* Ⅰ produced fragments of 169 and 102 bp, corresponding to genotype 1, in 53 of 55 cases. Digestion with *Pst* Ⅰ produced fragments of 124 and 147 bp, corresponding to genotype 2, in 2 of 55 cases. The digestion with other restriction enzymes, *Nla*Ⅲ and *Mse* Ⅰ, could not produce any fragments, thus indicating the the absence of other genotypes. The results of genotype distribution are shown in Table 3 and Figure 4.

Genotypes of TTV in these cases were also confirmed by phylogenetic analysis of DNA sequences obtained from above. The results are shown in Figure 5. This analysis shows the maximum alignment of the sample sequence with TRM1 isolate of genotype 1 indicating a major presence of TRM1 isolate genotypes 1 and supporting the results of RFLP analysis. From these results, it is clear that genotype 1 is predominantly prevalent in all these cases with TTV infection.

DISCUSSION

TTV was assumed to be one of the possible agents

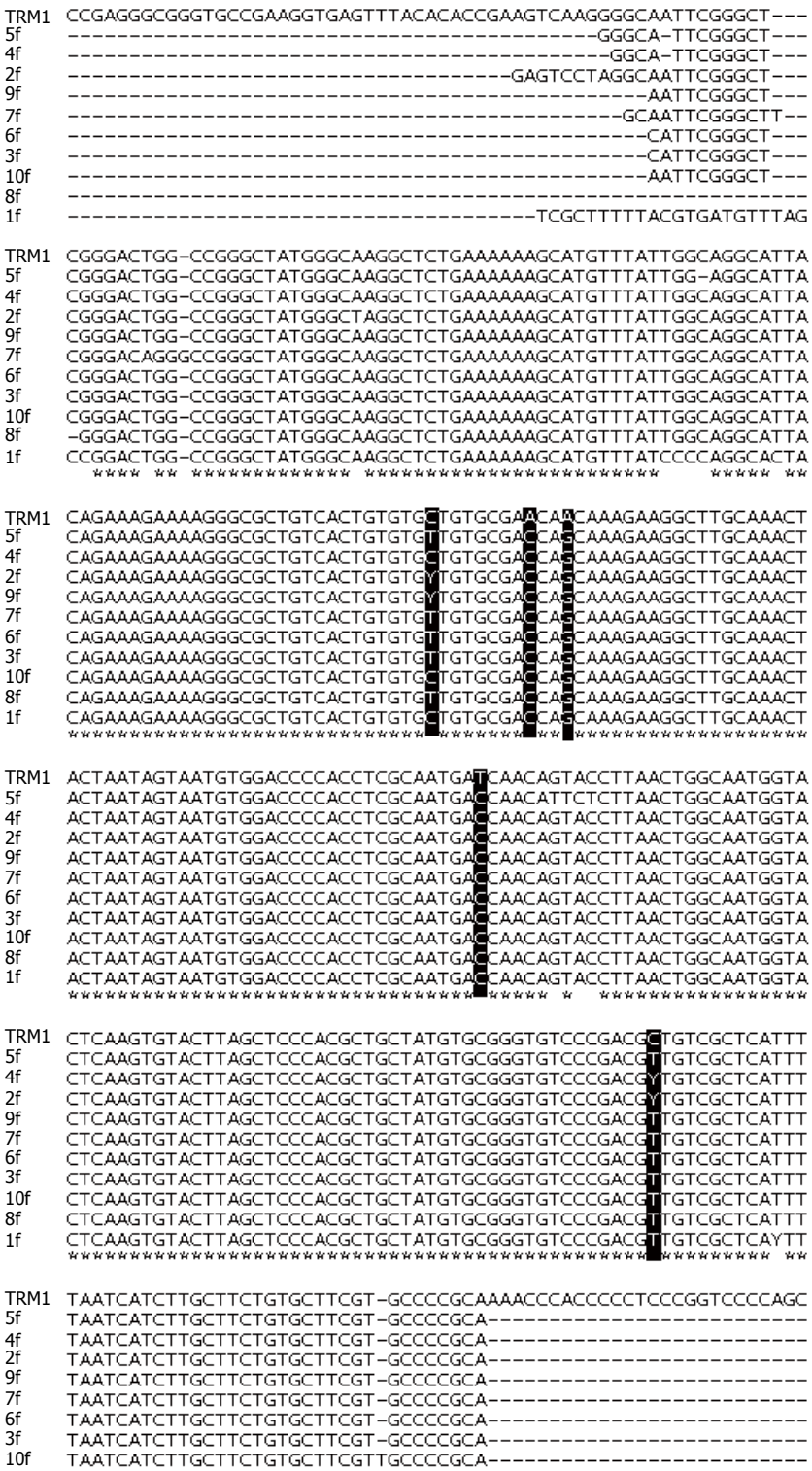


Figure 3 Sequence analysis of amplicons and their comparison with TRM1 isolates of Genotype 1 (ORF2: nt 107-712).

causing non A-G hepatitis. Its characterization and significance in causing hepatitis became more interesting with the findings by Okamoto *et al*[27], showing level of TTV-DNA in liver tissue to be 10-100 times higher than those in serum. A similar report by Nishizawa *et al*[1], who reported the appearance of TTV-DNA in the sera of patients with post-transfusion hepatitis of unknown etiology and displayed a close correlation with ALT levels, triggered an additional interest in this virus for its potential relationship with hepatitis. In India, viral hepatitis is a common disease in all parts of the country, with an established endemicity of all known hepatitis viruses. Previous studies from India have reported the existence of a group of viruses other than A-G hepatitis viruses, causing liver diseases[28]. This remains an enigma to investigate and characterize the responsible agent for non A-G hepatitis in this country. TTV attracted our attention to study this virus for its prevalence and

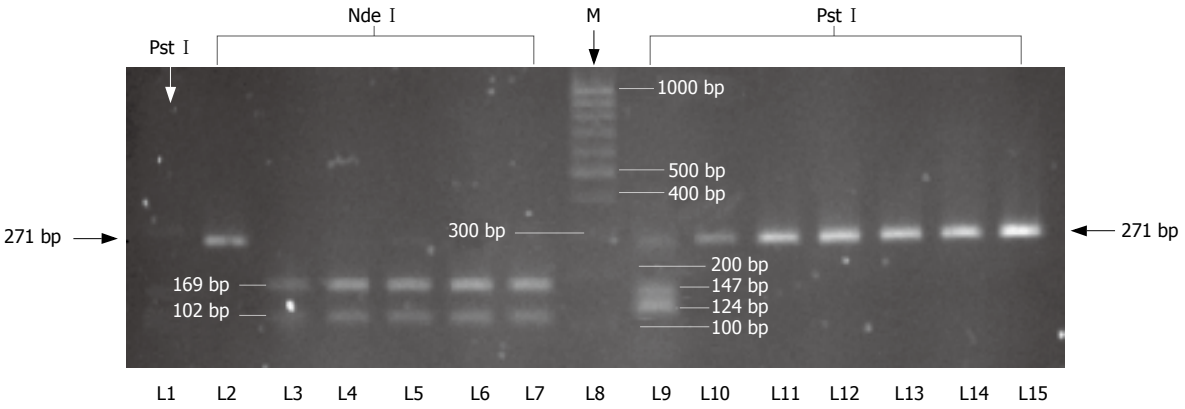


Figure 4 RFLP pattern of N22 region of TTV-DNA. L1-15: Lane 1 to lane 15. L1 is Pst I undigested product (271 bp), L2 is Nde I undigested product (271 bp), L3-7 is Nde I degested product resulting in 102 bp & 169 bp fragments, L8 is marker, L9 is Pst I digested product resulting in fragments of 124 bp & 147 bp size, L10-15 is Pst I undigested product (271 bp).

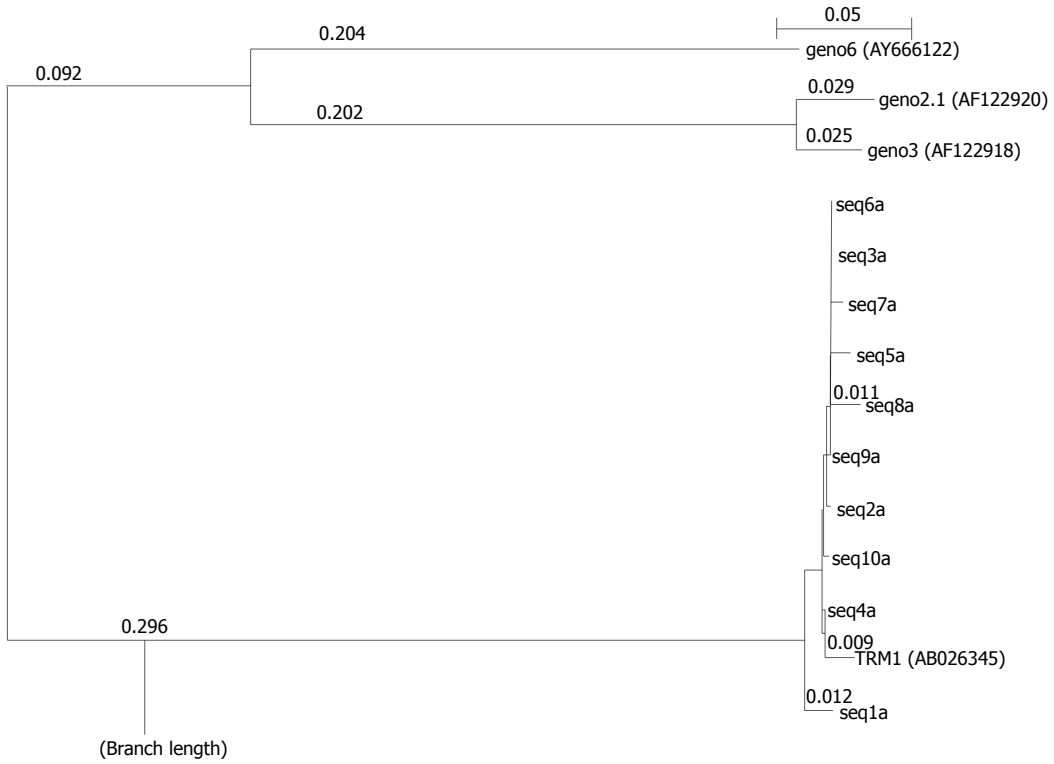


Figure 5 Phylogenetic tree analysis of amplicons with known TTV genotypes.

molecular form in Indian populations to understand its possible role in causing hepatitis as well as other blood transmitted diseases.

The prevalence of TTV infections in India has not been well documented. Only a few reports are available[19,29] that demonstrate the presence of TTV in patients population randomly selected from various studies. In this study, we found the presence of TTV-DNA in 22 to 35% of patients with different liver diseases. In CRF, on the contrary, its prevalence was relatively higher, reaching the level of 58.5%. A low prevalence of TTV in liver diseases, comparable to that reported in healthy persons (27%) supports our earlier preliminary report demonstrating a little role of TTV in liver diseases[19]. TTV infection has been found to be common in humans with prevalence which may exceed 90%. Its prevalence in healthy populations in India is lower than those previously reported for Turkish (51.6%), Japanish (92.0%) and Polish (78%) blood donors[30].

Our findings indicate that TTV prevalence in all tested groups was comparable to that among healthy controls, with the exception of a higher value in chronic renal failure patients, thus indicating that TTV infection in both the healthy population and liver disease patients is not very frequent in the northern part of India. Moreover, presence of TTV in liver diseases does not necessarily indicate its role in the etiology of liver damage. This is supported by the low level of ALT detected in all liver disease groups carrying TTV infection. Several other studies have similarly suggested that TTV does not seem to cause disease, simply acting as a by-stander virus. High prevalence in CRF patients

may be attributed to repeated blood transfusion or procedural transmission in these patients, due to the fact that TTV is frequently transmitted *via* parenteral routes.

The results of the sequence analysis in these cases has shown a point mutation at a fixed position of TTV genome, corresponding to ORF2. A nucleotide sequence heterogeneity is not very frequent, but is premature to conclude from these data about the impact of the genomic variation on its pathogenicity or change in etiological potency. It was interesting to find here that the heterogeneity in nucleotide sequence was at the same position in all amplicons studied. This suggests the likelihood of a single isotype circulating in all of the patients studied.

In this study, the genotyping of TTV was based on a RFLP procedure involving a set of four restriction enzymes targeting the N22 nucleotide region. The results indicate that most isolates assessed in our study belong to genotype 1, with only a minor contribution of genotype 2. Since there are very few reports available from our country, it is not possible to support or contradict other observations and therefore this report should be taken as accepted at this stage. While comparing TTV genotypes circulating in India with those reported from other countries, there are reports available showing evidence for the existence of 4 major genotypes, G1, G2, G3 and G4 with most belonging to G1 and G2. There are three major genotypes, i.e. G1, G2, G3, that are prevalent worldwide. Different countries have reported different genotypes in their population. G2 was reported from Western Anatolia, where appears to be very common[31]. Similarly, G3 & G4 genotypes were reported from Turkey, whereas G3 is detected primarily from Europe[32]. Asian countries have reported predominantly G4 genotype[24]. All these findings indicate diversity in the prevalence of TTV genotypes in different countries of the world. The reason(s) why G1 is predominant in India is difficult to explain and needs further investigations. The predominance of G1 was also supported by phylogenetic tree analysis of the amplicons studied in our patients' population.

In conclusion, our results indicate a low prevalence of TTV infections in North India. This also demonstrates that the prevailing genotype in North India is G1 with minor prevalence of G2 genotype. The presence of strains belonging to G1 reveals the limited genetic diversity in TTV genome circulating in this country.

ACKNOWLEDGMENTS

The authors thank and appreciate the financial aid provided by ICMR, New Delhi, India to conduct this study. Authors are also thankful to Mrs. Suman Rawat for preparing this manuscript and Mr. Surendra Singh to help in test performance.

COMMENTS

Background

Our results indicate that Torque teno virus (TTV) infection is moderately present in North India. Analysis of different genotypes of TTV demonstrate that G1 is the predominant genotype prevailing in North India. The presence of strains belonging mostly to G1 reveals that there is a limited genetic diversity in the TTV genome circulating in this part of country.

Research frontiers

There are only preliminary informations available on the status of TTV in India. There is a need to study and understand the endemicity, epidemiology and etiological potential of TTV infection in various diseases. The present study was planned to determine the prevalence of TTV in North India and which was the prevailing genotype(s).

Innovations breakthrough

The prevalence of TTV infection was observed in various categories of liver and renal diseases. In particular, the rate of TTV infection in liver diseases was comparable to that of a normal healthy populations, although it was significantly higher in chronic renal failure patients. In all these cases genotype 1 (G1) was the predominant genotype with a minor contribution of genotype 2 (G2).

Applications

The results of prevalence and genotyping of TTV infection in Indian patients' will be helpful in understanding the role of TTV in causing various diseases.

Peer review

Authors have studied TTV infection and found that TTV infection has minor role in causation of liver diseases. Its significant prevalence in renal diseases needs further investigations.

REFERENCES

1 **Nishizawa T**, Okamoto H, Konishi K, Yoshizawa H, Miyakawa Y, Mayumi M. A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. *Biochem Biophys Res Commun* 1997; **241**: 92-97

2 **Miyata H**, Tsunoda H, Kazi A, Yamada A, Khan MA, Murakami J, Kamahora T, Shiraki K, Hino S. Identification of a novel GC-rich 113-nucleotide region to complete the circular, single-stranded DNA genome of TT virus, the first human circovirus. *J Virol* 1999; **73**: 3582-3586

3 **Mushahwar IK**, Erker JC, Muerhoff AS, Leary TP, Simons JN, Birkenmeyer LG, Chalmers ML, Pilot-Matias TJ, Dexai SM. Molecular and biophysical characterization of TT virus: evidence for a new virus family infecting humans. *Proc Natl Acad Sci USA* 1999; **96**: 3177-3182

4 **Okamoto H**, Nishizawa T, Ukita M, Takahashi M, Fukuda M, Iizuka H, Miyakawa Y, Mayumi M. The entire nucleotide sequence of a TT virus isolate from the United States (TUS01): comparison with reported isolates and phylogenetic analysis. *Virology* 1999; **259**: 437-448

5 **Saláková M**, Nemecek V, König J, Tachezy R. Age-specific prevalence, transmission and phylogeny of TT virus in the Czech Republic. *BMC Infect Dis* 2004; **4**: 56

6 **Peng YH**, Nishizawa T, Takahashi M, Ishikawa T, Yoshikawa A, Okamoto H. Analysis of the entire genomes of thirteen TT virus variants classifiable into the fourth and fifth genetic groups, isolated from viremic infants. *Arch Virol* 2002; **147**: 21-41

7 **Irving WL**, Ball JK, Berridge S, Curran R, Grabowska AM, Jameson CL, Neal KR, Ryder SD, Thomson BJ. TT virus infection in patients with hepatitis C: frequency, persistence, and sequence heterogeneity. *J Infect Dis* 1999; **180**: 27-34

8 **Maggi F**, Pistello M, Vatteroni M, Presciuttini S, Marchi S, Isola P, Fornai C, Fagnani S, Andreoli E, Antonelli G, Bendinelli M. Dynamics of persistent TT virus infection, as determined in patients treated with alpha interferon for concomitant hepatitis C virus infection. *J Virol* 2001; **75**: 11999-12004

9 **Okamoto H**, Takahashi M, Kato N, Fukuda M, Tawara A, Fukuda S, Tanaka T, Miyakawa Y, Mayumi M. Sequestration of TT virus of restricted genotypes in peripheral blood mononuclear cells. *J Virol* 2000; **74**: 10236-10239

10 **Hino S**. TTV, a new human virus with single stranded circular DNA genome. *Rev Med Virol* 2002; **12**: 151-158

11 **Handa A**, Dickstein B, Young NS, Brown KE. Prevalence of the newly described human circovirus, TTV, in United States blood donors. *Transfusion* 2000; **40**: 245-251
12 **Ott C**, Duret L, Chemin I, Trepo C, Mandrand B, Komurian-Pradel F. Use of a TT virus ORF1 recombinant protein to detect anti-TT virus antibodies in human sera. *J Gen Virol* 2000; **81**: 2949-2958
13 **Qiu J**, Kakkola L, Cheng F, Ye C, Soderlund-Venermo M, Hedman K, Pintel DJ. Human circovirus TT virus genotype 6 expresses six proteins following transfection of a full-length clone. *J Virol* 2005; **79**: 6505-6510
14 **Okamoto H**, Mayumi M. TT virus: virological and genomic characteristics and disease associations. *J Gastroenterol* 2001; **36**: 519-529
15 **Okamoto H**, Ukita M, Nishizawa T, Kishimoto J, Hoshi Y, Mizuo H, Tanaka T, Miyakawa Y, Mayumi M. Circular double-stranded forms of TT virus DNA in the liver. *J Virol* 2000; **74**: 5161-5167
16 **Kondili LA**, Pisani G, Beneduce F, Morace G, Gentili G, Ballati G, Rapicetta M. Prevalence of TT virus in healthy children and thalassemic pediatric and young adult patients. *J Pediatr Gastroenterol Nutr* 2001; **33**: 629-632
17 **Niel C**, Diniz-Mendes L, Devalle S. Rolling-circle amplification of Torque teno virus (TTV) complete genomes from human and swine sera and identification of a novel swine TTV genogroup. *J Gen Virol* 2005; **86**: 1343-1347
18 **Pistello M**, Morrica A, Maggi F, Vatteroni ML, Freer G, Fornai C, Casula F, Marchi S, Ciccorossi P, Rovero P, Bendinelli M. TT virus levels in the plasma of infected individuals with different hepatic and extrahepatic pathology. *J Med Virol* 2001; **63**: 189-195
19 **Irshad M**, Sharma Y, Dhar I, Singh J, Joshi YK. Transfusion-transmitted virus in association with hepatitis A-E viral infections in various forms of liver diseases in India. *World J Gastroenterol* 2006; **12**: 2432-2436
20 **Tandon BN**, Joshi YK, Tandon M. Acute liver failure. Experience with 145 patients. *J Clin Gastroenterol* 1986; **8**: 664-668
21 **Acute and chronic hepatitis revisited**. Review by an international group. *Lancet* 1977; **2**: 914-919
22 **Agarwal SK**, Dash SC, Irshad M, Raju S, Singh R, Pandey RM. Prevalence of chronic renal failure in adults in Delhi, India. *Nephrol Dial Transplant* 2005; **20**: 1638-1642
23 **Biagini P**, Gallian P, Attoui H, Cantaloube JF, de Micco P, de Lamballerie X. Determination and phylogenetic analysis of partial sequences from TT virus isolates. *J Gen Virol* 1999; **80** (Pt 2): 419-424
24 **Tanaka Y**, Mizokami M, Orito E, Ohno T, Nakano T, Kato T, Kato H, Mukaide M, Park YM, Kim BS, Ueda R. New genotypes of TT virus (TTV) and a genotyping assay based on restriction fragment length polymorphism. *FEBS Lett* 1998; **437**: 201-206
25 **Thompson JD**, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 1997; **25**: 4876-4882
26 **Felsenstein J**. PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle, 1993
27 **Okamoto H**, Nishizawa T, Kato N, Ukita M, Ikeda H, Iizuka H, Miyakawa Y, Mayumi M. Molecular cloning and characterization of a novel DNA virus (TTV) associated with posttransfusion hepatitis of unknown etiology. *Hepatol Res* 1998; **10**: 1-16
28 **Irshad M**, Chaudhuri PS, Joshi YK. Superoxide dismutase and total anti-oxidant levels in various forms of liver diseases. *Hepatol Res* 2002; **23**: 178-184
29 **Chattopadhyay S**, Das BC, Gupta RK, Kar P. Presence of TT virus infection in chronic hepatitis patients from a hospital in New Delhi, India. *Indian J Med Res* 2005; **122**: 29-33
30 **Kalkan A**, Ozdarendeli A, Bulut Y, Saral Y, Ozden M, Kelestimur N, Toraman ZA. Prevalence and genotypic distribution of hepatitis GB-C/HG and TT viruses in blood donors, mentally retarded children and four groups of patients in eastern Anatolia, Turkey. *Jpn J Infect* Dis 2005; **58**: 222-227
31 **Erensoy S**, Sayiner AA, Turkoglu S, Canatan D, Akarca US, Sertoz R, Ozacar T, Batur Y, Badur S, Bilgic A. TT virus infection and genotype distribution in blood donors and a group of patients from Turkey. *Infection* 2002; **30**: 299-302
32 **Erensoy S**, Zeytinoglu A, Goksel S, Ozacar T, Ozkahya M, Ok E, Tuumlrkoglu S, Bilgic A. GB virus C/hepatitis G virus infection among renal transplant recipients in Izmir, Turkey: Molecular analysis of phylogenetic groups. *Int J Infect Dis* 2002; **6**: 242-243

S- Editor Zhong XY **L- Editor** Negro F **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6052

World J Gastroenterol 2008 October 21; 14(39): 6052-6059
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Overall expression of *beta-catenin* outperforms its nuclear accumulation in predicting outcomes of colorectal cancers

Worrawit Wanitsuwan, Samornmas Kanngurn, Teeranut Boonpipattanapong, Rassamee Sangthong, Surasak Sangkhathat

Worrawit Wanitsuwan, Teeranut Boonpipattanapong, Surasak Sangkhathat, Tumor Biology Research Group and Department of Surgery, Faculty of Medicine, Prince of Songkla University, Thailand
Samornmas Kanngurn, Tumor Biology Research Group and Department of Pathology, Faculty of Medicine, Prince of Songkla University, Thailand
Rassamee Sangthong, Tumor Biology Research Group and Epidemiology Unit, Faculty of Medicine, Prince of Songkla University, Thailand
Author contributions: Sangkhathat S designed the methodology; Wanitsuwan W, Boonpipattanapong T and Kanngurn S performed clinical work and histopathological study; Sangthong R analyzed data.
Supported by The Faculty of Medicine, Prince of Songkla University
Correspondence to: Dr. Surasak Sangkhathat, Tumor Biology Research Group and Department of Surgery, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla, 90110 Thailand. surasak.sa@psu.ac.th
Telephone: +66-7445-1401 Fax: +66-7442-9384
Received: July 1, 2008 Revised: August 17, 2008
Accepted: August 24, 2008
Published online: October 21, 2008

Abstract

AIM: To examine the expression of *beta-catenin* in colorectal cancer and look for association with other clinico-pathological parameters.

METHODS: Tumor samples from 163 cases of colorectal cancer (CRC) who had undergone primary colectomy between May, 1998 and November, 2002 with complete follow-up data for either 5 years or until death were recruited for a beta-catenin immunohistochemical study. The percentage of immunoreacted tumor cells was defined as overall staining density (OSD) and percentage of cells having nuclear localization was counted as nuclear staining density (NSD). Univariate exploration used log-rank test and multivariate survival analysis used Cox's hazard regression model.

RESULTS: Beta-catenin immunoreactivity was detected in 161 samples (98.8%), of which 131 cases had nuclear staining. High OSD (≥ 75%), detected in 123 cases (75.5%), was significantly associated with earlier clinical staging ($P < 0.01$), lower nodal status ($P = 0.02$), non-metastatic status ($P < 0.01$) and better differentiation ($P = 0.02$). Multivariate analysis found that high OSD was independently associated with better survival [Cox's hazard ratio 0.51, 95% confidence interval (CI) 0.31-0.83]. Although high NSD (≥ 75%) was correlated with high pre-operative serum CEA ($P = 0.03$), well differentiation ($P < 0.01$), and increased staining intensity ($P < 0.01$), the parameter was not significantly associated with survival.

CONCLUSION: Unlike previous reports, the study did not find a predictive value of nuclear beta-catenin in CRC. Instead, the overall expression of beta-catenin in CRC showed an association with better differentiation and earlier staging. Moreover, the parameter also independently predicted superior survival.

Key words: Colorectal carcinoma; *beta-catenin*; *CTNNB1*; Wnt-signaling pathway; Prognosis

Peer reviewer: Finlay A Macrae, MD, Professor, Royal Melbourne Hospital, Po Box 2010, Victoria 3050, Australia

Wanitsuwan W, Kanngurn S, Boonpipattanapong T, Sangthong R, Sangkhathat S. Overall expression of *beta-catenin* outperforms its nuclear accumulation in predicting outcomes of colorectal cancers. *World J Gastroenterol* 2008; 14(39): 6052-6059 Available from: URL: http://www.wjgnet.com/1007-9327/14/6052.asp DOI: http://dx.doi.org/10.3748/wjg.14.6052

INTRODUCTION

According to the recent World Cancer Report[1], colorectal cancer (CRC) ranks as the third most common malignancy, following breast and lung cancers. The incidence of CRC in developing countries, including Thailand, has been increasing in recent years[2]. The increasing adoption of Western life style habits in these countries is believed to be responsible for the growing magnitude of the CRC problem. Although surgical therapy is the mainstay treatment for early stage CRC, there is an expanding role for multidisciplinary treatment. As adjuvant treatments are considered based on an individual patient risk, prognosticating factors

are essential for risk stratification. Well accepted factors influencing outcome in CRC patients are tumor invasion, nodal status, lymphovascular invasion and serum carcinoembryonic antigen level[3,4]. Various histological parameters and molecular markers have been investigated and some have shown promising results in CRC, such as microvascular density[5] and microsatellite instability[6]. However, none of those biological factors have yet been integrated into the treatment algorithm.

Beta-catenin, a central molecule of the Wnt-signaling system, expresses in epithelial cells as two main forms; membrane localization and nuclear accumulation. Membrane localization can be detected in normal cells and tumor lineage, whereas nuclear beta-catenin is exclusively detected in immature cells and tumor cells[7]. Evidence of somatic mutations and nuclear accumulation of beta-catenin in various pediatric cancers signifies a role of the Wnt-signaling pathway in their tumorigenesis[8,9]. Accumulation of beta-catenin is a result of defects in its degradation process, which usually takes place in the cytoplasm by an interaction between *beta-catenin* and a complex of *APC*, *AXIN* and *GSK-3-beta*. Stabilized beta-catenin translocates into the nucleus where the protein acts as a transactivation factor and promotes tumor growth. In CRC, the molecular pathology involving members of the pathway has been elucidated[10]. Inactivation of APC, the tumor suppressor gene which regulates intracellular level of beta-catenin, is one of the earliest events observed in CRC development. Loss of APC function leads to pathologically increased cytoplasmic beta-catenin, which can be translocated into the nucleus or to the cell membranes[11]. At cell membranes, beta-catenin forms a complex with E-cadherin which plays a role as an adhesion molecule. Furthermore, Wnt-signaling may contribute to the process of colonic epithelial cells differentiation[12].

Recent studies have focused on the clinical meaning of nuclear beta-catenin accumulation in CRC and demonstrated its diagnostic as well as prognostic significance[13-19]. A high density of beta-catenin nuclear accumulation was associated with higher mortality in selected groups of CRC patients[14,18,19]. However, data from different series have been inconsistent. We conducted a study of beta-catenin immunohistochemistry in our clinical CRC series. With awareness that beta-catenin does not have only a growth promoting role, we did not limit our analysis to nuclear accumulation only, but also examined overall staining density against outcomes. The study failed to find evidence that nuclear beta-catenin accumulation could be a risk factor for unfavorable outcome in CRC. However, the study found a strong correlation between overall beta-catenin expression and better survival probability.

MATERIALS AND METHODS

CRC patients

Archival tumor samples from 163 non-consecutive patients with histologically proven colonic adenocarcinoma who underwent primary resection at Songklanagarind Hospital, a tertiary teaching hospital in southern Thailand, between May, 1998 and November, 2002, were examined for this immunohistochemical study. CRC patients who were treated elsewhere, received a non-curative excision so that accurate pathological staging could not be determined, or were lost to follow-up after surgical treatment were not included in this study. All patients were evaluated for at least 5 years after surgery or until death. Survival status was evaluated by the institutional Tumor Registry Unit on December, 2007.

Before the surgical treatment, all patients had routine pre-operative investigations, including chest-X-rays, blood chemistry including serum albumin and carcinoembryonic antigen (CEA), and an evaluation for liver metastasis by ultrasonography and/or computerized tomography. Primary tumor staging followed the sixth edition of TNM staging system of the American Joint Committee on Cancer (AJCC)[20]. Adjuvant chemotherapy was given to stage Ⅲ, colonic cancer patients, and adjuvant chemo-radiation was reserved for stages Ⅱ and Ⅲ rectal cancer cases. In stage Ⅳ CRC patients, chemoradiation therapy for advanced disease was considered for patients who were in status 0-2, according to the Eastern Cooperative Oncology Group. Follow-up visits were scheduled at 1-mo intervals during the first year after surgery, every 3-mo period during the second year, and every 6 mo, thereafter. Access to pathological samples and clinical records was approved by the institutional research ethics committee.

For analysis, age of the patients was stratified to less than 65 years and 65 years or more. Pre-operative serum CEA level was stratified at 5 ng/mL.

Beta-catenin immunohistochemistry

Hematoxylin and eosin stained slides were re-examined by a gastrointestinal pathologist and selected for the beta-catenin immunohistochemical study. From formalin-fixed paraffin embedded tissue, sections of 3 micrometer thickness were cut, deparaffinized, and rehydrated. Beta-catenin monoclonal antibody (1:500, Abcam Plc., UK) was used as the primary antibody. The staining protocol followed the manufacturers' instructions of Dako EnVision + System (Dako). Briefly, antigen retrieval was performed in a microwave oven using Tris-EDTA buffer. Endogenous peroxidase activity was blocked with 0.03% hydrogen peroxide containing sodium azide. Slides were incubated with non-immune serum for 30 minutes and were then incubated with the primary antibody for 120 minutes in a moist chamber, followed by a 30 minute incubation with peroxidase labeled polymer conjugated to goat anti-mouse immunoglobulins. Color was then developed by the liquid 3,3'-diaminobenzidine chromogen solution. Light counterstaining was done with hematoxylin. All immunohistological staining in this study was performed by one technician.

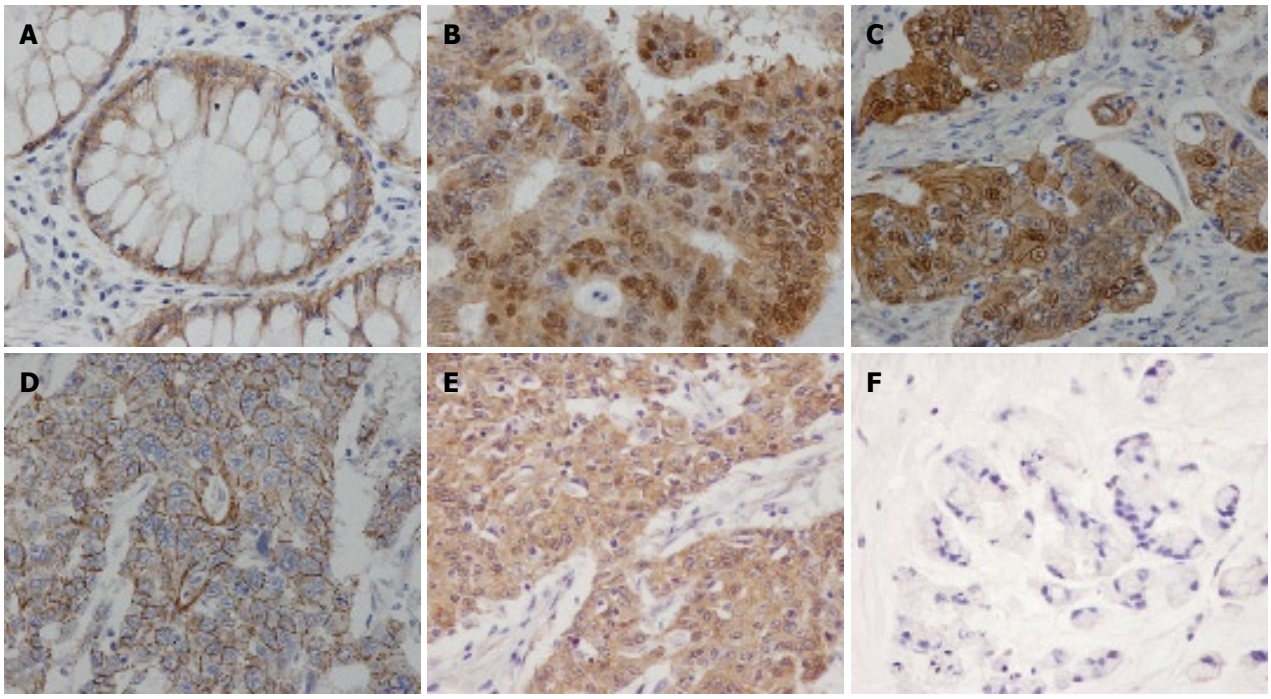


Figure 1 Immunohistochemistry study of beta-catenin in CRC. **A**: Membranous staining pattern in normal colonic mucosa; **B**: Nuclear staining pattern and high OSD in a case with well differentiation histology; **C**: Nuclear and membranous staining pattern; **D** Membranous staining pattern; **E** Cytoplasmic staining pattern; **F**: Very weak staining intensity in a case of poorly differentiated CRC. (40 × magnification).

Staining intensity of beta-catenin was graded as weak, moderate, strong and very strong intensity. Intracellular localization patterns of beta-catenin were categorized according to the dominant pattern as nuclear staining, nuclear together with membrane staining, cytoplasm staining and membrane staining.

Beta-catenin nuclear staining density (NSD) was defined as the number of tumor cells with nuclear staining per 100 cells examined. Overall staining density (OSD) meant number of beta-catenin immunoreacted cell per 100 tumor cells examined. Tumors were defined as having high NSD when NSD was 75% or more, and OSD at 75% or more indicated high OSD. The pathologists responsible for histological slide examination were blinded to the clinical outcomes before data analysis.

Statistical analysis

Death from cancer was assigned as failure in the overall survival analysis. Local recurrence and distant metastasis were counted as failure in disease-free survival, which were analyzed only for cases in AJCC stage Ⅰ-Ⅲ. Univariate analysis used Chi-square test to evaluate any association between parameters and Log rank test for survival analysis. Multivariate survival analysis used a multiple Cox's regression model. Parameters with *P*-value less than 0.3 from the Log rank test were included for analysis. Those with a *P*-value $\geqslant$ 0.05 in Cox's regression were excluded until every parameter in the model was independently associated with survival. A *P*-value of less than 0.05 was considered statistically significant.

Calculations used the R-program (Foundation for Statistical Computing, Vienna, Austria).

RESULTS

The clinical characteristics and pathological descriptions of the 163 patients are shown in Table 1. The mean age of the patients was 61.6 years (range 20-88 years). Median pre-operative serum CEA was 4 ng/mL (range 0.5-1215 ng/mL). Eleven of the primary tumors (6.7%) were at the cecum, 13 (8.0%) at the ascending colon, 5 (3.0%) at the hepatic flexure colon, 11 (6.7%) at the transverse colon, 2 (1.2%) at the splenic flexure colon, 3 (1.8%) at the descending colon, 37 (22.7%) at the sigmoid colon and 81 (49.7%) at the rectum.

Staining characteristics of beta-catenin

Beta-catenin immunoreactivity was detected in 161 cases (98.8%). Among these, 131 cases (80.3% of all cases) were positive for nuclear beta-catenin staining while the remaining cases showed limited immunoreactivity within the cell membranes and/or cytoplasm. Among cases with positive nuclear beta-catenin, 40 (30.5%) were also positive for membrane immunoreactivity. Concerning staining intensity, 74 cases (45.4%) displayed very strong immunoreactivity, 35 (21.5%) showed strong intensity, 27 (16.6%) had moderate intensity, and 25 (15.5%) cases exhibited weak staining (Figure 1). Interestingly, nuclear beta-catenin was detected in a significantly higher proportion in cases with strong or very strong intensity (99.1%), compared to cases with moderate or weak immunoreactivity (44.23%). Cases with positive nuclear staining had a higher incidence of lymph node metastasis (50.3%) when compared to cases with only membranes or cytoplasm staining (33.3%), however, the difference was not statistically significant (Chi-square *P*-value = 0.09).

Table 1 Demographic data of the study population and association of studied parameters and overall staining density, *n* (%)

Parameter	Cases	Overall staining density		*P*
		Low	High	
Sex				
Male	80 (49.3)	18 (45.0)	62 (50.8)	0.52
Female	83 (50.6)	22 (55.0)	60 (49.2)	
Age (yr)				
< 65	87 (53.4)	21 (52.5)	66 (53.7)	0.90
> 65	76 (46.6)	19 (47.5)	57 (47.3)	
CEA				
< 5 ng/mL	83 (50.9)	25 (62.5)	58 (47.1)	0.09
⩾ 5 ng/mL	80 (49.1)	15 (37.5)	65 (52.9)	
AJCC staging				
Stage 1	20 (12.3)	4 (10.0)	16 (13.0)	0.004
Stage 2	61 (37.4)	11 (27.5)	50 (40.7)	
Stage 3	63 (38.7)	14 (35.0)	49 (39.8)	
Stage 4	19 (11.7)	11 (27.5)	8 (6.5)	
Tumor size				
T 0-1	25 (15.3)	5 (12.5)	20 (16.3)	0.57
T 2-3	138 (84.7)	35 (87.5)	103 (83.7)	
Nodal status				
N0	86 (52.8)	17 (42.5)	69 (56.1)	0.02
N1	39 (23.9)	7 (17.5)	32 (26.0)	
N2	38 (23.3)	16 (40.0)	22 (17.8)	
Metastatic status				
M0	144 (88.3)	29 (72.5)	115 (93.5)	< 0.001
M1	19 (11.7)	11 (27.5)	8 (6.5)	
Differentiation				
Well	83 (50.9)	19 (47.5)	64 (52.0)	0.02
Moderate	60 (36.8)	11 (27.5)	49 (39.8)	
Poor	20 (12.3)	10 (25.0)	10 (8.1)	
Staining localization				
Nuclear	122 (50.3)	19 (47.5)	63 (51.2)	0.68
Non-nuclear	31 (49.6)	21 (52.5)	60 (48.8)	
Staining intensity				
Weak to moderate	54 (33.1)	18 (45.0)	34 (28.1)	0.05
Strong to very strong	109 (66.9)	22 (55.0)	87 (71.9)	
NSD				
Low (< 75%)	101 (47.2)	33 (82.5)	44 (35.8)	< 0.001
High (⩾ 75%)	62 (52.8)	7 (17.5)	9 (64.2)	

Overall staining density ranged from 5% to 100% with an average value of 86%. One hundred and twenty-three cases (75.5%) were rated as high OSD. On univariate analysis, high OSD was significantly associated with earlier AJCC staging, lower nodal status, non-metastatic status and better differentiation status (Table 1).

Among the 131 cases in which the nuclear staining appeared positive, nuclear accumulation density ranged from 1% to 99 % with an average value of 65.6%. Eighty-six cases (52.8%) had nuclear accumulation density at 75% or more and were counted as high NSD. High NSD had significantly positive correlation with pre-operative serum CEA ($P = 0.03$), well differentiation ($P < 0.001$), and staining intensity ($P < 0.001$). In addition, cases with NSD at 25% or more had significantly higher incidence of nodal metastasis at the operation ($P = 0.01$).

Survival analysis

There were no missing data in this study. The mean follow-up period was 56.2 mo. Overall survival and disease-free survival at 5-years were 57.7% and 54.0%, respectively. Univariate analysis found that AJCC staging, nodal status, metastatic status and tumor location were among the clinical parameters that had significant association with both overall survival and disease-free survival. Tumor differentiation was correlated with disease-free survival and the overall survival only at borderline significance (Log rank *P*-values 0.05 and 0.06, respectively) (Table 2). CEA at 5 ng/mL or more predicted significantly poorer outcome only in the AJCC stage I subgroup (Log rank *P*-value 0.045).

Five-year overall survival in patients with high NSD (59.3%) was not different from cases with low density (55.8%) when compared by Log-rank analysis ($P = 0.59$).

Table 2 Time-points of 5-yr overall survival and disease free survival according to each parameter and *P*-value of univariate survival analysis with log-rank test

Parameter	5-yr DFS (%)	5-yr OS (%)	Log-rank *P*
Overall	54	57.7	-
Sex			0.77
Male	50.6	56.3	
Female	56.9	58.5	
Age (yr)			0.3
< 65	55.7	59.7	
⩾ 65	52.1	55.3	
CEA			0.09
< 5 ng/mL	59.7	65.1	
⩾ 5 ng/mL	48.2	50.0	
AJCC staging			< 0.001
Stage 1	68.6	75.0	
Stage 2	81.2	86.9	
Stage 3	39.2	41.3	
Stage 4	0	0	
Tumor size			0.38
T 0-1	61.9	72.0	
T 2-3	52.4	55.1	
Nodal status			< 0.001
N0	73.1	79.1	
N1	42.6	46.1	
N2	21	21.1	
Metastatic status			< 0.001
M0	61.1	65.2	
M1	0	0	
Location			0.03
Colon	61.6	65.8	
Rectum	46.1	49.4	
Differentiation			0.06
Well	61.9	62.0	
Moderate	49.4	49.5	
Poor	34.2	34.3	
Staining localization			0.09
Nuclear	69.2	73.3	
Non-nuclear	50.5	54.1	
Staining intensity			0.61
Weak to moderate	60.9	65.4	
Strong to very strong	50.7	54.1	
OSD			< 0.001
Low (< 75%)	34.7	63.4	
High (⩾ 75%)	60.5	40.0	
NSD			0.59
Low (< 75%)	51.1	55.8	
High (⩾ 75%)	56.5	59.3	

OSD: Overall staining density; NAD: Nuclear staining density; OS: Overall survival; DFS: Disease free survival.

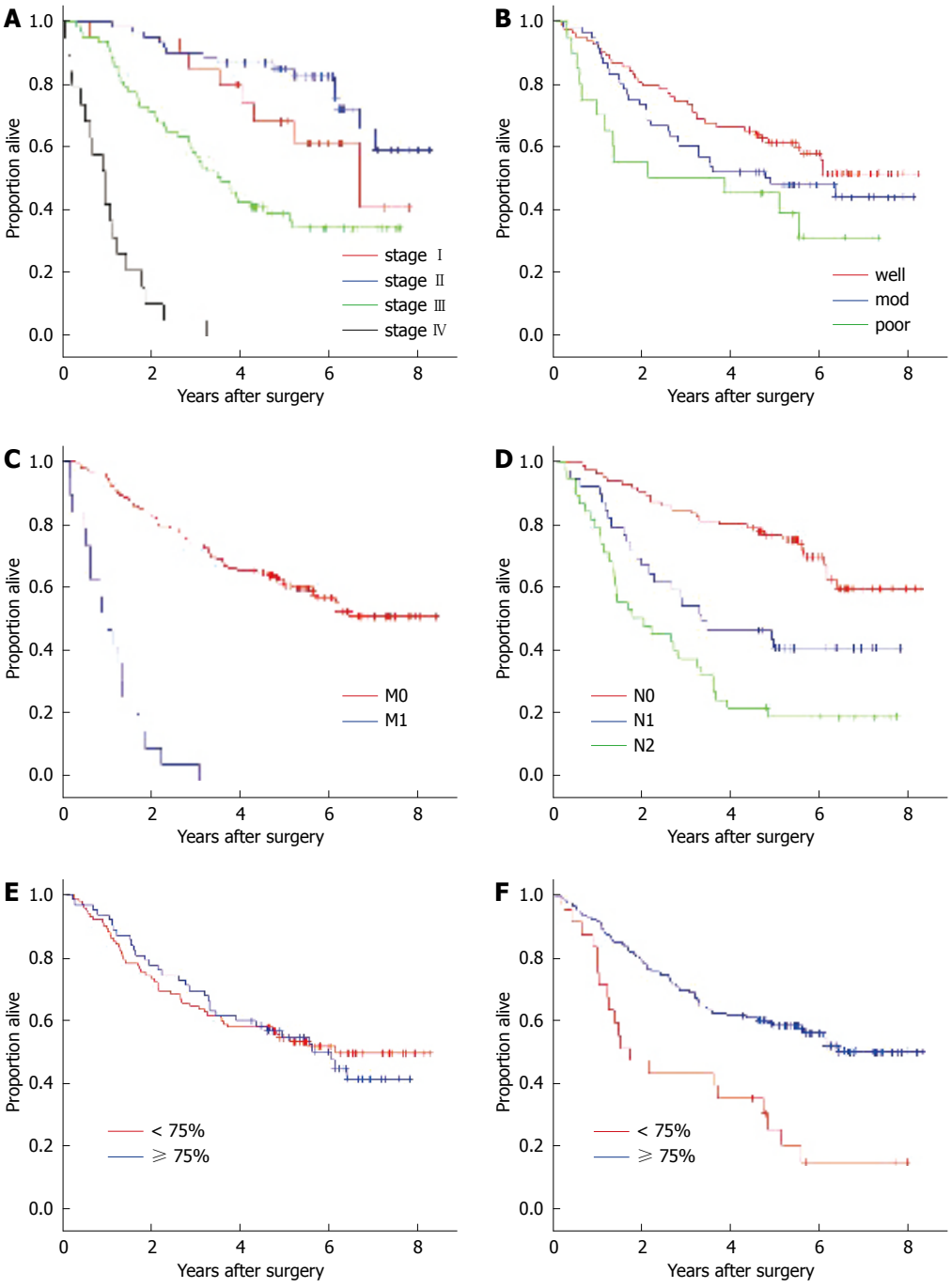


Figure 2 Kaplan-Meier survival curves and log-rank analysis. **A**: AJCC staging ($P < 0.001$); **B**: Tumor differentiation ($P = 0.06$); **C**: Metastatic status ($P < 0.001$); **D**: Nodal status ($P < 0.001$); **E**: Nuclear staining density ($P = 0.59$); **F**: Overall staining density ($P < 0.001$). Well: Well differentiation; mod: Moderate differentiation; poor: Poorly differentiation.

Table 3 Intensity-adjusted 5-yr overall survival and disease-free survival by overall staining density and nuclear staining density

Parameter	Intensity-adjusted 5-yr survival	
	5-yr DFS (%)	5-yr OS (%)
OSD		
Low (< 75%)	34.9	38.2
High (⩾ 75%)	73.2	77.8
NSD		
Low (< 75%)	55.3	58.4
High (⩾ 75%)	68.7	75.7

OS: Overall survival; DFS: Disease free survival; OSD: Overall staining density; NSD: Nuclear staining density.

On the other hand, OSD showed a significant influence on both overall ($P < 0.001$) and disease-free survival probability ($P < 0.001$) (Figure 2). Two and five-year overall survival rates in cases with high OSD (84.6% and 60.5%, respectively) were obviously higher than low OSD (50.0% and 34.7%, respectively). Adjusting for the staining intensity resulted in a greater difference of 5-year overall survival probability between the high OSD (77.8%) and low OSD group (38.2%) (Table 3).

With Cox's hazard multivariate analysis, the final model demonstrated that perioperative parameters that were independently associated with survival probability in colorectal cancer were nodal status, metastatic status, and OSD (Table 4). When subgroup analysis was performed for cases without metastasis at the time of diagnosis, nodal status (hazard ratio 2.0, $P < 0.001$), OSD (hazard ratio 0.36, $P < 0.001$), serum CEA at 5 ng/mL or more (hazard ratio 1.8, $P < 0.001$) and differentiation status (hazard ratio 1.4, $P < 0.02$) were significantly

Table 4 Final Cox's regression model (*P*-value of the model < 0.001), Log likelihood = -347.75

Parameter	Hazard ratio	95% CI	*P*
Nodal status	1.87	1.43-2.44	< 0.001
Metastatic status	7.50	3.89-14.43	< 0.001
OSD	0.51	0.31-0.83	0.007

OSD: Overall staining density.

associated with disease-free survival. Within the same non-metastatic subgroup, factors that significantly fit the final regression model analyzing overall survival were nodal status (hazard ratio 2.3, $P < 0.001$), OSD (hazard ratio 0.4, $P < 0.001$), and age more than 65 years (hazard ratio 1.6, $P < 0.04$).

Excluding OSD from the model, the Cox's hazard analysis showed that high NSD had a hazard ratio of 2.04 [95% confidence interval (CI) 1.0-4.2], compared with NSD less than 50, when adjusted for tumor stage, differentiation, nodal and metastatic status.

DISCUSSION

An outcome prognosticating factor is an essential component for risk categorization in utilizing the risk-based therapy concept. Defining an individual at-risk of unfavorable prognosis helps in selecting patients who are most likely to benefit from intensive adjuvant treatment. Earlier successful examples of risk-based therapy in biological factors have been integrated into the management scheme are neuroblastoma[21], breast cancer[22] and gastrointestinal stromal tumors[23]. In CRC, although various biological outcome predictors have been discovered, those parameters have not yet been adopted into a standard treatment protocol of CRC.

The Wnt-signaling pathway plays several roles in humans; a physiological role in normal development and a pathological role in tumorigenesis[7]. A common denominator of the pathway activation is the intracellular amount and localization of beta-catenin, the central molecule. The carcinogenic process of CRC could be linked to the Wnt-signaling cascade through the loss of APC function, which has been attributed to up to 60% of sporadic tumors[24]. Because APC is essential for clearance of excessive intracellular beta-catenin, accumulation of beta-catenin occurs as a result of APC down-regulation. Positive nuclear accumulation has been reported between 21%-100% in sporadic CRC[15-17,19], depending on the characteristics of the patients in the series, the staining protocol and their histological parameter. In our study, tumor cells with nuclear staining was detected in at least 80% of the cases, however, only 66% of these cases had high NSD. In a series of 136 patients with CRC, Cheah and colleagues identified a negative survival influence of nuclear beta-catenin[15]. In their study, the nuclear beta-catenin was covariate with tumor stage; however, it could be a strong prognosticator when combined with another factor p27b12. Two publications by Wong and colleagues reported that progression of nuclear beta-catenin immunohistochemistry scores was correlated with advances in the malignant potential of colonic neoplasms[13,14]. The studies also showed survival differences between cases with high and low expression density of nuclear beta-catenin. Outcome prognosticating ability of non-membranous beta-catenin was also supported by another two other separate works from Europe[16,17]. Lines of epidemiological evidence suggested that nuclear accumulation of beta-catenin could be employed as a factor identifying cases at risk of treatment failure. However, consensus histological criteria had not been established at the time of there studies and most of them focused only on nuclear localization.

Our study aimed to evaluate the roles of beta-catenin in our clinical series of CRC patients in whom the therapeutic process was homogenously performed by the same multidisciplinary team and the follow-up period was long enough to give reliable data. Apart from nodal status, pre-operative CEA and chronological age predicted disease-free survival and overall survival non-metastatic CRC in the series. Although NSD showed no significant survival function, hazard analysis demonstrated that the factor was associated with mortality risk when other factors were adjusted. Moreover, high NSD was linked to lymph node metastasis and high serum CEA, a finding consistent with previous reports which found that nuclear-accumulated beta-catenin predicted an unfavorable outcome. However, in our series, the influence of nuclear beta-catenin was not independent from other major prognosticators. On the other hand, when we analyzed the overall expression of beta-catenin in term of OSD, it was found that the factor was strongly associated with survival advantage. This conflicting data could not be simply explained by using the mainstream theory of the Wnt-signaling cascade. A positive correlation between NSD, OSD and staining intensity was one of the interesting findings in our study. If NSD is regarded as a localization parameter of beta-catenin and OSD represents an overall expression level of the protein, it appears that the two parameters are co-variant and OSD has much stronger influence on survival.

Besides the growth promoting function, alternative roles of beta-catenin should not be overlooked. Beta-catenin performs necessary physiologic tasks in cellular differentiation and cell-to-cell adhesion[25]. Our study detected a significant correlation between beta-catenin expression and tumor differentiation. Poorly differentiated tumors harbored a significantly smaller proportion of high NSD and OSD. This association was also shown in previous series, although the survival correlation appeared in an opposite direction[15]. A recent study from our group demonstrated that beta-catenin promotes differentiation in another malignancy, neuroblastoma[26]. If these things are taken together, it might be hypothesized that beta-catenin plays a role in

maintaining a good differentiation status in cancer cells. Furthermore, the physiologic function of beta-catenin at the cell membranes is related to cell-to-cell adhesion, which possibly prevents metastasis. A study by Lugli[18] and colleagues found that membranous beta-catenin co-localized with E-cadherin and loss of membranous or cytoplasmic beta-catenin characterized a higher stage disease. In our series, loss of OSD was evident in advanced stage CRC, and there was also a significantly higher OSD proportion in early nodal status. The physiologic functions of beta-catenin in tumor cell differentiation and adhesion may explain our findings. However, to conclude that beta-catenin expression provides a protective role in CRC, further functional genetics research needs to be performed.

In summary, expression and localization of beta-catenin immunohistochemistry in a series of CRC were analyzed. The study did not find a prognosticating role of the nuclear localization; however, overall expression of beta-catenin was found as a strong and independent predictor of favorable outcome.

ACKNOWLEDGEMENT

This work was partially supported by the Faculty of Medicine, Prince of Songkla University. The authors thank Kanda Tongmitr for her assistance in pathological techniques. Arinda Ma-a-lee contributed greatly to the statistical analysis. English language in the manuscript was kindly edited by Dave Patterson.

COMMENTS

Background

The authors examined the expression of beta-catenin in colorectal cancer and looked for association with other clinicopathological parameters, and outcomes.

Research frontiers

Beta-catenin immunohistochemistry was performed in 163 cases of colorectal cancer in whom the outcome data was clearly available.

Innovations and breakthroughs

The study found certain data that was not in-line with previous reports. Instead of nuclear beta-catenin that was associated with survival, the overall staining density of this protein showed strong and independent correlation with overall survival and disease-free survival. Moreover, the parameter (overall staining density) also had positive association with tumor differentiation.

Applications

The data suggested that beta-catenin may have an alternative role in colorectal cancer that was associated with differentiation of tumor cells.

Terminology

Beta-catenin nuclear staining density (NSD) was defined as the number of tumor cells with nuclear staining per 100 cells examined. Overall staining density (OSD) meant number of beta-catenin immunoreacted cell per 100 tumor cells examined. Tumors were defined as having high NSD when NSD was 75% or more, and OSD at 75% or more indicated high OSD.

Peer review

This is a well analyzed paper and provides a new insight into the importance of beta catenin staining as a prognostic marker in colorectal cancer.

REFERENCES

1 **Stewart BW**, Kleihues P. World cancer report. Lyon: IARC Press, 2003
2 **Khuhaprema T**, Srivatanakul P. Colon and rectum cancer in Thailand: an overview. *Jpn J Clin Oncol* 2008; **38**: 237-243
3 **Compton C**, Fenoglio-Preiser CM, Pettigrew N, Fielding LP. American Joint Committee on Cancer Prognostic Factors Consensus Conference: Colorectal Working Group. *Cancer* 2000; **88**: 1739-1757
4 **Boonpipattanapong T**, Chewatanakornkul S. Preoperative carcinoembryonic antigen and albumin in predicting survival in patients with colon and rectal carcinomas. *J Clin Gastroenterol* 2006; **40**: 592-595
5 **Acikalin MF**, Oner U, Topcu I, Yasar B, Kiper H, Colak E. Tumour angiogenesis and mast cell density in the prognostic assessment of colorectal carcinomas. *Dig Liver Dis* 2005; **37**: 162-169
6 **Lurje G**, Zhang W, Lenz HJ. Molecular prognostic markers in locally advanced colon cancer. *Clin Colorectal Cancer* 2007; **6**: 683-690
7 **Polakis P**. Wnt signaling and cancer. *Genes Dev* 2000; **14**: 1837-1851
8 **Koesters R**, von Knebel Doeberitz M. The Wnt signaling pathway in solid childhood tumors. *Cancer Lett* 2003; **198**: 123-138
9 **Sangkhathat S**, Kusafuka T, Miao J, Yoneda A, Nara K, Yamamoto S, Kaneda Y, Fukuzawa M. In vitro RNA interference against beta-catenin inhibits the proliferation of pediatric hepatic tumors. *Int J Oncol* 2006; **28**: 715-722
10 **Chung DC**. The genetic basis of colorectal cancer: insights into critical pathways of tumorigenesis. *Gastroenterology* 2000; **119**: 854-865
11 **Behrens J**, Lustig B. The Wnt connection to tumorigenesis. *Int J Dev Biol* 2004; **48**: 477-487
12 **de Lau W**, Barker N, Clevers H. WNT signaling in the normal intestine and colorectal cancer. *Front Biosci* 2007; **12**: 471-491
13 **Wong SC**, Lo ES, Lee KC, Chan JK, Hsiao WL. Prognostic and diagnostic significance of beta-catenin nuclear immunostaining in colorectal cancer. *Clin Cancer Res* 2004; **10**: 1401-1408
14 **Wong SC**, Lo ES, Chan AK, Lee KC, Hsiao WL. Nuclear beta catenin as a potential prognostic and diagnostic marker in patients with colorectal cancer from Hong Kong. *Mol Pathol* 2003; **56**: 347-352
15 **Cheah PY**, Choo PH, Yao J, Eu KW, Seow-Choen F. A survival-stratification model of human colorectal carcinomas with beta-catenin and p27kip1. *Cancer* 2002; **95**: 2479-2486
16 **Bondi J**, Bukholm G, Nesland JM, Bukholm IR. Expression of non-membranous beta-catenin and gamma-catenin, c-Myc and cyclin D1 in relation to patient outcome in human colon adenocarcinomas. *APMIS* 2004; **112**: 49-56
17 **Baldus SE**, Monig SP, Huxel S, Landsberg S, Hanisch FG, Engelmann K, Schneider PM, Thiele J, Holscher AH, Dienes HP. MUC1 and nuclear beta-catenin are coexpressed at the invasion front of colorectal carcinomas and are both correlated with tumor prognosis. *Clin Cancer Res* 2004; **10**: 2790-2796
18 **Lugli A**, Zlobec I, Minoo P, Baker K, Tornillo L, Terracciano L, Jass JR. Prognostic significance of the wnt signalling pathway molecules APC, beta-catenin and E-cadherin in colorectal cancer: a tissue microarray-based analysis. *Histopathology* 2007; **50**: 453-464
19 **Martensson A**, Oberg A, Jung A, Cederquist K, Stenling R, Palmqvist R. Beta-catenin expression in relation to genetic instability and prognosis in colorectal cancer. *Oncol Rep* 2007; **17**: 447-452
20 **Greene FL**, Page DL, Fleming ID, Fritz AG, Balch CM, Haller DG, Morrow M. AJCC Cancer Staging Manual, 6th ed. New York: Springer-Verlag, 2002
21 **Maris JM**, Hogarty MD, Bagatell R, Cohn SL. Neuroblastoma. *Lancet* 2007; **369**: 2106-2120
22 **Acharya CR**, Hsu DS, Anders CK, Anguiano A, Salter KH, Walters KS, Redman RC, Tuchman SA, Moylan CA,

Mukherjee S, Barry WT, Dressman HK, Ginsburg GS, Marcom KP, Garman KS, Lyman GH, Nevins JR, Potti A. Gene expression signatures, clinicopathological features, and individualized therapy in breast cancer. *JAMA* 2008; **299**: 1574-1587

23 **Takahashi T**, Nakajima K, Nishitani A, Souma Y, Hirota S, Sawa Y, Nishida T. An enhanced risk-group stratification system for more practical prognostication of clinically malignant gastrointestinal stromal tumors. *Int J Clin Oncol* 2007; **12**: 369-374

24 **Segditsas S**, Tomlinson I. Colorectal cancer and genetic alterations in the Wnt pathway. *Oncogene* 2006; **25**: 7531-7537

25 **Gavert N**, Ben-Ze'ev A. beta-Catenin signaling in biological control and cancer. *J Cell Biochem* 2007; **102**: 820-828

26 **Sangkhathat S**, Nara K, Kusafuka T, Yoneda A, Fukuzawa M. Artificially accumulated beta-catenin inhibits proliferation and induces neurite extension of neuroblastoma cell line NB-1 via up-regulation of trkA. *Oncol Rep* 2006; **16**: 1197-1203

S- Editor Xiao LL **L- Editor** Rippe RA **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6060

World J Gastroenterol 2008 October 21; 14(39): 6060-6064
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Therapeutic effect of traditional Chinese medicine on coagulation disorder and accompanying intractable jaundice in hepatitis B virus-related liver cirrhosis patients

Yang-Mei Li, Hong-Zhi Yang, Wei-Bing Guan, Qian-Shan Ke, Min Dai, He-Ping Xie, Shi-Jun Zhang

Yang-Mei Li, Hong-Zhi Yang, Wei-Bing Guan, Qian-Shan Ke, Min Dai, He-Ping Xie, Department of TCM, The Third Affiliated Hospital of Sun Yat-Sen University, 600# Tianhe Road, Guangzhou 510630, Guangdong Province, China
Shi-Jun Zhang, Department of TCM, The First Affiliated Hospital of Sun Yat-Sen University, 58# Zhongshan Er Road, Guangzhou 510080, Guangdong Province, China
Author contributions: Li YM and Yang HZ contributed equally to this work, designed and overviewed the research; Li YM, Zhang SJ and Xie HP analyzed the data; Li YM, Ke QS, Guan WB and Dai M performed the research; Li YM wrote the paper.
Supported by Science and Technology Agency of Guangdong Province, NO. 2008B030301041
Correspondence to: Hong-Zhi Yang, Department of TCM, The Third Affiliated Hospital of Sun Yat-Sen University, 600# Tianhe Road, Guangzhou 510630, Guangdong Province, China. hzyang1960@163.com
Telephone: +86-13318815024 Fax: +86-20-85253336
Received: June 26, 2008 Revised: August 5, 2004
Accepted: August 12, 2008
Published online: October 21, 2008

Abstract

AIM: To observe the therapeutic effects of new traditional Chinese medicine (TCM) therapy on coagulation disorder and accompanying intractable jaundice in HBV-related liver cirrhosis patients.

METHODS: Using stratified random sampling according to fibrinogen (Fib) levels, 145 liver cirrhosis patients due to hepatitis B complicated by coagulation disorder were treated. Of them, 70 in research group were treated with TCM by "nourishing yin, cooling blood and invigorating blood circulation" and Western medicine, 75 in control group were treated with conventional Western medicine. The indexes of liver function, coagulation function and bleeding events were observed and compared.

RESULTS: The prothrombin time (PT) was shorter and the fibrinogen (Fib) level was higher in the research group than in the control group (Fib = 1.6-2.0 g/L, 1.1-1.5 g/L, and ≤ 1.0 g/L). The total bilirubin (TBIL) level was significantly lower in the research group than in the control group, except for the subgroup of FIB ≤ 1.0 g/L.

CONCLUSION: TCM therapy can improve coagulation function and decrease TBIL.

Key words: Combination of traditional Chinese and Western medicine; Liver cirrhosis; Coagulation disorder; Nourishing yin; Cooling blood and invigorating blood circulation

Peer reviewers: Dr. Yukihiro Shimizu, Kyoto Katsura Hospital, 17 Yamada-Hirao, Nishikyo, Kyoto 615-8256, Japan; James Neuberger, Professor, Liver Unit, Queen Elizabeth Hospital, Birmingham B15 2TH, United Kingdom

Li YM, Yang HZ, Guan WB, Ke QS, Dai M, Xie HP, Zhang SJ. Therapeutic effect of traditional Chinese medicine on coagulation disorder and accompanying intractable jaundice in hepatitis B virus-related liver cirrhosis patients. *World J Gastroenterol* 2008; 14(39): 6060-6064 Available from: URL: http://www.wjgnet.com/1007-9327/14/6060.asp DOI: http://dx.doi.org/10.3748/wjg.14.6060

INTRODUCTION

About 25% of hepatitis B virus (HBV)-infected patients would die of severe chronic liver diseases such as liver cirrhosis and liver failure[1]. Coagulation disorder is prevalent in patients with chronic liver disease which is usually detected in laboratory tests and characterized by prolonged prothrombin time (PT), decreased fibrinogen (Fib, coagulation factor Ⅰ) level and thrombocytopenia[2]. For the lack of blood products (plasma and coagulation factor) and high medical expenditure, economical and effective treatment modalities for coagulation disorder are demanded. Moreover, intractable jaundice accompanying coagulation disorder in HBV-related liver cirrhosis patients is also a puzzle and there is no effective treatment for it. We have proved in our prophase researches that coagulation function indexes are significantly related to total bilirubin (TBIL). Therefore, this study was to observe the therapeutic effects of traditional Chinese medicine (TCM) on coagulation disorder and accompanying intractable jaundice in HBV-related liver cirrhosis patients.

MATERIALS AND METHODS

Inclusion criteria

Patients with HBV-related liver cirrhosis[3,4], patients with

coagulation disorder (PT > 14.5 s, fib < 2.0 g/L with or without platelets < 100×10^9/L before admission), patients with no bleeding events (such as epistaxis, gum bleeding, hematemesis and hematochezia before admission), and those at the age of 20-75 years, were included in the study.

Exclusion criteria

Patients with coagulation disorders accompanying liver cirrhosis due to different reasons (such as parasitic infection, autoimmune liver disease, intrahepatic cholestasis, alcoholic liver disease, drug-induced liver disease, fatty liver disease, liver hereditary diseases and liver vascular diseases), patients with other hepatovirus superinfection, haemolysis, disseminated intravascular coagulation (DIC), complications of severe diseases (such as cardio-cerebrovascular disease, hematological disease, respiratory disease, urinary disease and psychosis), and those with pregnancy and lactation, patients with poor compliance, incomplete clinical data, hospitalization time < 14 d, were excluded from the study.

All patients were given their informed consent before therapy.

Information about patients

All the 145 patients with HBV-related liver cirrhosis accompanying coagulation disorder were randomly chosen according to their Fib levels from the Third Affiliated Hospital of Sun Yat-Sen University from January 2002 to February 2008. The data were collected and analyzed retrospectively. The 145 patients were assigned to three subgroups

Subgroup A: Sixty patients (Fib = 1.6-2.0 g/L) were divided into in research group and control group (n = 30). There were 20 males and 10 females in the research group, their average age was 49.83 ± 12.32 years and the average hospitalization time was 35.73 ± 24.20 d. There were 21 males and 9 females in the control group, their average age was 44.67 ± 10.34 years and the average hospitalization time was 36.83 ± 18.15 d.

Subgroup B: Sixty patients (Fib = 1.1-1.5 g/L) were divided into research group and control group (n = 30). There were 20 males and 10 females in the research group, their average age was 50.27 ± 11.71 years and the average hospitalization time was 37.10 ± 19.94 d. There were 21 males and 9 females in the control group, their average age was 49.60 ± 10.45 years and the average hospitalization time was 30.37 ± 16.81 d.

Subgroup C: Twenty-five patients (Fib ⩽ 1.0 g/L) were divided into research group (n = 10) and control group (n = 15). There were 7 males and 3 females in the research group, their average age was 40.80 ± 8.92 years and the average hospitalization time was 41.70 ± 27.57 d. There were 10 males and 5 females in the control group, their average age was 43.20 ± 10.17 years and the average hospitalization time was 54.93 ± 37.10 d.

Methods

Control group: Patients in the control group were treated with conventional Western medicine supplemented with coagulation factors and platelets. Artificial liver system therapy and liver transplantation were not performed.

Research group: Patients in the research group were treated with TCM by nourishing yin, cooling blood and invigorating blood circulation (basic prescription: Yiwei Decoction and Dahuang Zhechong Pills: shashen 15 g, maidong 15 g, shengdi 30 g, yuzhu 15 g, dahuang 6-30 g, huangqin 12 g, gancao 6 g, taoren 9 g, xingren 12 g, shaoyao 12 g, shuizhi 6 g, tubiechong 6 g), in combination with conventional Western medicine. The prescription was modified if symptoms changed.

The herbal decoction was taken half an hour after each meal, one dose a day for 2-3 wk according to the severity of liver cirrhosis.

Observation indexes

Observations included serological index, coagulation function (PT, Fib and PLT), liver function [alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB) and TBIL], bleeding events and other complications, death rate and side effects during the treatment.

Statistic analysis

Statistical analysis was performed using Spss11.5. Data were expressed as mean ± SD and analyzed by t-test. Numeration data were analyzed by chi square test. $P < 0.05$ (two-sided test) was considered statistically significant.

RESULTS

Analysis of comparability

Fib level was used as the standard for all the patients who were divided into three subgroups. Chi square test and t-test showed that the general conditions of patients in the research and control groups were similar (Table 1). The serological indexes of coagulation function, liver function and the severity of liver cirrhosis were similar in the two groups before treatment (Tables 2-4). Improvement in coagulation function of the three subgroups was comparable.

Analysis of data

PT, Fib and TBIL levels were significantly higher in subgroups (Fib = 1.6-2.0 g/L and Fib = 1.1-1.5 g/L) of the research group than those in subgroup of the control group after treatment. There was no significant difference in ALB and PLT between the groups (Tables 5 and 6).

PT and Fib levels were significantly higher in subgroups (Fib ⩽ 1.0 g/L) of the research group were significantly higher than those in subgroups of the control group after treatment. There was no significant difference in ALB, PLT and TBIL between the two groups (Table 7).

Bleeding events occurred in 3 patients of the

Table 1 Balance test for general information

		Cases (*n*)	Sex (male/female)	Age (mean ± SD)	Days of hospitalization (mean ± SD)
Fib (1.6-2.0 g/L) level	Research group	30	20/10	49.83 ± 12.32	35.73 ± 24.20
	Control group	30	21/9	44.67 ± 10.34	36.83 ± 18.15
	P value		0.781	0.084	0.843
Fib (1.1-1.5 g/L) level	Research group	30	20/10	50.27 ± 11.71	37.10 ± 19.94
	Control group	30	19/11	49.60 ± 10.45	30.37 ± 16.81
	P value		0.787	0.817	0.128
Fib (≤ 1.0 g/L) level	Research group	10	7/3	46.80 ± 8.92	41.70 ± 27.57
	Control group	15	10/5	43.20 ± 10.71	54.93 ± 37.10
	P value		1.000	0.389	0.346

Anyone in the three subgroups divided by the standard Fib level, age, sex and days of hospitalization was balanced between research and control groups before treatment.

Table 2 Balance test for indexes of coagulation function before treatment (mean ± SD)

		Fib (g/L)	PT (s)	PLT (10^9/L)
Fib (1.6-2.0 g/L) level	Research group	1.68 ± 0.18	20.52 ± 3.12	69.07 ± 32.57
	Control group	1.72 ± 0.15	21.01 ± 3.31	83.60 ± 56.41
	P value	0.279	0.563	0.227
Fib (1.1-1.5 g/L) level	Research group	1.25 ± 0.13	22.27 ± 2.18	59.87 ± 29.90
	Control group	1.29 ± 0.11	22.60 ± 5.75	71.67 ± 24.24
	P value	0.138	0.765	0.099
Fib (≤ 1.0 g/L) level	Research group	0.77 ± 0.19	26.59 ± 5.39	62.80 ± 33.19
	Control group	0.79 ± 0.18	31.49 ± 8.68	73.27 ± 57.34
	P value	0.861	0.127	0.608

Anyone in the three subgroups divided by the standard Fib level and the indexes of coagulation function was balanced between research and control groups before treatment.

Table 3 Balance test for indexes of liver function before treatment (mean ± SD)

		ALT (U/L)	AST (U/L)	TBIL (μmol/L)	ALB (g/L)
Fib (1.6-2.0 g/L) level	Research group	180.90 ± 255.59	163.17 ± 176.13	104.51 ± 65.26	32.79 ± 4.69
	Control group	241.87 ± 349.01	190.87 ± 191.11	126.97 ± 61.69	33.47 ± 3.72
	P value	0.089	0.562	0.176	0.537
Fib (1.1-1.5 g/L) level	Research group	118.57 ± 121.99	144.37 ± 113.62	129.71 ± 95.70	31.74 ± 4.69
	Control group	234.07 ± 392.07	232.87 ± 265.04	169.95 ± 156.22	29.59 ± 5.54
	P value	0.129	0.098	0.234	0.111
Fib (≤ 1.0 g/L) level	Research group	74.40 ± 30.89	92.40 ± 78.44	198.40 ± 123.37	29.60 ± 3.82
	Control group	181.67 ± 283.59	142.80 ± 128.34	245.57 ± 193.69	30.15 ± 6.61
	P value	0.249	0.280	0.503	0.817

Anyone in the three subgroups divided by the standard of Fib level and the indexes of liver function was balanced between research and control groups before treatment.

Table 4 Balance test for related clinical materials before treatment

		Combined with ascites liquid	Combined with hepatic encephalopathy	Combined with infection	Combined with liver cancer
Fib (1.6-2.0 g/L) level	Research group	10	0	8	5
	Control group	15	3	15	2
	P value	0.190	0.236	0.063	0.421
Fib (1.1-1.5 g/L) level	Research group	17	1	10	6
	Control group	20	0	13	2
	P value	0.426	1.000	0.426	0.255
Fib (≤ 1.0 g/L) level	Research group	8	1	4	0
	Control group	12	1	5	1
	P value	1.000	1.000	0.734	1.000

Anyone in the three subgroups divided by standard Fib level, and clinical materials such as complications was balanced between research and control groups before treatment.

Table 5 Fib (1.6-2.0 g/L) level and serum index before and after treatment (mean ± SD)

		Fib (g/L)	PT (s)	PLT (10^9/L)	TBIL (μmol/L)	ALB (g/L)
Research group 30 cases	Before treatment	1.68 ± 0.18	20.52 ± 3.12	69.07 ± 32.57	104.51 ± 65.26	32.79 ± 4.69
	After treatment	1.95 ± 0.43	17.66 ± 2.38	80.10 ± 42.12	34.44 ± 17.10	36.32 ± 3.98
Control group 30 cases	Before treatment	1.72 ± 0.15	21.01 ± 3.31	83.60 ± 56.41	126.97 ± 61.69	33.47 ± 3.72
	After treatment	1.64 ± 0.44	19.07 ± 7.13	67.63 ± 42.65	113.60 ± 163.86	35.69 ± 5.21
P value		< 0.0001	< 0.0001	0.259	0.008	0.604

PT, Fib and TBIL were significantly higher in the research group than in the control group after treatment.

Table 6 Fib (1.1-1.5 g/L) level before and after treatment (mean ± SD)

		Fib (g/L)	PT (s)	PLT (10^9/L)	TBIL (μmol/L)	ALB (g/L)
Research group 30 cases	Before treatment	1.25 ± 0.13	22.27 ± 2.18	59.87 ± 29.90	129.71 ± 95.70	31.74 ± 4.69
	After treatment	1.72 ± 0.33	18.45 ± 2.11	59.50 ± 27.86	46.75 ± 19.83	36.46 ± 3.83
Control group 30 cases	Before treatment	1.29 ± 0.11	22.60 ± 5.75	71.67 ± 24.24	169.95 ± 156.22	29.59 ± 5.54
	After treatment	1.29 ± 0.41	20.56 ± 9.99	68.37 ± 27.20	130.95 ± 180.92	35.51 ± 4.75
P value		< 0.0001	0.032	0.217	0.014	0.399

PT, Fib and TBIL were significantly higher in the research group than in the control group after treatment.

Table 7 Fib (≤ 1.0 g/L) level before and after treatment (mean ± SD)

		Fib (g/L)	PT (s)	PLT (10^9/L)	TBIL (μmol/L)	ALB (g/L)
Research group 10 cases	Before treatment	0.77 ± 0.19	26.59 ± 5.39	62.80 ± 33.19	198.40 ± 123.37	29.60 ± 3.82
	After treatment	1.29 ± 0.35	23.29 ± 5.35	54.80 ± 37.42	77.85 ± 39.21	35.31 ± 5.07
Control group 10 cases	Before treatment	0.79 ± 0.18	31.49 ± 8.68	73.27 ± 57.34	245.57 ± 193.69	30.15 ± 6.61
	After treatment	0.90 ± 0.36	39.08 ± 22.78	70.93 ± 54.06	173.47 ± 149.30	35.86 ± 5.71
P value		0.013	0.043	0.421	0.061	0.807

PT and Fib were significantly higher in the research group than in the control group after treatment.

research group and in 19 patients of the control group ($P < 0.0001$).

DISCUSSION

Fib, which has coagulation function, is a kind of protein that is synthesized in the liver. Fib, the most important coagulation factor in human body, is transformed into fibrin in the coagulation process. Fib decrease is a sensitive change in chronic hepatitis patients, which means that the biological enzyme is declined and the coagulation function is abnormal[5]. Fib can also be used to diagnose DIC caused by liver diseases. It was reported that Fib contents are closely related with the damage degree of hepatocytes, the severity and prognosis of liver cirrhosis[6-10]. Therefore, Fib was chosen as a criterion in this research.

TCM believes that the original etiological factor for HBV infection is "damp-heat", which belongs to the category of warm pathogens. By analyzing and differentiating the development of an epidemic febrile disease and by studying conditions of the four systems (Wei, Qi, Ying, Xue) of patients with coagulation disorder, Yingfen syndrome and Xuefen syndrome are diagnosed. As one of the febrile disease characteristics, warm pathogen can injure yin easily, meanwhile "cooling the blood and invigorating blood circulation" is the traditional therapeutic method for Xuefen syndrome. Therefore, we chose TCM to treat liver cirrhosis accompanying coagulation disorder by nourishing yin, cooling the blood and invigorating blood circulation.

This study showed that TCM therapy for liver cirrhosis could improve PT and Fib, and reduce occurrence of bleeding events by improving microcirculation, increasing blood and oxygen supply to the liver, thus promoting regeneration and restoration of hepatocytes. It was reported that this new TCM therapy has anti-thrombosis effects by relieving the microangium spasm and hypercoagulable state in the liver[11-15]. Heat-clearing and blood-cooling drugs can stimulate pituitary-adrenal axis, enhance stress capability, dredge microcirculation, protect vessel wall, and maintain the balance between coagulation and anti-substance[14], suggesting that such drugs can promote cell proliferation and speed up cell cycle progression. This new TCM therapy can alleviate hepatocellular immune injury caused by HBV infection and degenerative necrosis of hepatocytes. It was reported that blood circulation promoting therapy can inhibit cellular and humoral immunity. Herbal medicine for cooling the blood and invigorating blood circulation can alleviate immune injury by inhibiting autoimmune effect and γ-globulin[12,14,15], and damaged hepatocytes and vascular endothelial cells caused by endotoxmia and inflammatory factors. Studies showed that nourishing yin, cooling the blood and invigorating blood circulation can antagonize apoptosis of vascular endothelial cells

induced by endotoxin[16-18]. The reason why PLT does not ameliorate is that coagulation disorder in patients with HBV-related liver cirrhosis is usually accompanied with hypersplenia and PLT is severely destroyed and phagocytosed by the spleen. In addition, PLT does not come from liver but from bone marrow megakaryocytes.

This study also showed that the new TCM therapy could significantly decrease TBIL. TCM believes that jaundice would not regress easily if only the blood circulation is fluent. Since the pathogenesis of jaundice is blood stasis which is one of the pathogenic factors for coagulation disorder in liver cirrhosis patients, the new TCM therapy can achieve the purpose of treating different diseases with the same method. On the one hand, it can improve hepatocyte function by exerting its anti-thrombosis microcirculation effect and by improving the blood circulation of liver. On the other hand, it can promote biliary excretion by inhibiting immunologic reaction, alleviating inflammation of intrahepatic bile ducts, which can improve the coagulation disorder in liver cirrhosis patients and decrease jaundice. The reason why TBIL can be decreased only when Fib > 1.0 g/L, may be due to the impaired liver function, a short course of treatment and a relative small sample.

In conclusion, this new TCM therapy can improve coagulation function indexes, such as PT and Fib in patients with HBV-related liver cirrhosis and reduce bleeding events which can also decrease TBIL.

COMMENTS

Background

Hepatitis B virus (HBV) infection is prevalent all over the world and 2000 million people have been infected with HBV, 350 million of them are chronic HBV carriers and 25% of HBV-infected individuals will die of chronic severe liver diseases. Coagulation disorder is an important clinical feature of chronic liver disease characterized by prolonged PT, decreased Fib and thrombocytopenia. HBV infection usually leads to bleeding, anaemia, decreased granulocytes, thrombosis and even multiple organ failure,*etc*. Plasma infusion can improve coagulation disorder. We performed this study to find a new traditional Chinese medicine (TCM) therapy for coagulation disorder in patients with HBV-related liver cirrhosis.

Research frontiers

Conventional treatment modalities for coagulation disorders in Western medicine are to improve liver function, avoid using drugs which can affect platelet function and aggravate coagulation disorder, and supply vitamin K, coagulation factors and platelets. Due to the disadvantages of blood products, such as limited supply, allergic reaction and virus infection during infusion, the third generation recombinant coagulation factor VIIa (rhVIa) is a highlight and has been used in clinical practice. In the field of TCM, promoting blood circulation to remove blood stasis for coagulation disorder can increase fibrinogen (Fib), improve prothrombin time (PT) and eliminate complications.

Innovations and breakthroughs

Fib level was used as a criterion to observe the new TCM therapeutic effects on coagulation disorder in patients with HBV-related liver cirrhosis. Early treatment of coagulation disorder by nourishing yin, cooling the blood and invigorating blood circulation before occurrence of bleeding events can reduce bleeding events and prevent disseminated intravascular coagulation (DIC). Meanwhile, this TCM therapy could improve coagulation function and decrease total bilirubin (TBIL).

Applications

The present study confirmed that the TCM therapy by nourishing yin, cooling the blood and invigorating blood circulation focusing on the pathogenic factors and pathogenesis of coagulation disorders in patients with HBV-related liver cirrhosis, could improve coagulation function, decrease TBIL. Therefore, it can be used in the treatment of chronic liver diseases.

Peer review

Deterioration of coagulation function is a serious problem in liver cirrhosis patients. This new TCM therapy is encouraging and interesting with satisfactory therapeutic effects on serum prothrombin, fibrinogen and TBil in patients with HBV-related liver cirrhosis.

REFERENCES

1 **Peng WW**. Communicable Diseases. The 6th edition. People's Medical Publishing House, 2004: 28
2 **Lv YF**. The coagulation disorder of liver disease. *Zhonghua Xiandai Shiyong Yixue Zazhi* 2006; **5**: 47-50
3 **The prevention and control plan of viral hepatitis**. *Zhonghua chuanranbing Zazhi* 2001; **19**: 56-61
4 **The standard of clinic diagnosis of Liver Cirrhosis, syndrome differentiation of TCM and therapeutic evaluation (Tentative Scheme)**. *Xin Xiaohuabingxue Zazhi* 1994; **2**: 126
5 **Ren GB.** Clicical Hepatology. The 1st edition. Shanghai: Shanghai Scientific & Technical Publishers, 2004: 311
6 **Wang WJ**, Shi NY. Study on clinical significance of coagulation mechanisim in the diagnosis of liver disease. *Shiyong Yiji Zazhi* 2006, **13**: 4163
7 **Chu HY**, Wang HL, Wang XF, Qu B, Wang ZY. The Study on Coagulation-Fibrinolytic System of Viral Hepatitis. *Xueshuan Yu Zhixue ZaZhi* 2001; **7**: 62-80
8 **Lin JH**, Wen ZF. Multivariate proportional hazards regression analysis of 216 hepatic cirrhosis patients. *Zhongguo Xiandai Yixue Zazhi* 2007; 17: 718-724
9 **Cui CJ**, Jin TR, Jin X, Pu XX, Pei FY. The meaning and changes of contents of Fib in patients with liver cirrhosis. *Zhongguo Yishi Zazhi* 2003; **5**: 1664
10 **Wang HL**, Zhi LM, Shao HZ, Zhou H, Li SM, Wang ZY. Research on the changing of coagulation factors in patients with viral hepatitis. *Zhonghua chuanranbing Zazhi* 1992; **10**: 124
11 **Jiang LH**, Liu YW, Wu X, Cao GJ. Clinical observation on the impact of Dansen injection solution on blood stream in portal hypertension patients. *Zhongguo Redai Yixue Zazhi* 2006; **6**: 500
12 **Liu GQ**, Sun SC. The animal experiment study of the four stages og acute seasonal febrile disease. Xi'an: Shaanxi People's Education Press, 1992: 127-149
13 **Gong JN**, Wei KF, Bian HM, Liu XF. Mechanism research on the function of nourishing-yin and activating-blood circulation recipe in prevention and treatment of thrombosis. *Zhong Cheng Yao Zazhi* 2003; **25**: 1012-1014
14 **Li J**. Over-dose madder and siegesbeckiae grass in the therapy of hyperglobulinemia in chronic viral hepatitis. *Zhongyi Zazhi* 1993; **34**: 60
15 **Ye HJ**. The effect of salvia injection on immune function of animal with liver fibrosis. *Linchuang Gandanbing Zazhi* 1995; **11**: 142
16 **Pu XD**. The Study on Endo Toxemia's Path Ogenesis and the Treatment of TCM. *Zhongguo Zhongyi Jizheng Zazhi* 2005; **14**: 1190-1191
17 **Xu LJ**, Lu FE, Wang KF, Zou X, Yang MW, Li MZ, Ye WY. Comparative study on therapeutic effects of three Chinese herbal preparations of antifebrile principles on endotoxemic rabbits. *Zhongguo Zhongxiyi Jiehe Jijiu Zazhi* 2002; **9**: 132-134
18 **Wang EN**, Huang ZR, Jiang M, Chen YR, Lin T. The Experimental Study of the Prevention and Treatment Effects of Yiqi Yangyin Jiedu Huoxue Prescription and Its Minor Components on immunological liver injury in mice. *Fujian Yixueyuan Xuebao* 2003; **10**: 119-121

S- Editor Zhong XY **L- Editor** Wang XL **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6065

World J Gastroenterol 2008 October 21; 14(39): 6065-6071
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Drug utilization of clarithromycin for gastrointestinal disease treatment

Quan Zhou, Ling-Ling Zhu, Xiao-Feng Yan, Wen-Sheng Pan, Su Zeng

Quan Zhou, Xiao-Feng Yan, Department of Clinical Pharmacy, the 2nd Affiliated Hospital, School of Medicine, Zhejiang University, Zhejiang Province 310009, China
Ling-Ling Zhu, Geriatric Ward, the 2nd Affiliated Hospital, School of Medicine, Zhejiang University, Zhejiang Province 310009, China
Wen-Sheng Pan, Department of gastroenterology, the 2nd Affiliated Hospital, School of Medicine, Zhejiang University, Zhejiang Province 310009, China
Su Zeng, Department of Pharmaceutical Analysis & Drug Metabolism, College of Pharmaceutical Sciences, Zhejiang University, Zhejiang Province 310058, China
Author contributions: Zhou Q and Pan WS contributed equally to this work; Zhou Q and Yan XF put forward the viewpoint and designed this survey; Zhou Q, Zhu LL and Zeng S performed literature review and data analysis/interpretation; and Zhou Q and Pan WS wrote the paper.
Supported by Zhejiang Provincial Bureau of Education, No. 20070227; Zhejiang Medical Association, No. 2007ZYC18 and Association of Zhejiang Hospital Administration, No. 2007AZHA-KEB312
Correspondence to: Quan Zhou, Associate Professor, Department of Clinical Pharmacy, the 2nd Affiliated Hospital, School of Medicine, Zhejiang University, Zhejiang Province 310009, China. zhouquan142602@zju.edu.cn
Telephone: +86-571-87784615 Fax: +86-571-87213864
Received: September 1, 2008 Revised: September 12, 2008
Accepted: September 29, 2008
Published online: October 21, 2008

Abstract

AIM: To evaluate the patterns of use of clarithromycin for gastrointestinal disease treatment and promote its rational use.

METHODS: Using a structured pro forma, we conducted a two-month survey of the electronic prescriptions containing immediate-release (IR) or sustained-release (SR) product of clarithromycin for outpatients with gastrointestinal diseases in a 2200-bed general hospital. Suitability of the prescription was audited retrospectively.

RESULTS: One hundred and sixty-four prescriptions of SR product and 110 prescriptions of IR product were prescribed for gastrointestinal disease treatment. Among prescriptions for anti-*Helicobacter pylori* (*H pylori*) therapy, triple therapy take the dominant position (91.8%), followed by quadruple therapy (4.3%) and dual therapy (3.9%). Amoxicillin was the most frequently co-prescribed antibiotic. Furazolidone and levofloxacin are used more widely than metronidazole or tinidazole. Clarithromycin SR was administered at inappropriate time points in all prescriptions. Fifty percent of all prescriptions of clarithromycin SR, and 6.4% of prescriptions of clarithromycin IR, were prescribed at inappropriate dosing intervals. Surprisingly, disconcordance between diagnoses and indications was observed in all prescriptions of clarithromycin SR which has not been approved for treating *H pylori* infection although off-label use for this purpose was reported in literature. On the contrary, only one prescription (0.9%) of clarithromycin IR was prescribed for unapproved indication (i.e. gastro-oesophageal reflux disease). 1.4% of prescriptions for chronic gastritis or peptic ulcer treatment were irrational in that clarithromycin was not co-prescribed with gastric acid inhibitors. Clinical significant CYP3A based drug interactions with clarithromycin were identified.

CONCLUSION: There is a great scope to improve the quality of clarithromycin prescribing in patients with gastrointestinal disease, especially with regard to administration schedule, concordance between indications and diagnoses and management of drug interactions.

Key words: Clarithromycin; Drug utilization; Prescriptions; *Helicobacter pylori*; Gastrointestinal diseases; Drug administration schedule; Drug interactions; Polypharmacy

Peer reviewers: Tadashi Shimoyama, MD, Hirosaki University, 5 Zaifu-cho, Hirosaki 036-8562, Japan; Vasiliy Ivanovich Reshetnyak, Professor, Scientific Research Institute of General Reanimatology RAMS, 25-2, Petrovka Str. Moscow 107031, Russia

Zhou Q, Zhu LL, Yan XF, Pan WS, Zeng S. Drug utilization of clarithromycin for gastrointestinal disease treatment. *World J Gastroenterol* 2008; 14(39): 6065-6071 Available from: URL: http://www.wjgnet.com/1007-9327/14/6065.asp DOI: http://dx.doi.org/10.3748/wjg.14.6065

INTRODUCTION

Clarithromycin is a semi-synthetic macrolide antibiotic

that inhibits bacterial protein synthesis. It is more acid-stable, better absorbed, and is widely used as a component of anti-*Helicobacter pylori* (*H pylori*) regimens[1,2]. The oral clarithromycin formulations available on the market include immediate-release (IR) clarithromycin and sustained-release (SR) clarithromycin. The two formulations have different administration schedule, clinical indications and therapeutic cost. The SR clarithromycin has obvious advantages over the IR product when they are prescribed for the same indications. These advantages are as follows: (1) higher antimicrobial activity in that clarithromycin is a time-dependent antibiotic; (2) better tolerability, fewer gastrointestinal adverse reactions and reports of abnormal taste[3,4]; (3) bioequivalence between the SR (1000 mg *qd*) and IR (500 mg *bid*)[4]; and (4) enhanced medication compliance due to its convenience.

The patterns of combination use of clarithromycin for *H pylori* infection have not been reported in literature. Meanwhile, many patients with *H pylori* infection also suffer from other diseases and hence may receive polytherapy regimens, which may exert complex, significant drug interactions[5]. Up to now, drug utilization of clarithromycin for gastrointestinal disease treatment has not been available. Targeting inappropriate prescribing is one means of trying to reduce drug costs and promote rational use of drug. The aim of this two-month drug utilization study was to assess the extent and appropriateness of clarithromycin by examining prescribing practice for outpatients in a general hospital.

MATERIALS AND METHODS

The setting of this study is a 2200-bed general hospital in Zhejiang Province, China. The pharmacy has two products of clarithromycin [Klaci® (clarithromycin IR tablets, Abbott S.P.A.) and Nuobang® (clarithromycin SR tablets, Jiangsu Hengrui Medicine Co., Ltd, China)]. Each tablet of BIAXIN® contains 250 mg of clarithromycin. Each tablet of Nuobang® contains 500 mg of clarithromycin. Prescribing information for these products along with BIAXIN® XL Filmtab® (clarithromycin SR tablets, Abbott S.P.A.) were referenced[6]. Relevant literature was identified by performing Pubmed searches until the end of 2007. A structured pro forma was used to perform a survey of electronic prescriptions containing IR or SR product for outpatients with alimentary disease covering the period from December 2007 to January 2008. The pro forma included details of the patient's age and sex, indication for clarithromycin therapy, the type of clarithromycin product prescribed, the dose and duration of therapy and details of other prescribed medications. Suitability of the prescription was audited retrospectively.

Differences between patient groups were tested for statistical significance using χ^2 analysis. A P-value < 0.05 was considered significant.

RESULTS

Over a two-month period clarithromycin SR was prescribed for 949 patients whereas clarithromycin IR product was prescribed for 197 patients (Table1). With respect to use for alimentary disease treatment by gastroenterologists, 164 patients (17.3%) were on SR product compared to 110 patients (55.8%) on IR product ($P < 0.05$). The mean age of these patients was 45.9 years (range: 18-82 years). The number of male patients was equal to that of female patients. Among prescriptions for anti-*H pylori* therapy, triple therapy take the dominant position (91.8%), followed by quadruple therapy (4.3%) and dual therapy (3.9%).

Table 1 Details of prescriptions of the two clarithromycin products

	Clarithromycin	
	Sustained-release	Immediate release
Total number of prescriptions	949	197
Number of prescriptions for alimentary disease	164	110
Mean age (range) yr	45.9 (18-82)	
Male:Female	137:137	
Number of prescriptions for anti-*H pylori* therapy	159	96
Triple therapy	151	83
PPI/Clarithromycin/Amoxicillin	43	58
PPI/Clarithromycin/Furazolidone	44	21
PPI/Clarithromycin/Levofloxacin	63	1
PPI/Clarithromycin/Metronidazole	1	1
PPI/Clarithromycin/Tinidazole	0	2
Quadruple therapy	5	6
PPI/Bismuth/Amoxicillin/Clarithromycin	0	2
PPI/Bismuth/ Furazolidone/Clarithromycin	1	3
PPI/Bismuth/Levofloxacin/Clarithromycin	4	1
Dual therapy	3	7
Clarithromycin/PPI	2	7
Clarithromycin/Ranitidine bismuth citrate	1	0

PPI: Proton pump inhibitor.

Administration schedule

Post-meal dosing of clarithromycin was specified in all investigated electronic prescriptions. According to the prescribing information, Nuobang® should be taken with food whereas Klaci® may be given irrespective of food intake. Thus, Nuobang® in all prescriptions was administered at inappropriate time.

Eighty-two prescriptions of Nuobang® (50%) were prescribed twice daily, which was inconsistent with the once-daily dosing method according to its prescribing information. Klaci® was given twice daily according to all prescriptions, which met the requirements for triple therapy. However, dual therapy requires clarithromycin IR 500 mg to be given three times daily[6]. Thus, 7 prescriptions of dual therapy containing clarithromycin IR tablet (500 mg *bid*) and PPI were identified as irrational.

Diagnoses of patients on clarithromycin-based therapy

For patients with alimentary disease receiving clarithromycin-based therapy, diagnoses were summarized in Table 2. The diagnoses were various. Combining the results of upper gastrointestinal

Table 2 Diagnoses of patients on clarithromycin-based therapy

Diagnoses	Clarithromycin	
	SR	IR
Chronic gastritis	90	87
Peptic ulcer	40	5
Duodenal ulcer	9	5
Gastro-oesophageal reflux disease	5	1
Chronic gastritis, *H pylori* infection	4	3
Gastric ulcer	1	4
Chronic gastritis, Gastric ulcer	2	1
Gastro-oesophageal reflux disease, Gastric ulcer	1	
H pylori infection	2	
Chronic gastritis, Peptic ulcer	1	3
Peptic ulcer, *H pylori* infection		1
Mesenteric lymphadenitis	7	
Abdominal pain of unknown origin	2	

SR: Sustained-release; IR: Immediate release.

Table 3 Concomitant drugs used in clarithromycin-based triple therapy

Comedicated drugs	Clarithromycin	
	SR	IR
PPI		
Omeprazole	17	6
Lansoprazole	68	6
Pantoprazole	30	66
Esomeprazole magnesium	15	11
Rebeprazole	23	21
Antibiotics		
Amoxicillin	38	63
Furazolidone	47	24
Levofloxacin	67	2
Metronidazole	1	1
Tinidazole	0	13
Amoxicillin/clavulanate potassium	5	0

SR: Sustained-release; IR: Immediate release.

Table 4 CYP3A based clinical significant drug interactions with clarithromycin

Concurrent medications primarily metabolized by CYP3A	Clarithromycin		References
	SR	IR	
Alprazolam and zolpidem	1		[6,7]
Midazolam	1		[6,8]
Amlodipine	1	2	[9]
Levoamlodipine		1	
Nifedipine		1	[10]
Carbamazepine		1	[11]
Nifedipine, clopidogrel and atorvastatin		1	[10,12-14]
Amlodipine and ergoloid mesylate sustained release capsules		1	[9,15]
Prednisone		1	[16]

SR: Sustained-release; IR: Immediate release.

endoscopy or ^{13}C-urea breath test, concordance between diagnoses and indications were examined. Except for patients with mesenteric lymphadenitis or abdominal pain of unknown origin, 90.6% of other patients test positive for *H pylori* infection prior to initiation of anti-*H pylori* regimen. Surprisingly, disconcordance between diagnoses and indications was observed in all prescriptions of clarithromycin SR. On the contrary, only one prescription (0.9%) of clarithromycin IR was prescribed for unapproved indication (i.e. gastro-oesophageal reflux disease).

Drug interactions

Concomitant PPIs and anti-*H pylori* agents used in clarithromycin-based triple therapy were listed in Table 3. The PPIs included omeprazole, lansoprazole, pantoprazole, esomeprazole magnesium and rebeprazole. The antibiotics co-presribed with clarithromycin included amoxicillin, furazolidone, levofloxacin, metronidazole, tinidazole and amoxicillin/clavulanate potassium. Amoxicillin was the most frequently co-prescribed antibiotic. Furazolidone and levofloxacin were used more widely than metronidazole or tinidazole. The CYP3A dependent clinical significant drug interactions with clarithromycin in this survey were summarized in Table 4.

Clarithromycin plays its role of anti-*H pylori* only under the circumstance of pH more than 4.0 and thus it usually needs concomitant use of anti-gastric-secretion drugs. However, 4 prescriptions for chronic gastritis or peptic ulcer treatment did not contain gastric acid inhibitors, and thus were judged as irrational.

DISCUSSION

Administration schedule

Food has no significant effects on pharmacokinetics of IR clarithromycin and thus the product may be given irrespective of food intake. With regard to Nuobang®, administration under fasting conditions is associated with approximately 30% lower area under the plasma concentration-time curve (*AUC*) for clarithromycin relative to administration with food. Therefore, it should be taken with food to maximize bioavailability. Physicians and pharmacists should pay attention to this biopharmaceutical requirement and strengthen patient education.

Compared to the triple therapy, the dual therapy has a lower eradication rate of *H pylori*. Moreover, regimens which contain clarithromycin as the single antibiotic are more likely to be associated with the development of clarithromycin resistance among patients who fail therapy. When the IR clarithromycin tablet is combined with PPI as dual therapy, the dose needs to be tailored to 500 mg three times daily[6].

The SR clarithromycin has obvious advantages over the IR product when they are prescribed for the same indications[3,4]. However, the novelty of the SR product and its administration of only once a day would decrease the benefit for patients and their compliance if given twice daily as detected in most prescriptions.

Concordance between indications and diagnoses

Chronic gastritis is an inflammation of the lining of the stomach that occurs gradually and persists for a prolonged time. It can be classified based on the underlying etiologic agent (e.g. *H pylori*, bile reflux,

nonsteroidal anti-inflammatory drugs, autoimmunity, allergic response) and the histopathological pattern. Diagnosis of chronic gastritis is broad and disconcordant with indications described in the package insert of clarithromycin. It should be further specified if patients test positive for *H pylori* (i.e. *H pylori*-associated chronic gastritis instead of chronic gastritis) and the rationale also applies to diagnoses of gastric ulcer, peptic ulcer and duodenal ulcer.

Gastro-oesophageal reflux disease (GERD) is an unapproved indication for IR or SR clarithromycin. Although a significant proportion of patients with GERD have *H pylori* infection, it is unclear whether or not *H pylori* should be treated. Eradication therapy is currently not recommended for most of GERD patients with *H pylori* infection[1,2]. Relief of abdominal pain of unknown origin was also an unapproved indication for clarithromycin-based therapy. Seven prescriptions for mesenteric lymphadenitis treatment included monotherapy with clarithromycin (n = 1), dual therapy with clarithromycin-levofloxacin (n = 3), clarithromycin-cefdinir (n = 1), clarithromycin-amoxicillin/clavulanate potassium (n = 2). Given the predominance of *Y. enterocolitica* in mesenteric lymphadenitis infection, initial oral antibiotic selection from third-generation cephalosporins, broad spectrum penicillins, fluoroquinolones and doxycycline may be considered. Recently, association of mesenteric lymphadenitis with mycoplasma was revealed by Tao *et al*[17]. Among 108 patients with mesenteric lymphadenitis in that study, 36 patients (33%) were Mycoplasma-IgM positive. The switch to macrolide azithromycin provided a benefit for patients with an unsatisfactory response to third-generation cephalosporins or broad spectrum penicillins. In our survey, follow-up indicated that the combination of macrolide clarithromycin with levofloxacin, amoxicillin/clavulanate potassium or cefdinir showed satisfactory results in patients suffering from mesenteric lymphadenitis.

Clarithromycin IR based triple therapy or dual therapy is indicated for the treatment of patients with *H pylori* infection. However, the efficacy and safety of clarithromycin SR treatment for *H pylori* infection have not been established, as indicated in the prescribing information for Biaxin Xl Filmtab®. There have been three studies on clinical efficacy of clarithromycin SR -based triple therapy to cure *H pylori* infection. Coelho *et al*[18] observed that the combination of lansoprazole 30 mg, clarithromycin SR 500 mg and furazolidone 400 mg, once daily for 7 d, was inexpensive, safe and an effective alternative for anti-*H pylori* therapy in family members of gastric cancer patients. Chu *et al*[19] proved that one-week once-daily course of lansoprazole 30 mg, clarithromycin SR 500 mg and metronidazole 800 mg was a safe, well-tolerated, easy to comply with, and efficacious treatment for *H pylori* infection. A randomized controlled trial study by Liou *et al*[20] provided the direct evidence that clarithromycin SR 1000 mg once daily can be used as an alternative to clarithromycin IR 500 mg twice daily for the treatment of *H pylori*-associated peptic ulcer disease. In that study, 161 patients with *H pylori*-associated peptic ulcer were randomized to receive one-week triple therapy with either clarithromycin SR 1000 mg once daily or clarithromycin IR 500 mg twice daily combination with amoxicillin 1000 mg twice daily and esomeprazole 40 mg once daily. The eradication rates were comparable in the two groups. Further clinical trials with a larger sample size are required to establish the efficacy and safety of clarithromycin SR. Effective communication between patients and gastroenterologists are rather necessary prior to initiation of off-label use of clarithromycin SR.

Drug interactions

The combination of clarithromycin with omeprazole has a synergic effect. The C_{max}, AUC0-24, and $T_{1/2}$ derived from omeprazole increased by 30%, 89%, and 34%, respectively by the concomitant administration. The mean 24-h gastric pH value was 5.2 when omeprazole was administered alone and 5.7 when co-administered with clarithromycin[6]. On the other hand, by concomitant administration of omeprazole, clarithromycin concentrations in the gastric tissue and mucus increased (e.g. clarithromycin mucus concentrations 2 h after application increased by about 9-fold)[21]. Simultaneous administration of lansoprazole, amoxicillin and clarithromycin increases the serum concentrations of lansoprazole and the active 14-OH-clarithromycin metabolite significantly[22]. Compared to treatment with esomeprazole alone, the mean steady state AUC and C_{max} of esomeprazole increased by 70% and 18%, respectively, during triple therapy (esomeprazole magnesium 40 mg *qd*, clarithromycin 500 mg *bid* and amoxicillin 1000 mg *bid* for 7 days)[23]. The AUC and C_{max} of rabeprazole and 14-hydroxyclarithromycin (active metabolite of clarithromycin) increased, although the AUC and C_{max} for clarithromycin were not different following combined administration consisting of rabeprazole, amoxicillin and clarithromycin compared to values following single administration[24]. Although there is no significant pharmacokinetic interaction between clarithromycin and pantoprazole, clarithromycin has a better effect in *H pylori* treatment when pantoprazole is used concomitantly[25].

Clarithromycin is a potent inhibitor of CYP3A4 and P-gp. Concomitant administration of clarithromycin and any of the following CYP3A4 substrates is contraindicated: cisapride, pimozide, astemizole, terfenadine, and ergotamine or dihydroergotamine, as described in standard information sources. In this survey, such prescriptions were not found. Coadministration of clarithromycin and a drug primarily metabolized by CYP3A may be associated with elevations in drug concentrations that could increase or prolong both the therapeutic and adverse effects of the concomitant drug. Dosage adjustments may be considered, and when possible, plasma concentrations of drugs primarily metabolized by CYP3A should be monitored closely in patients concurrently receiving clarithromycin.

Triazolobenziodidiazepines (e.g. triazolam

and alprazolam) and related benzodiazepines (e.g. midazolam) have been observed of CYP3A based drug interactions with erythromycin products and/or with clarithromycin in postmarketing experience. For example, intestinal and hepatic CYP3A inhibition by clarithromycin can significantly reduce the clearance of midazolam, resulting in an increase in the *AUC* of midazolam by 8-fold following oral dose in the elderly[8]. Zolpidem is extensively metabolized, mainly by CYP3A4[7]. Thus, a prescription containing alprazolam, zolpidem and clarithromycin has a high risk for excessive sedation (Table 4). Pharmacotherapy monitoring and dosage adjustment for these sedative drugs should be implemented accordingly.

Clarithromycin may increase the levels/effects of amlodipine[9]. Levoamlodipine is an eutomer of amlodipine and the first enantiomerically pure dihydropyridine calcium channel blockers. Levoamlodipine is also mainly metabolized by CYP3A4, so its levels/effects may also be affected by clarithromycin. A case of vasodilatory shock possibly resulting from a clarithromycin-nifedipine interaction was reported by Gerónimo-Pardo *et al*[10]. A potentially significant pharmacokinetic interaction between clarithromycin and carbamazepine was identified in two patients with long-standing epilepsy who were given omeprazole/clarithromycin therapy for *H pylori* gastritis[9]. Serum carbamazepine levels were augmented by clarithromycin and returned to the therapeutic range following cessation of clarithromycin therapy. Empirically in such cases carbamazepine dose need to be tailored by 30% to 50%.

Clarithromycin did have a significant effect on atorvastatin pharmacokinetic parameters. When coadministered, clarithromycin raised atorvastatin *AUC* by 82% and C_{max} by 56%. Hence, clarithromycin should be avoided in patients taking atorvastatin and similarly metabolized HMG-CoA inhibitors[12]. Sipe *et al*[13] reported a case of rhabdomyolysis causing AV blockade due to possible atorvastatin, esomeprazole, and clarithromycin interaction. The antiplatelet effects of the prodrug clopidogrel can be reduced by concomitant administration of erythromycin or troleandomycin. The proposed mechanism is inhibition of CYP3A4 activity, which is responsible for the conversion of clopidogrel to its active metabolite. Clarithromycin also inhibits CYP3A4 activity and is also expected to affect clopidogrel metabolism[14]. Until more information is available, monitoring for altered efficacy of clopidogrel may be advisable if clarithromycin is co-administered with clopidogrel.

In a combination of clarithromycin, nifedipine, clopidogrel and atorvastatin (Table 4), at least 4 clinical significant pharmacokinetic interactions are involved, e.g. clarithromycin-nifedipine, clarithromycin-clopidogrel, clarithromycin-atorvastatin and atorvastatin-clopidogrel[26]. Such a prescription with high risk of adverse drug interactions is irrational. Considering the short course of clarithromycin therapy, close monitoring and proper dose adjustment may be more practicable than to switch to alternatives not mainly metabolized by CYP3A4.

Clarithromycin may increase levels of ergoloid mesylate by inhibiting CYP3A4 metabolism, resulting in toxicity (ischemia, vasospasm) and the combined use is contraindicated[15]. So the combination therapy of clarithromycin with amlodipine and ergoloid mesylate at conventional dosage is irrational. A case of mania due to prednisone-clarithromycin interaction was reported by Finkenbine *et al*[16], suggesting that pharmacotherapy monitoring should be performed during the concurrent therapy.

Triple therapy with a PPI, clarithromycin and either amoxicillin or metronidazole is the first-line treatment regimen to eradicate *H pylori* infection[1,2,27]. Significant differences are observed in the prevalence of metronidazole resistance between developed and developing countries[28-30]. High levels of resistance to metronidazole mainly relates to the wide application in parasite infection, dental infection and gynecological diseases in developing countries. Antimicrobial susceptibility tests performed in Zhejiang Province of China indicated that the antibiotic resistance rate increased perceptibly during the period of 2003-2007[31,32]. Among six antibiotics (metronidazole, amoxicilin, gentamycin, levofloxacin, furazolidone and clarithromycin), the rate of resistance to metronidazole (99.32%) appeared to be the highest and the levofloxacin resistance rate (0.51%) was the lowest. Amoxicillin rarely induces resistance[33]. Fluoroquinolones are active against *H pylori* in vitro and have a synergistic effect with PPIs[34]. Strains resistant to furazolidone are rare. Furthermore, there is no cross-resistance to metronidazole and furazolidone is effective in populations with a high prevalence of metronidazole resistance[35]. The resistance status may explain the pattern of antibiotic use in this hospital, i.e. furazolidone and levofloxacin are used more widely than are metronidazole or tinidazole. Guo *et al*[28] reported that *H pylori* eradication rates were significantly different in patients receiving OAC (omeprazole/amoxicillin/clarithromycin) and OFC (omeprazole/furazolidone/clarithromycin) compared to those receiving OMC (omeprazole/metronidazole/clarithromycin). The eradication rate for *H pylori* infection was 90.3%, 90.9% and 70.9% in OAC, OFC and OMC groups, respectively. Based on these results, one-week of triple therapy with OAC or OFC were recommended for Chinese patients with duodenal ulcers and chronic gastritis. Since furazolidone is cheap and the *H pylori* eradication rate is high, OFC regimen is recommended to be one of choices for *H pylori* eradication.

PPI-based double combinations were clearly inferior to triple regimens, which is in accordance with the evidence-based data and they are not recommended in the first-line treatment. However, concurrent therapy of ranitidine bismuth citrate and clarithromycin have a similar efficacy compared to the triple regimens[36-38]. Thus, the prescription of a combination of clarithromycin and ranitidine bismuth citrate in our survey is rational. A 7 d

quadruple therapy based on PPI, bismuth, tetracycline and metronidazole is the more frequently accepted[1,2]. Our survey found that some patients received quadruple therapy regimens containing PPI, bismuth, clarithromycin, and one of antibiotics including amoxicillin, furazolidone or levofloxacin.

In conclusions, a retrospective utilization study of clarithromycin for gastrointestinal disease treatment was conducted. There is a great scope to improve the quality of clarithromycin prescribing, especially with regard to administration schedule, concordance between indications and diagnoses and management of drug interactions.

COMMENTS

Background

The oral clarithromycin formulations available on the market include immediate-release (IR) clarithromycin and sustained-release (SR) clarithromycin. Due to difference in pharmaceutical forms, the IR and SR formulations have different administration schedule, clinical indications and therapeutic cost. Meanwhile, the patterns of combination use of clarithromycin for *Helicobacter pylori* (*H pylori*) infection have not been reported in literature. Many patients with *H pylori* infection also suffer from other diseases and hence they may receive polytherapy regimens, which may exert complex, significant drug interactions if clarithromycin is used. In order to promote its rational use in gastrointestinal disease treatment, it is essential to assess the extent and appropriateness of clarithromycin by examining prescribing practice.

Research frontiers

Drug utilization studies can provide useful information to improve the appropriate and effective use of pharmaceuticals in populations. In recent years many such studies have been performed to monitor prescribing patterns and assess adherence to standard therapeutic guidelines in clinical practice.

Innovations and breakthroughs

This is the first drug utilization study of clarithromycin for gastrointestinal disease treatment and the article critically compares the prescribing patterns of clarithromycin with different pharmaceutical forms.

Applications

The significance of this article is: (1) it provides insights into the aspects of drug use and prescribing pattern; (2) it helps to improve the quality of clarithromycin prescribing, especially with regard to administration schedule, concordance between indications and diagnoses and management of drug interactions; (3) it helps doctors to attach equal importance to other medicines for gastrointestinal disease treatment and finally promote rational drug use in clinical practice.

Terminology

Drug utilization: The study to describe the extent, nature and determinants of drug exposure, consider clinical appropriateness and cost effectiveness, and facilitate rational use of drugs in populations. Drug administration schedule: time schedule for administration of a drug in order to achieve optimum effectiveness and convenience. Drug interactions: The action of a drug that may affect the activity, metabolism, or toxicity of another drug. Polypharmacy: The use of multiple drugs administered to the same patient, most commonly seen in elderly patients.

Peer review

This retrospective utilization study is well designed. It is of particular interest to the practical medicine and can improve the quality of clarithromycin prescribing in patients with gastrointestinal disease.

REFERENCES

1 **Alberta Medical Association**. Guideline for treatment of Helicobacter Pylori infection in adults, 2007. Available from: URL: http://www.topalbertadoctors.org/cpgs/hpylori.html

2 **Chey WD**, Wong BC. American College of Gastroenterology guideline on the management of Helicobacter pylori infection. *Am J Gastroenterol* 2007; **102**: 1808-1825

3 **Gotfried M**, Notario G, Spiller J, Palmer R, Busman T. Comparative efficacy of once daily, 5-day short-course therapy with clarithromycin extended-release versus twice daily, 7-day therapy with clarithromycin immediate-release in acute bacterial exacerbation of chronic bronchitis. *Curr Med Res Opin* 2005; **21**: 245-254

4 **Guay DR**, Gustavson LE, Devcich KJ, Zhang J, Cao G, Olson CA. Pharmacokinetics and tolerability of extended-release clarithromycin. *Clin Ther* 2001; **23**: 566-577

5 **Zhou Q**, Yan XF, Zhang ZM, Pan WS, Zeng S. Rational prescription of drugs within similar therapeutic or structural class for gastrointestinal disease treatment: drug metabolism and its related interactions. *World J Gastroenterol* 2007; **13**: 5618-5628

6 **Abbott Laboratories, USA**. BIAXIN® prescribing information, 2005. Available from: URL: http://www.fda.gov/medwatch/SAFETY/2005/Oct_PI/Biaxin_PI.pdf

7 **A.D.A.M., Inc. Zolpidem information**. Available from: URL: http://www.umm.edu/ altmed/drugs/zolpidem-135400.htm

8 **Quinney SK**, Haehner BD, Rhoades MB, Lin Z, Gorski JC, Hall SD. Interaction between midazolam and clarithromycin in the elderly. *Br J Clin Pharmacol* 2008; **65**: 98-109

9 **A.D.A.M., Inc. Amlodipine information**. Available from: URL: http://www.umm.edu/altmed/drugs/amlodipine-005950.htm

10 **Gerónimo-Pardo M**, Cuartero-del-Pozo AB, Jiménez-Vizuete JM, Cortiñas-Sáez M, Peyró-García R. Clarithromycin-nifedipine interaction as possible cause of vasodilatory shock. *Ann Pharmacother* 2005; **39**: 538-542

11 **Metz DC**, Getz HD. Helicobacter pylori gastritis therapy with omeprazole and clarithromycin increases serum carbamazepine levels. *Dig Dis Sci* 1995; **40**: 912-915

12 **Amsden GW**, Kuye O, Wei GC. A study of the interaction potential of azithromycin and clarithromycin with atorvastatin in healthy volunteers. *J Clin Pharmacol* 2002; **42**: 444-449

13 **Sipe BE**, Jones RJ, Bokhart GH. Rhabdomyolysis causing AV blockade due to possible atorvastatin, esomeprazole, and clarithromycin interaction. *Ann Pharmacother* 2003; **37**: 808-811

14 **A.D.A.M., Inc. Clopidogrel information**. Available from: URL: http://www.umm.edu/altmed/drugs/clopidogrel-030450.htm

15 **A.D.A.M., Inc. Ergoloid mesylates information**. Available from: URL: http://www.umm.edu/altmed/drugs/ergoloid-mesylates-048700.htm

16 **Finkenbine RD**, Frye MD. Case of psychosis due to prednisone-clarithromycin interaction. *Gen Hosp Psychiatry* 1998; **20**: 325-326

17 **Tao RH**. Association of mycoplasma infection with clinical efficacy in 108 patients with mesenteric lymphadenitis. *Jiangxi Yiyao* 2007; **42**: 440-441

18 **Coelho LG**, Martins GM, Passos MC, Bueno ML, Sanches BS, Lopes LG, Miranda CH, Castro LP. Once-daily, low-cost, highly effective Helicobacter pylori treatment to family members of gastric cancer patients. *Aliment Pharmacol Ther* 2003; **17**: 131-136

19 **Chu KM**, Kwok KF, Law SY, Wong J. One-week once-daily triple therapy for Helicobacter pylori--a pilot study. *Hepatogastroenterology* 2000; **47**: 1624-1626

20 **Liou JM**, Chen CY, Wu MS, Hsu MT, Fang CC, Lin YL, Chang MC, Lin JT, Wang HP. Comparative study of modified-release clarithromycin and immediate-release clarithromycin in the treatment of Helicobacter pylori-associated peptic ulcer disease. *Hepatogastroenterology* 2006; **53**: 792-796

21 **Gustavson LE**, Kaiser JF, Edmonds AL, Locke CS, DeBartolo ML, Schneck DW. Effect of omeprazole on concentrations of clarithromycin in plasma and gastric tissue at steady state. *Antimicrob Agents Chemother* 1995; **39**: 2078-2083

22 **Mainz D**, Borner K, Koeppe P, Kotwas J, Lode H. Pharmacokinetics of lansoprazole, amoxicillin and

clarithromycin after simultaneous and single administration. *J Antimicrob Chemother* 2002; **50**: 699-706
23 **AstraZeneca, USA**. NEXIUM prescribing information, 2006. Available from: URL: http://www.fda.gov/cder/foi/label/2006/021153s022lbl.pdf
24 **RxList Inc. Aciphex information**. Available from: URL: http://www.rxlist.com/cgi/generic/aciphex.htm
25 **Calabresi L**, Pazzucconi F, Ferrara S, Di Paolo A, Tacca MD, Sirtori C. Pharmacokinetic interactions between omeprazole/pantoprazole and clarithromycin in health volunteers. *Pharmacol Res* 2004; **49**: 493-499
26 **Lau WC**, Waskell LA, Watkins PB, Neer CJ, Horowitz K, Hopp AS, Tait AR, Carville DG, Guyer KE, Bates ER. Atorvastatin reduces the ability of clopidogrel to inhibit platelet aggregation: a new drug-drug interaction. *Circulation* 2003; **107**: 32-37
27 **Cianci R**, Montalto M, Pandolfi F, Gasbarrini GB, Cammarota G. Third-line rescue therapy for Helicobacter pylori infection. *World J Gastroenterol* 2006; 12: 2313-2319
28 **Guo CY**, Wu YB, Liu HL, Wu JY, Zhong MZ. Clinical evaluation of four one-week triple therapy regimens in eradicating Helicobacter pylori infection. *World J Gastroenterol* 2004; **10**: 747-749
29 **Kumala W**, Rani A. Patterns of Helicobacter pylori isolate resistance to fluoroquinolones, amoxicillin, clarithromycin and metronidazoles. *Southeast Asian J Trop Med Public Health* 2006; **37**: 970-974
30 **Malfertheiner P**, Megraud F, O'Morain C, Hungin AP, Jones R, Axon A, Graham DY, Tytgat G. Current concepts in the management of Helicobacter pylori infection--the Maastricht 2-2000 Consensus Report. *Aliment Pharmacol Ther* 2002; **16**: 167-180
31 **Shi JM**, Li WP. Analysis of the antibiotic resistance rate of Helicobacter Pylori. *Yixue Yanjiu Zazhi* 2007; **36**: 98-100
32 **Dai N**, Zhou G, Yan J. [Correlation of rdxA gene mutation and metronidazole resistance of Helicobacter pylori] *Zhejiangdaxue Xuebao Yixueban* 2003; **32**: 37-40
33 **Megraud F**. H pylori antibiotic resistance: prevalence, importance, and advances in testing. *Gut* 2004; **53**: 1374-1384
34 **Tanaka M**, Isogai E, Isogai H, Hayashi S, Hirose K, Kimura K, Sugiyama T, Sato K. Synergic effect of quinolone antibacterial agents and proton pump inhibitors on Helicobacter pylori. *J Antimicrob Chemother* 2002; **49**: 1039-1040
35 **Kwon DH**, Lee M, Kim JJ, Kim JG, El-Zaatari FA, Osato MS, Graham DY. Furazolidone- and nitrofurantoin-resistant Helicobacter pylori: prevalence and role of genes involved in metronidazole resistance. *Antimicrob Agents Chemother* 2001; **45**: 306-308
36 **Bardhan KD**, Dallaire C, Eisold H, Duggan AE. Ranitidine bismuth citrate with clarithromycin for the treatment of duodenal ulcer. *Gut* 1997; **41**: 181-186
37 **Buzás GM**, Józan J. First-line eradication of H pylori infection in Europe: a meta-analysis based on congress abstracts, 1997-2004. *World J Gastroenterol* 2006; **12**: 5311-5319
38 **Mesquita MA**, Lorena SL, Almeida JR, Montes CG, Guerrazzi F, Campos LT, Zeitune JM. One-week dual therapy with ranitidine bismuth citrate and clarithromycin for the treatment of Helicobacter pylori infection in Brazilian patients with peptic ulcer. *World J Gastroenterol* 2005; **11**: 3566-3569

S- Editor Tian L **L- Editor** Kremer M **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6072

World J Gastroenterol 2008 October 21; 14(39): 6072-6077
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Distinct expression patterns in hepatitis B virus- and hepatitis C virus-infected hepatocellular carcinoma

Chun-Feng Lee, Zhi-Qiang Ling, Ting Zhao, Kuan-Rong Lee

Chun-Feng Lee, Kuan-Rong Lee, Institute of Molecular Medicine, National Tsing Hua University, 101, Section 2, Kuang-Fu Road, Hsinchu, Taiwan 30013, China
Zhi-Qiang Ling, Zhejiang Cancer Research Institute, 38 Guangji Road Banshanqiao, Hangzhou 310022, Zhejiang Province, China
Ting Zhao, Department of surgery, Zhejiang Provincial People Hospital, 158 Shangtang Road, Hangzhou 310014, Zhejiang Province, China
Author contributions: Lee CF performed most of the experiments, participated in most of the data analysis and drafted the manuscript; Lee KR was the leader of the project, who conceived the study, designed the research, guided the experiments and the data analyses, and suggested revisions in the manuscript; Ling ZQ carried out cDNA microarray, real-time RT-PCR experiments and provided critical comments and suggested revisions in the manuscript; Zhao T collected and prepared hepatocellular carcinoma samples, and participated in some experiments; All authors read and approved the final manuscript.
Correspondence to: Kuan-Rong Lee, Institute of Molecular Medicine, National Tsing Hua University, 101, Section 2, Kuang-Fu Road, Hsinchu, Taiwan 30013, China. krlee@mx.nthu.edu.tw
Telephone: +886-3-574-2755 Fax: +886-3-571-5934
Received: September 1, 2008 Revised: September 8, 2008
Accepted: September 15, 2008
Published online: October 21, 2008

Abstract

AIM: To identify biomarkers indicating virus-specific hepatocarcinogenic process, differential mRNA expression in 32 patients with hepatitis B virus (HBV)-/hepatitis C virus (HCV)-associated hepatocellular carcinoma (HCC) were investigated by means of cDNA microarrays comprising of 886 genes.

METHODS: Thirty two HCC patients were divided into two groups based on viral markers: hepatitis B virus positive and HCV positive. The expression profiles of 32 pairs of specimens (tumorous and surrounding non-tumorous liver tissues), consisting of 886 genes were analyzed.

RESULTS: Seven up-regulated genes in HBV-associated HCC comprised genes involved in protein synthesis (*RPS5*), cytoskeletal organization (*KRT8*), apoptosis related genes (*CFLAR*), transport (*ATP5F1*), cell membrane receptor related genes (*IGFBP2*), signal transduction or transcription related genes (*MAP3K5*), and metastasis-related genes (*MMP9*). The up-regulated genes in HCV-infected group included 4 genes: *VIM* (cell structure), *ACTB* (cell structure), *GAPD* (glycolysis) and *CD58* (cell adhesion). The expression patterns of the 11 genes, identified by cDNA microarray, were confirmed by quantitative RT-PCR in 32 specimens.

CONCLUSION: The patterns of all identified genes were classified based on the viral factor involved in HBV- and HCV-associated HCC. Our results strongly suggest that the pattern of gene expression in HCC is closely associated with the etiologic factor. The present study indicates that HBV and HCV cause hepatocarcinogenesis by different mechanisms, and provide novel tools for the diagnosis and treatment of HBV- and HCV-associated HCC.

Key words: Hepatocellular carcinoma; Hepatitis B virus; Hepatitis C virus-infected; cDNA microarray; Expression profiling

Peer reviewers: Peter Karayiannis, PhD, Associate Professor, Department of Medicine, Hepatology Section, St Mary's Hospital Campus, South Wharf Road, London W2 1NY, United Kingdom; Jose JG Marin, Professor, Head of the Departamento Physiology and Pharmacology, University of Salamanca, CIBERehd, Campus Miguel de Unamuno, ED-S09, Salamanca 37007, Spain

Lee CF, Ling ZQ, Zhao T, Lee KR. Distinct expression patterns in hepatitis B virus- and hepatitis C virus-infected hepatocellular carcinoma. *World J Gastroenterol* 2008; 14(39): 6072-6077 Available from: URL: http://www.wjgnet.com/1007-9327/14/6072.asp DOI: http://dx.doi.org/10.3748/wjg.14.6072

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide[1]. The major risk factors for HCC are chronic hepatitis resulting from infection with HBV and HCV, and exposure to various exogenous carcinogens, including aflatoxin B1[2]. Several studies have shown that the incidence of HCC has increased substantially in East Asia, including China, Korea and Japan[3,4]. More than 350 million people worldwide are known to be chronic carriers of HBV[5]. Moreover, the incidence of HCC is increasing in many countries in

parallel with the increase in chronic HCV infection[1,2]. Therefore, clarification of the genetic portrait of hepatocarcinogenesis caused by HBV or HCV infection may provide clues to help reduce the incidence of HCC, and establish effective treatments for HCC. However, the molecular nature of this association is poorly understood.

The phenotypic diversity of cancer is accompanied with a corresponding diversity in the gene expression patterns[6-10]. Honda *et al*[11] showed the presence of different gene expression profiles in the liver lesions of chronic hepatitis caused by HBV and HCV, and suggested that the molecular mechanisms responsible for the pathogenesis of HCC differ between HBV and HCV infections. In the present study, we investigated the gene expression patterns of 32 HCC samples, using cDNA microarrays containing 886 clones in order to gain additional insight into hepatocarcinogenesis or cancer progression related to HBV and HCV infections. The aim of the present study was to characterize the gene expression associated with HCC, with a view to better understand the molecular pathophysiology, which may lead to better methods of detection, diagnosis, and classification of HCC.

MATERIALS AND METHODS

Patient material

The Institutional Review Board on Medical Ethics, Zhejiang Provincial People Hospital (China), approved the method of tissue collection. The present study was conducted in the department of surgery, Zhejiang Provincial People Hospital, on 32 patients who underwent hepatectomy for sporadic HCC without preoperative radio- or chemotherapy. All of tissue samples were immediately frozen in liquid nitrogen, and stored at -80℃ until use. A total of 32 HCC samples from 15 lymph node negative and 17 lymph node positive cases were used (Table 1).

Table 1 Clincal data of patients with hepatocellular carcinoma

Case	Sex	Age	Hepatitis virus	Differentiated grade	TNM score
1	M	54	HBV	WD	T1N0M0
2	M	60	HCV	WD	T1N0M0
3	F	61	HBV	WD	T2N0M0
4	M	62	HBV	WD	T2N0M0
5	M	58	HBV	WD	T1N0M0
6	F	56	HCV	MD	T3N0M0
7	F	44	HBV	WD	T2N0M0
8	M	49	HCV	WD	T1N0M0
9	M	58	HBV	WD	T2N0M0
10	M	67	HCV	PD	T3N0M0
11	M	69	HBV	WD	T2N0M0
12	F	63	HCV	WD	T1N0M0
13	M	48	HCV	MD	T2N0M0
14	F	63	HBV	WD	T1N0M0
15	M	49	HCV	MD	T1N0M0
16	F	51	HBV	PD	T3N1M0
17	M	65	HCV	MD	T3N1M0
18	F	58	HBV	PD	T4N1M1
19	M	60	HBV	MD	T2N1M0
20	F	56	HCV	PD	T3N1M1
21	M	42	HCV	PD	T3N1M0
22	M	55	HBV	PD	T4N1M1
23	M	66	HBV	MD	T3N1M0
24	F	70	HCV	WD	T2N1M0
25	M	58	HBV	PD	T4N1M1
26	M	53	HCV	PD	T3N1M0
27	M	61	HBV	PD	T4N1M0
28	F	65	HBV	MD	T3N1M0
29	M	59	HCV	MD	T3N1M1
30	M	50	HBV	PD	T3N1M0
31	F	63	HCV	PD	T4N1M1
32	M	66	HCV	PD	T3N1M0

M: Male; F: Female; HBV: Hepatitis B virus infection; HCV: Hepatitis C virus infection; WD: Well differentiated HCC; MD: Moderately differentiated HCC; PD: Poorly differentiated HCC.

Laser microdissection

Eight μm-thick sections of the frozen tissue were cut at -20℃ and stained with HE. Under microscopic observation, parts of cancer cells nests in the invasive and intraductal components were microdissected, using the LM100 laser capture microdissection system (Arcturus Engineering, Mountain View, CA, USA). A 15 μm-diameter beam was used to capture the tumor cells and the corresponding non-cancerous liver tissues. The cell nests were transferred to a LCM transfer film (CapSure TF-100S transfer film carrier, 5 mm-diameter optical-grade transparent plastic; Arcturus Engineering).

RNA preparation and T7-based RNA amplification

Total RNA was isolated from the dissected specimens using Trizol reagent (Gibco BRL) and a modified acidic guanidinium phenol-chloroform method, following the manufacturer's recommendations. Total RNA was treated with DNase I for removal of genomic DNA, and the mRNA was purified using a poly(A) purification kit (Oligotex, Qiagen), according to the manufacturer's instructions. The quality of mRNA was assessed by $A_{260/280}$ ratios and the contamination of genomic DNA was checked using the PCR method. cDNA was synthesized with T7-oligo (dT) primer (Ambion) and Superscript II enzyme (Gibco BRL), as described in the instruction manual. cDNA was purified by cDNA clean-up column (DNA clear™ kit, Ambion). cRNA was generated by T7 MEGAscript™ kit (MEGAscript *in vitro* Transcription Kit, Ambion, AUSTIN, Tex), per the manufacturer's recommendations. Column purification of cRNA was performed with RNeasy kit (Qiagen), according to the manufacturer's protocol. The concentration and quality of cRNA were analyzed by GeneQuant pro RNA/DNA Calculator (Amersharmacia biotech).

Microarray hybridization and scanning

Human Cancer Chip version 4.0 (IntelliGene, TaKaRa) was used for these studies. This array was spotted on a glass slide with 886 cDNA fragments of human genes, which are composed of 588 human identified genes related to cancer, and 298 cDNA fragments prescreened by differential display method between cancer tissue and normal tissue. Three μg of cRNA from the tumor and

Table 2 Primers used to amplify cDNA at various genes in real time RT-PCR

Target gene	Objective	Forward primer sequence (5'-3')	Reverse primer sequence (3'-5')	Genebank accession no./Amplicon size
RPS5	qRT-PCR	GTATGCCGCCAAACGCTTC	CGCCTGTGAGCAGGTGTAT	NM_001009, 152 bp
KRT8	qRT-PCR	GGAGGCATCACCGCAGTTAC	GGTTGGCAATATCCTCGTACTGT	NM_002273, 637 bp
CFLAR	qRT-PCR	GACAGAGCTTCTTCGAGACAC	GCTCGGGCATACAGGCAAAT	AF009616, 116 bp
ATP5F1	qRT-PCR	ACTGGGCTTATCTTGTACGCT	GCAAAGTCTGCAACAAAGGGA	NM_001688, 131 bp
IGFBP2	qRT-PCR	GACAATGGCGATGACCACTCA	GCTCCTTCATACCCGACTTGA	NM_000597, 121 bp
MAP3K5	qRT-PCR	AAAAAGGCATTTGAATCTGAGCC	GCTTGAATGACTCTCATGTGGTC	NM_005923, 233 bp
MMP9	qRT-PCR	GGGACGCAGACATCGTCATC	TCGTCATCGTCGAAATGGGC	NM_004994, 139 bp
VIM	qRT-PCR	AGTCCACTGAGTACCGGAGAC	CATTTCACGCATCTGGCGTTC	AK093924, 98 bp
ACTB	qRT-PCR	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT	NM_001101, 250 bp
GAPD	qRT-PCR	CAACTGGTCGTGGACAACCAT	GCACGGACACTCACAATGTTC	AC002389, 260 bp
CD58	qRT-PCR	CTCATGGGATTGTCCTATGGAGC	GCTTGGGATACAGGTTGTCAAA	NM_001779, 154 bp

the matched normal tissue were labeled with Cy3-dUTP and Cy5-dUTP respectively (Amersham Pharmacia Biotech, Buckinghamshire, England), using a labeling kit (RNA Fluorescence Labeling Core kit, TaKaRa), according to the manufacturer's instructions. The labeled probe was purified by centrifugation in a spin column (Centrisep, Princeton Separations, Adelphia, NJ). Two separate probes were combined, and 2 μL of 5 × competitor containing Cot I (Gibco BRL), poly dA (Amersham Pharmaca Biotech), and tRNA (TaKaRa) were added. After addition of 50 μL of 100% ethanol and 2 μL of 3 mol/L sodium acetate (pH 5.2), the mixture was cooled at -80℃ for 30 min, followed by centrifugation at 15 000 *g* for 10 min, and pelleted down. For final probe preparation, the pellet was washed in 500 μL of 70% ethanol twice, and eluted in 10 μL hybridization buffer (6 × SSC, 0.2% SDS, 5 × Denhardt's solution, 0.1 mg/mL salmon sperm solution). The probe were denatured by heating for 2 min at 95℃, cooled at room temperature, and centrifuged at 15 000 *g* for 10 min (20-26℃). Supernatants were placed on the array and covered with a 22-mm × 22-mm glass coverslip. The coverslip was sealed with a glue, and the probes were incubated overnight at 65℃ for 16 h in a custom-made slide chamber with humidity maintained by underlying moist papers. After hybridization, the slides were washed in 2 × SSC with 0.1% SDS, 1 × SSC, and 0.05 × SSC, sequentially for 1 min each, and then spin dried. Hybridized arrays were scanned using a confocal laser-scanning microscope (Affymetrix 428 array scanner, Santa Clara, CA). Image analysis and quantification were performed with ImaGene 4.2 software (BioDiscovery), according to the manufacture's instructions.

Data processing

Each spot was defined by manual positioning of a grid of circles over the array image. For each fluorescent image, the average pixel intensity within each circle was determined, and a local background, outside of 3 pixel buffer range from the circle was computed for each spot. Net signal was determined by subtraction of the local background from the average intensity of each spot. Signal intensities between the two fluorescent images were normalized by the intensities of the house-keeping genes provided on the arrays. The fluorescence intensities of Cy5 (non-tumor) and Cy3 (tumor) for each target spot were adjusted so that the mean Cy3:Cy5 ratios of 32 housekeeping gene spots were equal to one. Because data derived from low signal intensities are less reliable, we first determined the cutoff values for signal intensities on each slide so that all of the filtered genes had greater S:N (signal to noise) ratios of Cy3 or Cy5 than three, and we excluded genes for further analysis when both Cy3 and Cy5 dyes gave signal intensities lower than the cutoff. To estimate the range of expression ratio within which the expression change could be considered as fluctuation in non-cancerous cells, we compared expression profiles of non-cancerous cells from 6 patients. Because 90% of expression ratios in non-cancerous cells fell within the range of 1.726 and 0.503, we categorized genes into three groups according to their expression ratios (Cy3:Cy5): up-regulated (ratio, 2.0); down-regulated (ratio 0.5); and unchanged expression (ratios, between 0.5 and 2.0); provided that signal counts of T (Cy3) and R (Cy5) were > 500. Genes with Cy3:Cy5 ratios > 2.0 or < 0.5 in more than 75% of the cases examined were defined as commonly up- or down-regulated genes, respectively.

Real-time reverse transcription PCR

LightCycler (Roche Diagnostics) technology was applied to confirm the data obtained by cDNA microarray. The primer sequences of 11 genes were obtained from the GDB Human Genome Database (http://www.gdb.org/gdb/) (Table 2). We used the same RNA from the dissected cells for the microarray analysis. First-strand cDNA was obtained by reverse transcription using a commercially available kit (first strand synthesis kit, Amersham). For each PCR, 2 μL (20 ng) first strand cDNA template, 50 pmol of each primer, 2.4 μL (3 mmol) $MgCl_2$, and 2 μL 10 × SYBR Green I (Roche Laboratories)were mixed in 20 μL of PCR mixture. The running protocol was programmed as follows. In the first step, initial denaturation, reaction mixture was incubated for 10 min at 95℃. In the second step, DNA was amplified for 45 cycles at 95℃ for 10 s, specific annealing temperature (the primer sequences dependent) for 0-10 s, and elongation at 72℃ for some seconds [amplicon (bp)/25 s]. Finally, the temperature was raised gradually (0.2℃/s) from the annealing temperature to 95℃ for the melting curve analysis. Twelve μL of PCR product were visualized by electrophoresis on 2% agarose gel stained with ethidium bromide.

The amount of gene expression was normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using Human GAPDH kit (GmbH Heidelberg, Heidelberg, Germany). The qRT-PCR analysis was carried out in triplicate for each cDNA sample, and the median values were used for the three experiments. Up- and down-regulation were defined as the median value > 2.0 and < 0.5, respectively.

Statistical analysis

Statistical analysis among mean values was performed on the association of lymph node metastasis with expression levels by applying non-parametric Kruskal-Wallis and Mann-Whitney *U* tests. Statistical significance was defined as a *P*-value of < 0.05. Differential expression between the groups of HBV-infected and HCV-infected HCC was considered significant, with $P < 0.05$.

RESULTS

Quality analysis of total RNA after LCM and cRNA after T7-based amplification

About 20 slides were prepared from each sample, and the target cells were captured with at least 1000 cells per slide. Consequently, we captured a total of approximately 25 000-30 000 tumor cells and normal cells for RNA extraction. The quality of total RNA extracted after LCM was assessed by A_{260}/A_{280} and electrophoresis. To be considered for microarray analysis, the RNA samples were required to pass quality control criteria, with integrity of 28S and 18S, and A_{260}/A_{280} greater than 2.0. Products of cDNA synthesis and cRNA were also checked by A_{260}/A_{280} and electrophoresis. The results showed that A_{260}/A_{280} of all the RNA samples met the quality control criteria for sample preparation. Clear image appearance of 28S and 18S of ribosomal RNA was seen under the electropherogram for each total RNA sample, which had to be intact and without degradation. RNA was subjected to two rounds of T7-based RNA amplification after removal of DNA contamination by RNase-free Dnase I treatment as described in the methods section. All RNA samples were successfully amplified by an estimated 250-fold, using T7 RNA polymerase. cDNA synthesis and cRNA showed satisfactory quality control criteria, with 1.5 kb < cDNA < 5.0 kb; 1.0 kb < cRNA < 4.5 kb); and A_{260}/A_{280} ratio of cDNA and cRNA greater than 2, respectively.

Identification of genes related to HBV-positive or HCV-positive status

After reverse transcription, each cDNA probe was labeled with Cy3- or Cy5-conjugated dyes and hybridized to microarray cDNAs with 886 genes. We compared the expression profiles of cancer cells and the corresponding normal cells in each case. A representative scatter plot of microarray analysis of carcinoma cells and non-cancerous tissue in case 20 (HCV-infected HCC) is shown in Figure 1. Up-regulated, down-regulated and unchanged genes are indicated by red, green and blue spots, respectively. We first arranged the relative expression of each gene (Cy3/Cy5 intensity ratio) into one of four categories: up-regulated (ratio > 2.0), down-regulated (ratio < 0.5), unchanged (ratio between 0.5 and 2.0), and not expressed (or slight expression but under the cutoff level for detection).

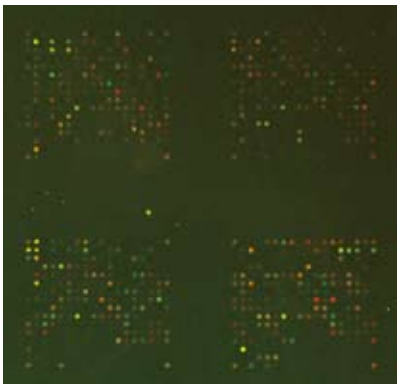


Figure 1 A representative of cDNA microarray expression pattern obtained from case 20. Up-, down-regulated and unchanged genes were indicated by red, green and blue spots, respectively.

To identify the genes related to HBV-positive and HCV-positive status, 32 patients were divided into two groups: HBV- associated HCC group in which HBV was positive in 17 patients, and HCV-associated HCC group in which HCV was positive in 15 patients (Table 1). When comparing gene expression profiles in the two groups, there were 7 genes that were commonly up-regulated, and expressed more than 2.09-fold in the HBV-infected group compared with in the HCV-infected group. On the other hand, 4 down-regulated genes in HBV-infected group correlated significantly with the HCV-infected group. Table 3 shows the list of differentially expressed genes and their respective category based on the GO (Gene Ontology) system and TreeView. The up-regulated genes in HBV-infected group were involved in protein synthesis (RPS5), cytoskeletal organization (KRT8), apoptosis related genes (CFLAR), transport (ATP5F1), cell membrane receptor related genes (IGFBP2), signal transduction or transcription related genes (MAP3K5), and metastasis-related genes (MMP9). The up-regulated genes in HCV-infected group included genes such as VIM (cell structure), ACTB (cell structure), GAPD (glycolysis) and CD58 (cell adhesion).

Verification of the genes related to HBV- and HCV-associated HCC using quantitative RT-PCR

To more quantitatively examine our data on hepatitis virus infection in HCC, we selected 7 up-regulated genes from the HBV-infected group, and 4 up-regulated genes from the HCV-infected group. The expression level of the selected genes was confirmed by quantitative RTPCR analysis in 32 patients. We used cDNA synthesized from 32 pair samples without amplification as template for real-time semiquantitative reverse transcription PCR. The results demonstrated that the samples obtained by means of T7-based amplification appropriately reflected the status of the original RNA in a proportional manner. The results of the DNA microarray were reproduced by reverse transcriptase PCR.

DISCUSSION

Genome-wide gene expression analysis of human cancer

Table 3 The 11 genes for which expression levels differed between HBV- and HCV-associated HCC

Gene name	Symbol[1]	Accession[2]	Fold change[3]	HBV:HCV[4]
7 genes up-regulated in HBV-associated HCC				
Ribosomal protein S5	RPS5	NM_001009	6.35	2.38
Keratin 8	KRT8	NM_002273	5.68	3.19
CASP8 and FADD-like apoptosis regulator	CFLAR	Y14039	2.86	2.09
ATP synthase, H+transporting, mitochondrial F0 complex, subunit b, isoform 1	ATP5F1	X60221	4.11	3.52
Insulin-like growth factor binding protein 2 (36 kDa)	IGFBP2	NM_000597	3.37	2.49
Mitogen-activated protein kinase kinase kinase 5	MAP3K5	NM_005923	3.76	2.33
Matrix metalloproteinase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type Ⅳ collagenase)	MMP9	NM_004994	7.43	3.74
4 genes up-regulated in HCV-associated HCC				
Vimentin	VIM	NM_003380	8.61	0.28
Actin-β	ACTB	X00351	4.13	0.37
Glyceraldehyde-3-phosphate_dehydrogenase	GAPD	NM_002046	5.27	0.29
CD58 antigen, (lymphocyte function-associated antigen 3)	CD58	NM_001779	4.68	0.31

[1]Symbol in LocusLink database; [2]GeneBank accession number; [3]Fold change, ratio of mean expression values in HBV- or HCV-associated cases (cancer cells *vs* non-cancerous cells); [4]HBV:HCV ratio of mean expression values (HBV positive cases to HCV positive cases).

may provide important clues in understanding HCC oncogenesis and may lead to improvement in predicting its clinical behavior[12]. Using cDNA microarray, we examined the difference in gene expression profiles between normal liver tissues and HCC cells, as well as between HBV positive and HCV-associated HCC. The data from cDNA microarray are consistent with RT-PCR data from HCC tissues and the corresponding non-tumor tissues. These expression profiles may be useful in elucidating the molecular carcinogenesis of HCC, especially HBV- and HCV-associated HCC.

In the present study, we attempted to establish a link between gene expression and the viral status of HCC. Comparative analysis of HBV- and HCV-associated HCC revealed that 11 genes, for which the expression levels differed between HBV- and HCV-associated HCC. Ribosomal-related genes such as RPS5 (RPL family genes) were up-regulated in HBV-associated HCC compared to HCV-associated HCC, suggesting the activation of protein translation in HBV-infected HCC. This observation is consistent with a previous report that major classes of genes encoding ribosomal proteins were up-regulated by the HBX protein[13]. Cytoskeletal organization, such as KRT8 was shown to be up-regulated in HBV-associated HCC, as well as genes such as ACTB in HCV-associated HCC. Our results support the hypothesis that the deregulation of genes encoding proteins associated with cytoskeleton play a role in liver carcinogenesis[14]. These findings also indicate that the pathway for liver carcinogenesis in the cytoskeleton may be different in HBV- and HCV-associated HCC. Cell adhesion genes such as CD58 were found to be up-regulated in HCV-associated HCC, but have not been reported to be related with human HBV-associated HCC. Xu *et al*[15] showed that several signal transduction related genes, including MAPK family genes were up-regulated in HBV-associated HCC. Up-regulation of MAPK has also been suggested as a common pathway for hepatocarcinogenesis caused by HBV and HCV infections[16]. In the present study, MAP3K5 was up-regulated in HBV-associated HCC compared with the non-tumorous liver tissue. However, MAP3K5 was down-regulated in HCV-associated HCC compared with the non-tumorous liver. Thus, additional studies are necessary to clarify the contribution of the MAPK pathway to each type of HCC. MMP9, which may promote metastasis, was up-regulated in HBV-associated HCC compared with HCV-associated HCC. Other genes such as IGFBP2, ATP5F1, VIM and GAPD, which are expressed differently in HBV- and HCV-associated HCC, were newly identified, although the findings of up-regulation of genes such as IGFBP2 and ATP5F1 in the HBV-associated HCC, and the up-regulation of genes such as VIM and GAPD in the HCV-associated HCC, were in agreement with previous observations[17]. It has been suggested that liver carcinogenesis induced by HBV and HCV, in addition to common genetic and epigenetic alterations, may involve distinct pathways[18]. Our expression profiles suggest that hepatitis viruses affect the expression of dozens of genes in HCC in a type-specific manner, thus invoking slightly different mechanisms of carcinogenesis. We believe that the results obtained in the present study will help our understanding of the molecular mechanisms underlying the pathogenesis of HBV- and HCV-associated HCC. The identification of genes defining virus type-specific expression profiles may contribute to our ability to develop virus type-dependent treatment regimens.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common fatal cancers worldwide. The major risk factors for HCC are chronic hepatitis resulting from infection with hepatitis B virus (HBV) and hepatocellular carcinoma (HCC), and exposure to various exogenous carcinogens, including aflatoxin B1. It has been reported that the incidence of HCC is increasing in several countries in parallel with the increase in chronic HBV and HCV infections. Therefore, clarification of the genetic portraits of hepatocarcinogenesis caused by HBV and HCV infection may provide clues to reducing the incidence of HCC, and establishing effective treatments for each type of HCC. However, the molecular nature of this association is poorly understood.

Research frontier

The aim of the present study was to identify any useful biomarkers indicating virus-specific hepatocarcinogenic process. The differential mRNA expression in 32 patients with HBV-/HCV-associated HCC was investigated by means of

cDNA microarrays comprising of 886 genes.

Innovations and breakthroughs

It has been suggested that liver carcinogenesis induced by HBV and HCV, in addition to common genetic and epigenetic alterations, may involve distinct pathways. The results of the present study suggest that hepatitis viruses affect the expression of dozens of genes in HCC in a type-specific manner, thus invoking slightly different mechanisms of carcinogenesis. Genome-wide gene expression analysis of human cancer may provide important clues to understanding HCC oncogenesis and lead to improvements in predicting its clinical behavior.

Applications

We believe that the results obtained in this study will provide greater understanding of the molecular mechanisms underlying the pathogenesis of HBV- and HCV-associated HCC. The identification of genes defining virus type-specific expression profiles may contribute to our ability to develop virus type-dependent treatment regimens.

Terminology

DNA microarray is a meticulous technology used in molecular biology and in the field of biomedicine. This technique involves an arrayed series of thousands of microscopic spots of DNA oligonucleotides. It may involve a short section of a gene or other DNA elements that are used as probes to hybridize cDNA or cRNA samples (called target) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by fluorescence-based detection of fluorophore-labeled targets to determine relative abundance of nucleic acid sequences in the target.

Peer review

This is a nice study on the changes in the expression patterns in cancer liver tissue associated with two different hepatitis viruses involved in hepatocarcinogenesis. The paper is well written and contains valuable data. The authors, using microarray technology, have compared gene expression between cancerous and non-cancerous liver tissue in both HBV and HCV infected patients. Seven genes were up-regulated in HBV and 4 genes, which were different, were up-regulated in HCV infected patients.

REFERENCES

1 **El-Serag HB**, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. *N Engl J Med* 1999; **340**: 745-750

2 **Kasai Y**, Takeda S, Takagi H. Pathogenesis of hepatocellular carcinoma: a review from the viewpoint of molecular analysis. *Semin Surg Oncol* 1996; **12**: 155-159

3 **Parkin DM**, Pisani P, Ferlay J. Global cancer statistics. *CA Cancer J Clin* 1999; **49**: 33-64, 1

4 **Okuda K**, Fujimoto I, Hanai A, Urano Y. Changing incidence of hepatocellular carcinoma in Japan. *Cancer Res* 1987; **47**: 4967-4972

5 **Lee WM**. Hepatitis B virus infection. *N Engl J Med* 1997; **337**: 1733-1745

6 **Perou CM**, Jeffrey SS, van de Rijn M, Rees CA, Eisen MB, Ross DT, Pergamenschikov A, Williams CF, Zhu SX, Lee JC, Lashkari D, Shalon D, Brown PO, Botstein D. Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. *Proc Natl Acad Sci USA* 1999; **96**: 9212-9217

7 **Alizadeh AA**, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, Boldrick JC, Sabet H, Tran T, Yu X, Powell JI, Yang L, Marti GE, Moore T, Hudson J Jr, Lu L, Lewis DB, Tibshirani R, Sherlock G, Chan WC, Greiner TC, Weisenburger DD, Armitage JO, Warnke R, Levy R, Wilson W, Grever MR, Byrd JC, Botstein D, Brown PO, Staudt LM. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 2000; **403**: 503-511

8 **Perou CM**, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D. Molecular portraits of human breast tumours. *Nature* 2000; **406**: 747-752

9 **Ross DT**, Scherf U, Eisen MB, Perou CM, Rees C, Spellman P, Iyer V, Jeffrey SS, Van de Rijn M, Waltham M, Pergamenschikov A, Lee JC, Lashkari D, Shalon D, Myers TG, Weinstein JN, Botstein D, Brown PO. Systematic variation in gene expression patterns in human cancer cell lines. *Nat Genet* 2000; **24**: 227-235

10 **Welsh JB**, Zarrinkar PP, Sapinoso LM, Kern SG, Behling CA, Monk BJ, Lockhart DJ, Burger RA, Hampton GM. Analysis of gene expression profiles in normal and neoplastic ovarian tissue samples identifies candidate molecular markers of epithelial ovarian cancer. *Proc Natl Acad Sci USA* 2001; **98**: 1176-1181

11 **Honda M**, Kaneko S, Kawai H, Shirota Y, Kobayashi K. Differential gene expression between chronic hepatitis B and C hepatic lesion. *Gastroenterology* 2001; **120**: 955-966

12 **Iizuka N**, Hamamoto Y, Tsunedomi R, Oka M. Translational microarray systems for outcome prediction of hepatocellular carcinoma. *Cancer Sci* 2008; **99**: 659-665

13 **Wu CG**, Forgues M, Siddique S, Farnsworth J, Valerie K, Wang XW. SAGE transcript profiles of normal primary human hepatocytes expressing oncogenic hepatitis B virus X protein. *FASEB J* 2002; **16**: 1665-1667

14 **Le Bail B**, Faouzi S, Boussarie L, Balabaud C, Bioulac-Sage P, Rosenbaum J. Extracellular matrix composition and integrin expression in early hepatocarcinogenesis in human cirrhotic liver. *J Pathol* 1997; **181**: 330-337

15 **Xu XR**, Huang J, Xu ZG, Qian BZ, Zhu ZD, Yan Q, Cai T, Zhang X, Xiao HS, Qu J, Liu F, Huang QH, Cheng ZH, Li NG, Du JJ, Hu W, Shen KT, Lu G, Fu G, Zhong M, Xu SH, Gu WY, Huang W, Zhao XT, Hu GX, Gu JR, Chen Z, Han ZG. Insight into hepatocellular carcinogenesis at transcriptome level by comparing gene expression profiles of hepatocellular carcinoma with those of corresponding noncancerous liver. *Proc Natl Acad Sci USA* 2001; **98**: 15089-15094

16 **Okabe H**, Satoh S, Kato T, Kitahara O, Yanagawa R, Yamaoka Y, Tsunoda T, Furukawa Y, Nakamura Y. Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: identification of genes involved in viral carcinogenesis and tumor progression. *Cancer Res* 2001; **61**: 2129-2137

17 **Yoon SY**, Kim JM, Oh JH, Jeon YJ, Lee DS, Kim JH, Choi JY, Ahn BM, Kim S, Yoo HS, Kim YS, Kim NS. Gene expression profiling of human HBV- and/or HCV-associated hepatocellular carcinoma cells using expressed sequence tags. *Int J Oncol* 2006; **29**: 315-327

18 **Delpuech O**, Trabut JB, Carnot F, Feuillard J, Brechot C, Kremsdorf D. Identification, using cDNA macroarray analysis, of distinct gene expression profiles associated with pathological and virological features of hepatocellular carcinoma. *Oncogene* 2002; **21**: 2926-2937

S- Editor Tian L **L- Editor** Anand BS **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6078

World J Gastroenterol 2008 October 21; 14(39): 6078-6082
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Endoscopic ultrasound-guided choledochoduodenostomy in patients with failed endoscopic retrograde cholangiopancreatography

Takao Itoi, Fumihide Itokawa, Atsushi Sofuni, Toshio Kurihara, Takayoshi Tsuchiya, Kentaro Ishii, Shujiro Tsuji, Nobuhito Ikeuchi, Fuminori Moriyasu

Takao Itoi, Fumihide Itokawa, Atsushi Sofuni, Toshio Kurihara, Takayoshi Tsuchiya, Kentaro Ishii, Shujiro Tsuji, Nobuhito Ikeuchi, Fuminori Moriyasu, Department of Gastroenterology and Hepatology, Tokyo Medical University Hospital, Tokyo 160-0023, Japan
Author contributions: Itoi T, Itokawa F, and Sofuni A were responsible for study design, data analysis, and script preparation; Itoi T and Itokawa F performed endoscopic diagnosis and treatment; Kurihara T, Tsuchiya T, Ishii K, Tsuji S and Ikeuchi N were responsible for the data collection; Moriyasu F reviewed this manuscript; and all authors have read and approved submitted version of the manuscript.
Correspondence to: Takao Itoi, MD, Department of Gastroenterology and Hepatology, Tokyo Medical University Shinjuku-ku, Nishishinjuku 6-7-1, Tokyo 160-0023, Japan. itoi@tokyo-med.ac.jp
Telephone: +81-3-33426111 Fax: +81-3-53816654
Received: June 26, 2008 Revised: September 16, 2008
Accepted: September 23, 2008
Published online: October 21, 2008

Abstract

Endoscopic ultrasonography (EUS)-guided biliary drainage was performed for treatment of patients who have obstructive jaundice in cases of failed endoscopic retrograde cholangiopancreatography (ERCP). In the present study, we introduced the feasibility and outcome of EUS-guided choledochoduodenostomy in four patients who failed in ERCP. We performed the procedure in 2 papilla of Vater, including one resectable case, and 2 cases of cancer of the head of pancreas. Using a curved linear array echoendoscope, a 19 G needle or a needle knife was punctured transduodenally into the bile duct under EUS visualization. Using a biliary catheter for dilation, or papillary balloon dilator, a 7-Fr plastic stent was inserted through the choledochoduodenostomy site into the extrahepatic bile duct. In 3 (75%) of 4 cases, an indwelling plastic stent was placed, and in one case in which the stent could not be advanced into the bile duct, a naso-biliary drainage tube was placed instead. In all cases, the obstructive jaundice rapidly improved after the procedure. Focal peritonitis and bleeding not requiring blood transfusion was seen in one case. In this case, pancreatoduodenectomy was performed and the surgical findings revealed severe adhesion around the choledochoduodenostomy site. Although further studies and development of devices are mandatory, EUS-guided choledochoduodenostomy appears to be an effective alternative to ERCP in selected cases.

Key words: Endoscopic ultrasonography-guided fine needle aspiration; Biliary drainage; Endoscopic retrograde cholangiopancreatography

Peer reviewer: Hsiu-Po Wang, Professor, Department of Emergency Medicine, National Taiwan University Hospital, No 7, Chung-Shan South Rd, Taipei 10016, Taiwan, China

Itoi T, Itokawa F, Sofuni A, Kurihara T, Tsuchiya T, Ishii K, Tsuji S, Ikeuchi N, Moriyasu F. Endoscopic ultrasound-guided choledochoduodenostomy in patients with failed endoscopic retrograde cholangiopancreatography. *World J Gastroenterol* 2008; 14(39): 6078-6082 Available from: URL: http://www.wjgnet.com/1007-9327/14/6078.asp DOI: http://dx.doi.org/10.3748/wjg.14.6078

INTRODUCTION

Endoscopic transpapillary biliary stenting is the most common procedure for biliary drainage in patients with obstructive jaundice. However, there are patients who failed to achieve bile duct access because of failed biliary cannulation or an inaccessible papilla due to severe duodenal stenosis caused by tumor invasion. Percutaneous transhepatic biliary drainage (PTBD) or surgical intervention is required in such cases. Both methods have a higher morbidity and mortality than endoscopic methods[1-5]. Recently, endoscopic ultrasonography (EUS)-guided choledochoduodenostomy has been reported as an alternative biliary drainage technique[6-10]. The aim of the study is to evaluate the potential role of EUS-guided choledochoduodenostomy in the biliary drainage.

CASE REPORT

This series includes all procedures performed at our institution between June 2005 and January 2008. At first,

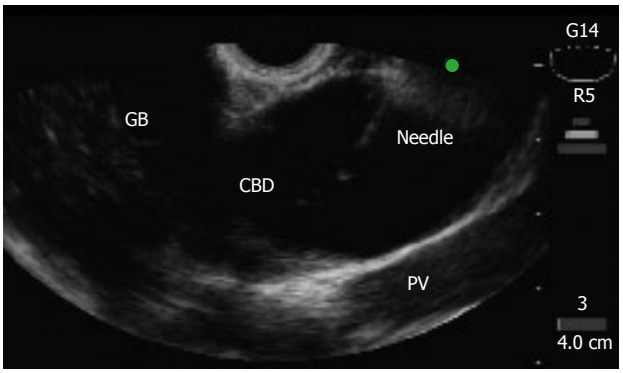


Figure 1 Convex echoendoscope clearly depicts the extrahepatic bile duct (green) (patient 3). GB: Gallbladder; CBD: Common bile duct; PV: Portal vein.

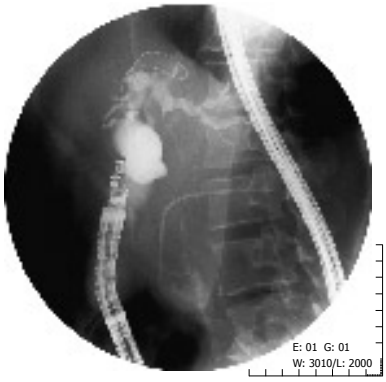
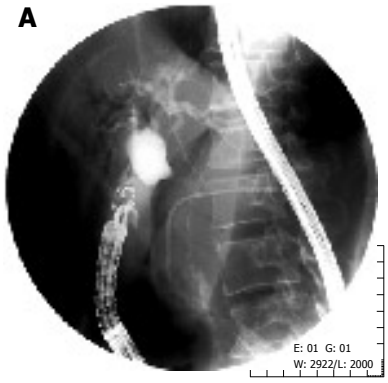


Figure 2 After EUS-guided puncture, contrast medium is injected into the bile duct for cholangiogram, a 0.035-inch guidewire was inserted into the outer sheath (patient 3).



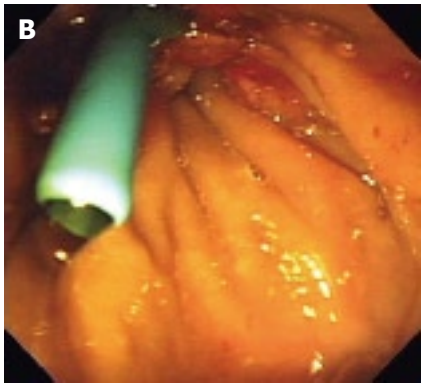


Figure 3 **A**: Choledochoduodenostomy was accomplished with a 7-Fr plastic stent in the apex of the duodenal bulb (patient 3); **B**: The stent was visible in the duodenal bulb (patient 3).

we attempted endoscopic retrograde cholangiography (ERCP) in all patients. If standard ERCP techniques failed, precut sphincterotomy was used for biliary cannulation. The EUS-guided choledochoduodenostomy was performed only in case of failed biliary cannulation or inaccessible papilla due to severe duodenal stenosis caused by tumor invasion. Using an echoendoscope with a curved linear array transducer, and a 3.7-mm accessory channel with an elevator (GF-UCT2000-OL5, Olympus Medical Systems Co. Ltd, Tokyo, Japan), the extrahepatic bile duct was visualized at the level of the duodenal bulb (Figure 1). A 19-gauge needle (EchoTip, Wilson-Cook, Winston-Salem, NC) without electrocoagulation, or a needle knife (Zimmon papillotomy knife, Wilson-Cook) with electrocoagulation (EndoCut ICC200, ERBE ELEKTROMEDIZIN GmbH, Tübingen, Germany) was inserted transduodenally into the bile duct under EUS visualization. After the central needle is removed, bile is aspirated and contrast medium is injected into the bile duct for cholangiography, and 450 cm long, a 0.035-inch guide wire is inserted into the outer sheath (Figure 2). If necessary, a biliary catheter for dilation, or papillary balloon dilator is used for dilation of duodenocholedochostomy site. Finally, a 7-Fr biliary plastic stent (FLEXIMA, Boston Scientific Japan, Tokyo, Japan) was inserted through the choledochoduodenostomy site into the extrahepatic bile duct (Figure 3).

After written informed consent was obtained from patients, all endoscopic procedures were performed with the patients under conscious sedation with intravenous flunitrazepam (5-10 mg). As for antibiotics, 1 g cefotiam hydrochloride was administered by intravenous drip infusion once on the test day and twice on the following day. This study was approved by the institutional review board of our institution.

Patient 1

An 80-year-old man was admitted for treatment of obstructive jaundice. Tumor invasion to the duodenal wall at the circumference of the major papilla was detected when the procedure was impossible. Biopsy specimens from the major duodenal papilla revealed adenocarcinoma of the papilla of Vater. Using a Zimmon needle knife, EUS-guide choledochoduodenostomy was performed. After dilation by a Soehendra dilator catheter (SBDC-7 and SBDC-9, Wilson-Cook), an indwelling 7-Fr plastic stent was placed across the choledochoduodenostomy site into the extrahepatic bile duct without any complications. The obstructive jaundice rapidly improved after insertion of the biliary stent. The stent did not occlude, and the patient died of pneumonia 3 mo after the procedure.

Patient 2

A 71-year-old man admitted for abdominal pain and obstructive jaundice. Computer tomography (CT)

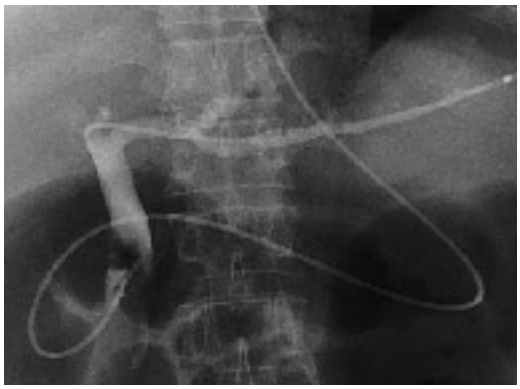


Figure 4 Choledochoduodenostomy was accomplished using a 5-Fr naso-biliary tube (patient 2).

showed a huge papilla of Vater tumor with a dilated bile duct and mild pancreatitis. Biopsy specimens from the major duodenal papilla revealed adenocarcinoma of the papilla of Vater. Imaging revealed that the tumor was resectable. However, since the biliary cannulation for biliary decompression was impossible because the tumor occupied the duodenal lumen, EUS-guided choledochoduodenostomy was performed. After puncture using a Zimmon needle knife, dilation of the choledochoduodenostomy site by a Soehendra dilator catheter was performed. Subsequently, we attempted to insert a 7-Fr plastic stent from the first portion of duodenum into the extrahepatic bile duct, but stent insertion was impossible because the tip of the stent became impacted in the bile duct site of choledochoduodenostomy. Although balloon dilation was performed across the choledochoduodenostomy site, a 7-Fr stent could not be inserted, therefore, a 5-Fr naso-biliary drainage tube was inserted into the left intrahepatic bile duct (Figure 4). Although the obstructive jaundice rapidly improved after the procedure, there was bleeding not requiring blood transfusion and smoldering focal peritonitis of choledochoduodenostomy site. No evidence of intra-abdominal bile leak was found by several cholangiography procedures via the naso-biliary drainage tube. Pancreatoduodenectomy 16 d after the procedure revealed severe adhesion around the choledochoduodenostomy site although choledochoduodenostomy was completed (Figure 5A). Histological examination revealed mild inflammatory cell infiltrate adjacent to the sinus tract in the duodenal and bile duct walls (Figure 5B-D). The patient is presently healthy 13 mo after surgery.

Patient 3

A 69-year-old man with a history of chronic pancreatitis and placement of pancreatic stent for the treatment of abdominal pain, was admitted with obstructive jaundice. ERCP was impossible because the duodenoscope could not pass through a severe stricture. EUS-FNA specimens from pancreas mass showed adenocarcinoma. Using a 19-gauge needle (EchoTip, Wilson-Cook), EUS-guided choledochoduodenostomy was performed. After dilation by a Soehendra dilator catheter, a 7-Fr plastic

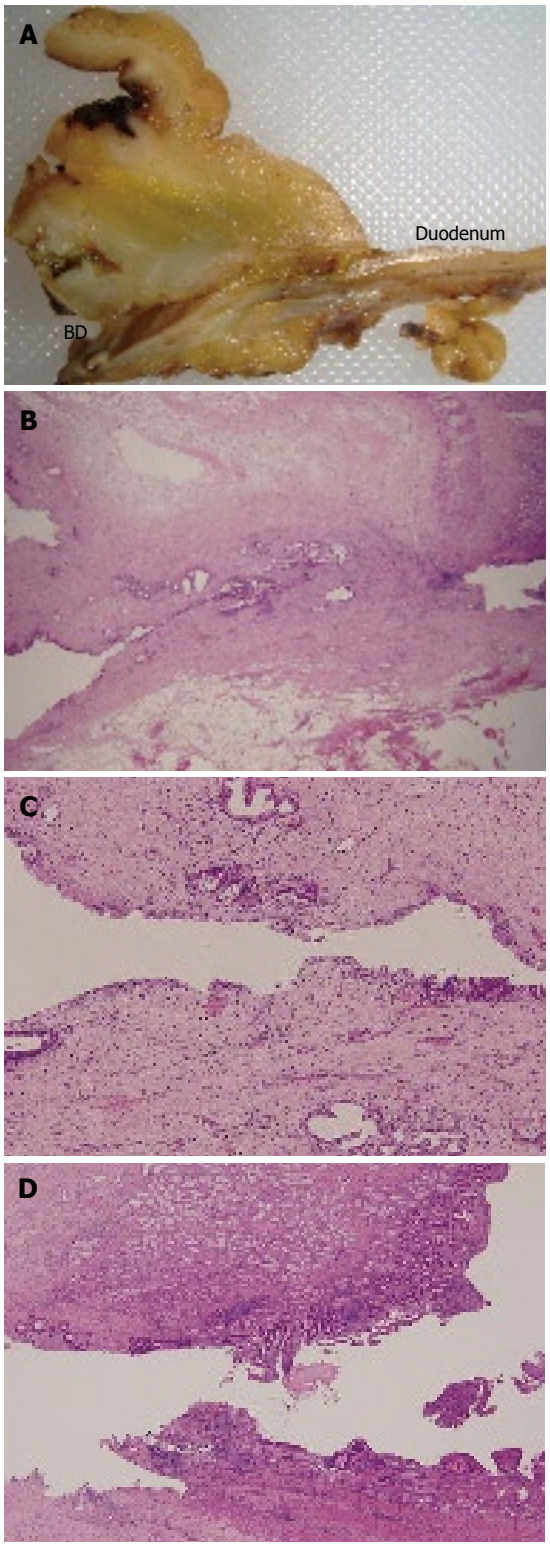


Figure 5 **A**: Macroscopic view of completed choledochoduodenostomy (patient 2); **B**: Surgical specimens showing the completed choledochoduodenostomy with mild inflammatory cell infiltrate adjacent to the sinus tract in the duodenal and bile duct walls (HE, × 20, patient 2); **C**: Magnification of bile duct site (HE, × 100, patient 2); **D**: Magnification of bile duct site (HE, × 100, patient 2). BD: Bile duct.

stent was placed into the extrahepatic bile duct without any complications. The obstructive jaundice rapidly improved after insertion of the biliary stent. Since acute cholangitis occurred 2 wk after the procedure due to stent clogging, we replaced the plastic stent

by a self-expandable metallic stent (SEMS) using a guide wire plus snare forceps technique because the choledochoduodenal fistula seemed to be incomplete. Actually, after placement of the first 10-Fr uncovered SEMS (Niti-S, Teung Medical Co. Ltd. Seoul, Korea), contrast medium flowed out of the bile duct to the intra-abdominal space. A second 10-Fr covered SEMS (Combi-S, Teung Medical Co. Ltd.), therefore, was placed in the first SEMS. No complications occurred and the patient had no symptoms 3 mo later at follow-up.

Patient 4

An 86-year-old woman with unresectable cancer of the head of the pancreas who had undergone biliary stenting 1 mo previously, was admitted with obstructive jaundice. At initial ERCP, an indwelling 10-Fr plastic stent (FLEXIMA, Boston Scientific Japan, Tokyo, Japan) was placed. ERCP was impossible because the duodenoscope could not pass through the tight stricture caused by cancer invasion. Then, EUS-guided choledochoduodenostomy was performed. Using a 19-gauge needle (EchoTip, Wilson-Cook), EUS-guided choledochoduodenostomy was performed. After dilation by a Soehendra dilator catheter, a 7-Fr plastic stent was placed into the extrahepatic bile duct without any complications. The obstructive jaundice rapidly improved after insertion of the biliary stent. No complications occurred and the patients had no related symptoms till she died 1 mo later.

DISCUSSION

Endoscopic biliary stent placement is the most well-established method for the treatment of obstructive jaundice[11]. When ERCP fails, usually, PTBD is chosen as an alternative method for treating biliary decompression[12]. Recently, EUS-guided biliary drainage using either direct access or a rendezvous technique, has attracted attention as an alternative procedure to ERCP or PTCS[6-10,13-17]. Until now, of these EUS-guided biliary drainage procedures, EUS-guided choledochoduodenostomy has been performed in 17 cases consisting of 11 pancreatic cancer, 4 papilla of Vater cancer, 1 bile duct cancer, and 1 bile duct stone[6-10].

The methodology and devices of EUS-guided choledochoduodenostomy are not yet fully established. Therefore, there are several important factors during the procedure to ensure technical success. First, as Yamao *et al*[9] mentioned, the scope position and puncture site are very important. Theoretically, the scope pushing position at which the tip of the convex transducer is directed at the hepatic hilum, is promising because the access route to the bile duct is shorter from the duodenal bulb to the bile duct and the echoendoscope is stable when several devices are advanced into the bile duct through the working channel[9]. However, when there is duodenal stenosis due to tumor invasion, anatomically abnormal situation after surgery, the same scope position may not be always possible. Second, the type of puncture needle also may be one of the most important factors. Several reports describe that various types of needle knife are used for puncture in all but two cases (88%, 15/17)[6-10]. Although a needle knife could make a larger hole compared to a fine-needle aspiration (FNA) needle which needs dilators or balloon dilation for the subsequent procedure, a larger hole may lead to possible intra-abdominal bile leak. In the present series, we used both a FNA needle and a needle knife with electrical coagulation. Unfortunately, one case using a needle knife failed in stent insertion despite making a comparatively large hole and also using a dilator and balloon. The main reason for this may be that the tip of the convex transducer was directed not at the hepatic hilum but the distal bile duct. In this case, the direction of the guidewire was changed from distal bile duct to hepatic hilum to enable technical success. Therefore, it may be possible that the choledochoduodenostomy site became kinked and the stent could not torque adequately because of the instabilization of the scope. These data suggest that technical success of EUS-guided choledochoduodenostomy may depend not on the choice of needle, but mainly the scope position and direction of puncture.

Several investigators used various diameter plastic stents[6,7,9,10,18] or a SEMS[8]. Although the stent diameter depends on the echoendoscope used, large bore plastic stent may be better for long patency, similar to the conventional transpapillary plastic stent. Recently, Yamao *et al*[19] has reported that the mean stent patency of 7- 8.5-Fr plastic stents for EUS-guided chodedocho-duodenostomy was 211.8 d. Since there are few data on SEMS in this procedure, the usefulness of SEMS should be evaluated from the aspect of cost effectiveness including the issue of the use of uncovered or covered SEMS.

Previous data revealed that the procedure was successful in all but 2 cases (a total success rate of 88%) and once stents were placed, all patients had successful resolution of biliary decompression. In the present study, in all patients, the obstructive jaundice rapidly improved after insertion of the biliary stent. These data suggest that this procedure may be as effective as conventional transpapillary biliary drainage once the indwelling stent is placed.

Surprisingly, no serious procedure-related complications were found in several reports although there was 1 case of mild focal bile peritonitis and 3 of pneumoperitoneum[6-10]. In the current study, we encountered 1 focal peritonitis with bleeding without severe complications. However, we must be cautious in evaluating the safety of the procedure because previous published data may be biased towards successful cases.

In conclusion, we report four cases of EUS-guided choledochoduodenostomy for the treatment of obstructive jaundice. This procedure was performed successfully, without severe complications and with highly effective biliary drainage. Although further studies and development of devices are necessary, EUS-guided choledochoduodenostomy can be an effective alternative to ERCP.

REFERENCES

1 **Guenther RW**, Vorwerk DR. Perkutane Gallenwegsdrainage. In: Guenther RW, Thelen M, editors. Interventionelle radiology. 2nd ed. New York: Thieme, 1996: 472-481
2 **Richter A**, Brambs HJ. Interventionen an Gallengängen. In: Görick J, Bramb HJ, editors. Interventionelle minimal invasive Radiologie. 1st ed. New York: Thieme, 2001: 58-64
3 **Smith AC**, Dowsett JF, Russell RC, Hatfield AR, Cotton PB. Randomised trial of endoscopic stenting versus surgical bypass in malignant low bileduct obstruction. *Lancet* 1994; **344**: 1655-1660
4 **Lai EC**, Mok FP, Tan ES, Lo CM, Fan ST, You KT, Wong J. Endoscopic biliary drainage for severe acute cholangitis. *N Engl J Med* 1992; **326**: 1582-1586
5 **Pessa ME**, Hawkins IF, Vogel SB. The treatment of acute cholangitis. Percutaneous transhepatic biliary drainage before definitive therapy. *Ann Surg* 1987; **205**: 389-392
6 **Giovannini M**, Moutardier V, Pesenti C, Bories E, Lelong B, Delpero JR. Endoscopic ultrasound-guided bilioduodenal anastomosis: a new technique for biliary drainage. *Endoscopy* 2001; **33**: 898-900
7 **Burmester E**, Niehaus J, Leineweber T, Huetteroth T. EUS-cholangio-drainage of the bile duct: report of 4 cases. *Gastrointest Endosc* 2003; **57**: 246-251
8 **Kahaleh M**, Hernandez AJ, Tokar J, Adams RB, Shami VM, Yeaton P. Interventional EUS-guided cholangiography: evaluation of a technique in evolution. *Gastrointest Endosc* 2006; **64**: 52-59
9 **Yamao K**, Sawaki A, Takahashi K, Imaoka H, Ashida R, Mizuno N. EUS-guided choledochoduodenostomy for palliative biliary drainage in case of papillary obstruction: report of 2 cases. *Gastrointest Endosc* 2006; **64**: 663-667
10 **Fujita N**, Noda Y, Kobayashi G, Ito K, Obana T, Horaguchi J, Takasawa O, Nakahara K. Histological changes at an endosonography-guided biliary drainage site: a case report. *World J Gastroenterol* 2007; **13**: 5512-5515
11 **Soehendra N**, Reynders-Frederix V. Palliative bile duct drainage-a new endoscopic method of introducing a transpapillary drain. *Endoscopy* 1980; **12**: 8-11
12 **Takada T**, Hanyu F, Kobayashi S, Uchida Y. Percutaneous transhepatic cholangial drainage: direct approach under fluoroscopic control. *J Surg Oncol* 1976; **8**: 83-97
13 **Giovannini M**, Dotti M, Bories E, Moutardier V, Pesenti C, Danisi C, Delpero JR. Hepaticogastrostomy by echo-endoscopy as a palliative treatment in a patient with metastatic biliary obstruction. *Endoscopy* 2003; **35**: 1076-1078
14 **Mallery S**, Matlock J, Freeman ML. EUS-guided rendezvous drainage of obstructed biliary and pancreatic ducts: Report of 6 cases. *Gastrointest Endosc* 2004; **59**: 100-107
15 **Kahaleh M**, Yoshida C, Kane L, Yeaton P. Interventional EUS cholangiography: A report of five cases. *Gastrointest Endosc* 2004; **60**: 138-142
16 **Bories E**, Pesenti C, Caillol F, Lopes C, Giovannini M. Transgastric endoscopic ultrasonography-guided biliary drainage: results of a pilot study. *Endoscopy* 2007; **39**: 287-291
17 **Will U**, Thieme A, Fueldner F, Gerlach R, Wanzar I, Meyer F. Treatment of biliary obstruction in selected patients by endoscopic ultrasonography (EUS)-guided transluminal biliary drainage. *Endoscopy* 2007; **39**: 292-295
18 **Puspok A**, Lomoschitz F, Dejaco C, Hejna M, Sautner T, Gangl A. Endoscopic ultrasound guided therapy of benign and malignant biliary obstruction: a case series. *Am J Gastroenterol* 2005; **100**: 1743-1747
19 **Yamao K**, Bhatia V, Mizuno N, Sawaki A, Ishikawa H, Tajika M, Hoki N, Shimizu Y, Ashida R, Fukami N. EUS-guided choledochoduodenostomy for palliative biliary drainage in patients with malignant biliary obstruction: results of long-term follow-up. *Endoscopy* 2008; **40**: 340-342

S- Editor Li DL **L- Editor** Ma JY **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6083

World J Gastroenterol 2008 October 21; 14(39): 6083-6086
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

A unique case of collagenous colitis presenting as protein-losing enteropathy successfully treated with prednisolone

Soichi Sano, Keiko Yamagami, Ayako Tanaka, Minako Nishio, Tomoyuki Nakamura, Yuki Kubo, Takeshi Inoue, Wataru Ueda, Kiyotaka Okawa, Katsunobu Yoshioka

Soichi Sano, Keiko Yamagami, Ayako Tanaka, Minako Nishio, Tomoyuki Nakamura, Katsunobu Yoshioka, Department of Internal Medicine, Osaka City General Hospital, Osaka 534-0021, Japan
Yuki Kubo, Takeshi Inoue, Department of Pathology, Osaka City General Hospital, Osaka 534-0021, Japan
Wataru Ueda, Kiyotaka Okawa, Department of Gastroenterology, Osaka City General Hospital, Osaka 534-0021, Japan
Author contributions: Sano S, Yamagami K, Tanaka A, Nishio M, Nakamura T and Yoshioka K were involved in treatment of this patient; Ueda W and Okawa K contributed to the gastroenterological examination; Kubo Y and Inoue T contributed to the pathological examination; and Sano S, Yamagami K and Yoshioka K wrote the paper.
Correspondence to: Keiko Yamagami, MD, Department of Internal Medicine, Osaka City General Hospital, 2-13-22, Miyakojima-hon-dori, Miyakojima-ku, Osaka 534-0021, Japan. yamasanz@qb3.so-net.ne.jp
Telephone: +81-6-69291221 Fax: +81-6-69291091
Received: September 1, 2008 Revised: September 9, 2008
Accepted: September 16, 2008
Published online: October 21, 2008

Abstract

A 76-year-old woman with a 5-mo history of recurrent diarrhea and generalized edema was admitted to our hospital. Colonoscopy revealed edematous mucosa, and histopathological examination was compatible with collagenous colitis. Protein leakage from the colon, particularly in the ascending portion, was identified on 99mTc-human serum albumin scintigraphy. Collagenous colitis associated with protein-losing enteropathy (PLE) without small bowel disease was diagnosed. Prednisolone treatment ameliorated diarrhea and hypoproteinemia. Collagenous colitis should be included in the differential diagnosis of chronic diarrhea with hypoproteinemia for appropriate management.

Key words: Collagenous colitis; Protein-losing enteropathy; Prednisolone; 99mTc-human serum albumin scintigraphy; chronic diarrhea

Peer reviewer: Robert Daniel Odze, PhD, Department of Gastrointestinal Pathology, Brigham and Women's Hospital, 75 Francis St., Boston MA, United States

Sano S, Yamagami K, Tanaka A, Nishio M, Nakamura T, Kubo Y, Inoue T, Ueda W, Okawa K, Yoshioka K. A unique case of collagenous colitis presenting as protein-losing enteropathy successfully treated with prednisolone. *World J Gastroenterol* 2008; 14(39): 6083-6086 Available from: URL: http://www.wjgnet.com/1007-9327/14/6083.asp DOI: http://dx.doi.org/10.3748/wjg.14.6083

INTRODUCTION

Since the first report in 1976[1], collagenous colitis has been found to be associated with a variety of conditions, including use of non-steroidal anti-inflammatory drugs[2] and proton pump inhibitors[3]. This condition is characterized by chronic watery diarrhea and abnormal deposition of collagen beneath the colonic epithelium[4]. Severe hypoproteinemia due to enteric protein loss is rare unless accompanied by small bowel malabsorption syndrome, such as celiac sprue. To the best of our knowledge, only 1 case of collagenous colitis associated with protein-losing enteropathy (PLE) in the absence of small bowel disease has been reported[5]. We report herein another case of collagenous colitis associated with PLE without small bowel disease, in which PLE was diagnosed by 99mTc-human albumin (HSA) scintigraphy.

CASE REPORT

A 76-year-old woman with a 5-mo history of recurrent diarrhea and generalized edema was admitted to our hospital. Repeated colonoscopies and gastrointestinal scope at a previous hospital had shown no abnormal changes in the gastrointestinal mucosa. The patient had been taking 180 mg of loxoprofen sodium and 30 mg of lansoprazole every day for the past 3 years, for osteoarthritis of the knees, but had otherwise been healthy with no prior history of gastrointestinal disease. Family history was unremarkable.

On admission, weight was 53 kg and height was 144 cm. She was afebrile, with a blood pressure of 128/84 mmHg and a heart rate of 82 beats/min. The right lower abdominal quadrant was slightly painful on palpation, the face was swollen, and pretibial pitting edema was also present. No superficial lymph nodes

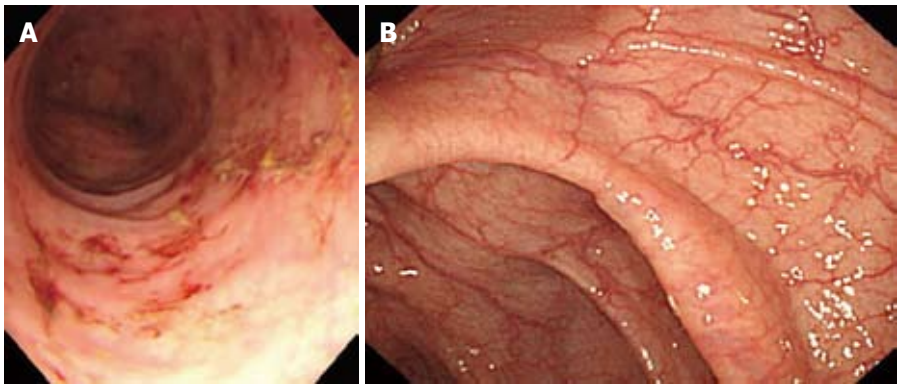


Figure 1 Colonoscopy shows an edematous mucosa, diminished vascular transparency throughout the colon on admission. Friability of mucosa and multiple erythema were observed (**A**). Endoscopic findings 1 mo after treatment showed normal mucosa (**B**).

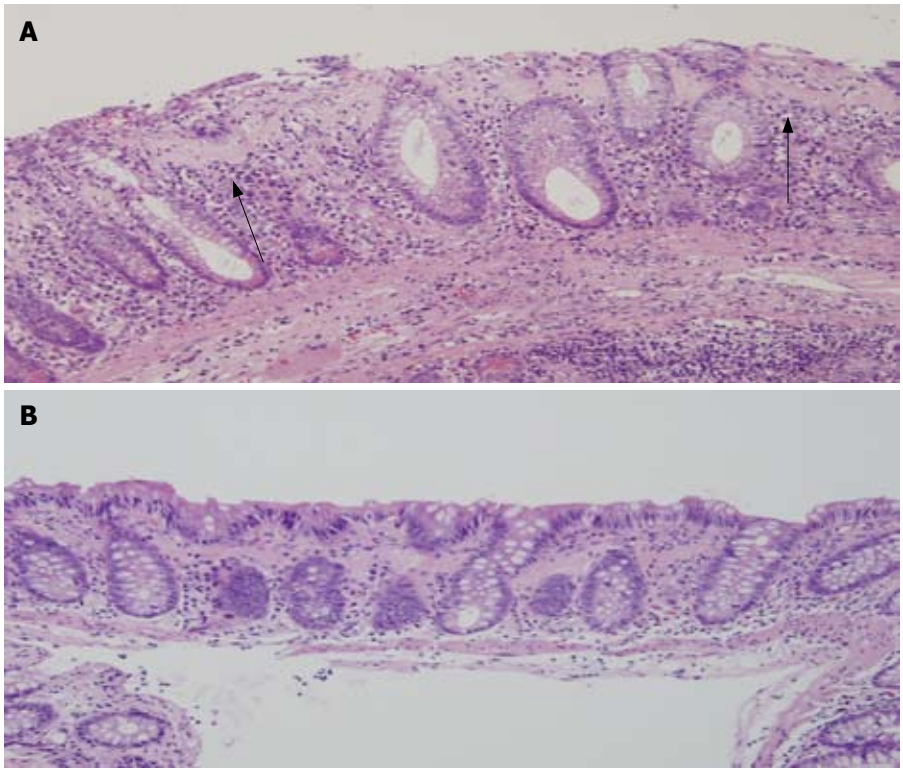


Figure 2 Histology of the biopsied specimen demonstrates subepithelial from eosinophilic band-like deposit (arrows), with increased lymphocytes and plasma cells. Sloughing of surface epithelium is also shown (**A**). Epithelial detachment and inflammatory cells decreased, although the collagen band beneath the mucosa was not reduced (**B**).

were palpable. Laboratory investigations revealed: white blood cells, 7960/mm^3; C-reactive protein, 0.31 mg/dL; total protein, 4.8 g/dL; plasma albumin, 2.8 mg/dL; no abnormalities in liver, renal, thyroid and adrenal function tests; negative serology for rheumatoid factor; normal results of urinalysis and no proteinuria. Moreover, no steatorrhea was identified, indicating that malabsorption syndrome was unlikely.

Endoscopic examination of the lower intestinal tracts showed edematous mucosa, diminished vascular transparency and multiple areas of erythema (Figure 1A). Multiple biopsies revealed changes consistent with collagenous colitis, showing prominent subepithelial eosinophilic band-like deposits with increased lymphocytes and plasma cells (Figure 2A). The distribution of the disease was whole colon and the findings were worse in the right compared to the left colon. The thickness of the collagen layer was 80-100 μm in the right and 40-50 μm in the left. There were no areas of cryptitis, crypt abscesses and no area of superimposed infection inflammatory bowel disease. Sloughing of surface epithelium was also identified. Endoscopic examination and biopsies of other portion of the gastrointestinal tract showed no obvious abnormalities in the stomach and the small intestine, and there was no evidence of celiac sprue. The duodenum showed normal villi and no obvious lymphocyte infiltration. Small bowel barium study showed no abnormalities. On 99mTc HSA scintigraphy, protein leakage was detected throughout the whole colon, particularly in the ascending portion (Figure 3A). No leakage was apparent from the stomach or small intestine. Neither chest/abdominal computed tomography (CT) nor ^{67}Ga scintigraphy showed any evidence of malignancy. On the basis of these findings, collagenous colitis associated with PLE was diagnosed.

After diagnosis, loxoprofen sodium and lansoprazole were discontinued. However, general condition remained unimproved. Administration of 1.8 g polycarbophil calcium, 2 g natural aluminum silicate, 2 g bifidobacterium, 20 mg scopolamine butylbromide, 4 mg loperamide hydrochloride and 2 g albumin tannate

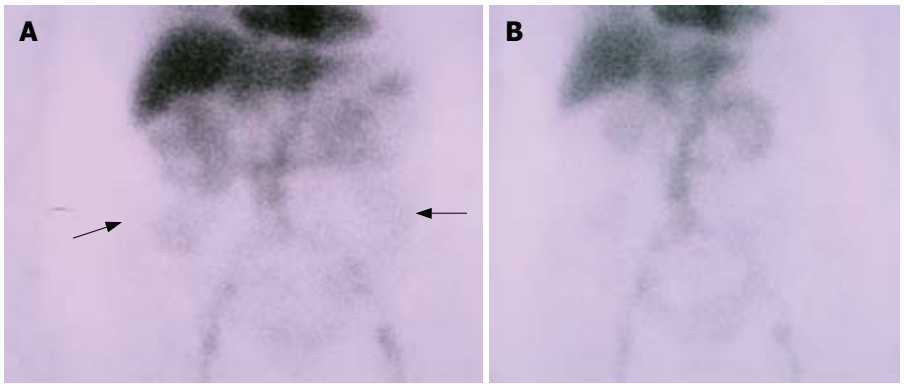


Figure 3 99mTc-human serum albumin scintigraphy shows leakage of the tracer in the large bowel (arrows) on admission (**A**) but no accumulation 1 mo after starting oral prednisolone (**B**).

likewise did not improve symptoms, and serum protein and albumin levels remained low despite administration of human serum albumin. Frequency of diarrhea increased and abdominal pain became extremely severe, and the condition of the patient deteriorated. We decided to start prednisolone therapy after hypovolemic shock developed. Soon after initiating prednisolone treatment at 30 mg/d, abdominal pain and diarrhea dramatically improved along with general condition. Total plasma protein and plasma albumin levels gradually increased to 5.4 g/dL and 3.4 g/dL, respectively, by 1 mo after starting prednisolone therapy. Follow-up endoscopy showed normal-appearing colonic mucosa (Figure 1B), and multiple biopsies showed decreased epithelial detachment and inflammatory cells, although the collagen band beneath the mucosa was not reduced (Figure 2B). Additional 99mTc HSA scintigraphy showed no accumulation of tracer in the large bowel, indicating remission of PLE (Figure 3B).

DISCUSSION

This patient presented with chronic diarrhea and anasarca with severe hypoproteinemia, and colonoscopic examination revealed collagenous colitis. We considered that hypoproteinemia was enteric in origin, as no proteinuria, liver disease, cardiac disease or other inflammatory disease was present. Severe enteric protein loss causing hypoproteinemia is reportedly rare in collagenous colitis unless accompanied by small bowel malabsorption syndrome. In the present case, coexistence of malabsorption syndrome was unlikely given the absence of steatorrhea, which is characteristic of malabsorption syndrome. Furthermore, histopathology of the duodenum revealed no evidence of celiac sprue.

The cause of hypoproteinemia was thus considered to be excessive enteric protein loss without malabsorption syndrome. Small bowel barium radiography showed no abnormality such as Crohn's disease or malignant lymphoma, or other diseases that could cause PLE. Furthermore, 99mTc HSA scintigraphy revealed no accumulation of tracer in the small bowel. Considering that no obvious small bowel disease was present, collagenous colitis itself might have been the cause of PLE and 99mTc HSA scintigraphy is useful in this case[6,7].

Although the mechanisms of enteric protein loss in patients with collagenous colitis have not been clarified, abnormalities of the surface epithelium[8], superficial capillaries[9] and pericryptal fibroblasts[10] have been hypothesized. Long-term use of loxoprofen sodium and lansoprazole may be associated in the pathogenesis of severity of collagenous colitis in this patient. In the present case, histological examination revealed marked reductions in epithelial detachment together with clinical improvement after prednisolone therapy, suggesting that abnormalities of surface epithelium play an important role in the development of PLE. The endoscopic findings of collagenous colitis are thought to be normal and nonspecific[11]. In the present case, however, endoscopic examination revealed edematous mucosa, diminished vascular transparency and multiple red spots, and histological examination revealed sloughing of surface epithelium, suggesting this was a severe case of collagenous colitis. This may be one reason why the patient developed PLE.

No established treatments have yet been defined for PLE associated with collagenous colitis[12]. Corticosteroids are occasionally used for the treatment of collagenous colitis and some studies have shown that prednisolone treatment is ineffective[13] and budesonide is effective for collagenous colitis without PLE[14,15]. As oral budesonide is unavailable in Japan, prednisolone treatment was selected because prednisolone has been reported to ameliorate PLE caused by other diseases through the reduction of submucosal edema and inflammation in the gastrointestinal tract. In the present case, oral prednisolone was effective for refractory diarrhea and exudative enteropathy. Corticosteroid was provided at a dose of 30 mg/d, and general status of the patient immediately improved in parallel with a decrease in the frequency of diarrhea and an increase in serum total protein.

Abnormal protein leakage on 99mTc HSA scintigraphy was diminished and biopsy specimens from the colon after treatment histopathologically reflected the efficacy of this therapy in terms of reduced epithelial sloughing and inflammatory cells, although the collagen band under the mucosa was largely unchanged. These results suggest that treatment with prednisolone may be effective for PLE associated with collagenous colitis.

In summary, we have reported a patient with collagenous colitis who presented with PLE. Prednisolone treatment ameliorated diarrhea and hypoproteinemia. Collagenous colitis should be included in the differential diagnosis of chronic diarrhea with hypoproteinemia to achieve appropriate management.

REFERENCES

1 **Lindstrom CG**. 'Collagenous colitis' with watery diarrhoea--a new entity? *Pathol Eur* 1976; **11**: 87-89
2 **Riddell RH**, Tanaka M, Mazzoleni G. Non-steroidal anti-inflammatory drugs as a possible cause of collagenous colitis: a case-control study. *Gut* 1992; **33**: 683-686
3 **Rammer M**, Kirchgatterer A, Hobling W, Knoflach P. Lansoprazole-associated collagenous colitis: a case report. *Z Gastroenterol* 2005; **43**: 657-660
4 **Mogensen AM**, Olsen JH, Gudmand-Hoyer E. Collagenous colitis. *Acta Med Scand* 1984; **216**: 535-540
5 **Stark ME**, Batts KP, Alexander GL. Protein-losing enteropathy with collagenous colitis. *Am J Gastroenterol* 1992; **87**: 780-783
6 **Chiu NT**, Lee BF, Hwang SJ, Chang JM, Liu GC, Yu HS. Protein-losing enteropathy: diagnosis with (99m)Tc-labeled human serum albumin scintigraphy. *Radiology* 2001; **219**: 86-90
7 **Hubalewska-Hola A**, Sowa-Staszczak A, Szczerbinski T, Lis G, Huszno B, Szybinski Z. [Protein losing enteropathy (PLE) detected by Tc99m-labelled human serum albumin abdominal scintigraphy--case report] *Przegl Lek* 2003; **60**: 606-608
8 **Jessurun J**, Yardley JH, Giardiello FM, Hamilton SR, Bayless TM. Chronic colitis with thickening of the subepithelial collagen layer (collagenous colitis): histopathologic findings in 15 patients. *Hum Pathol* 1987; **18**: 839-848
9 **Teglbjaerg PS**, Thaysen EH. Collagenous colitis: an ultrastructural study of a case. *Gastroenterology* 1982; **82**: 561-563
10 **Hwang WS**, Kelly JK, Shaffer EA, Hershfield NB. Collagenous colitis: a disease of pericryptal fibroblast sheath? *J Pathol* 1986; **149**: 33-40
11 **Freeman HJ**. Collagenous mucosal inflammatory diseases of the gastrointestinal tract. *Gastroenterology* 2005; **129**: 338-350
12 **Chande N**, McDonald JW, Macdonald JK. Interventions for treating collagenous colitis. *Cochrane Database Syst Rev* 2008: CD003575
13 **Munck LK**, Kjeldsen J, Philipsen E, Fischer Hansen B. Incomplete remission with short-term prednisolone treatment in collagenous colitis: a randomized study. *Scand J Gastroenterol* 2003; **38**: 606-610
14 **Bonderup OK**, Hansen JB, Madsen P, Vestergaard V, Fallingborg J, Teglbjaerg PS. Budesonide treatment and expression of inducible nitric oxide synthase mRNA in colonic mucosa in collagenous colitis. *Eur J Gastroenterol Hepatol* 2006; **18**: 1095-1099
15 **Lanyi B**, Dries V, Dienes HP, Kruis W. Therapy of prednisone-refractory collagenous colitis with budesonide. *Int J Colorectal Dis* 1999; **14**: 58-61

S- Editor Li DL **L- Editor** Ma JY **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6087

World J Gastroenterol 2008 October 21; 14(39): 6087-6092
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Pneumatosis cystoides intestinalis following alpha-glucosidase inhibitor treatment: A case report and review of the literature

Tatsuhiro Tsujimoto, Erika Shioyama, Kei Moriya, Hideto Kawaratani, Yasuyo Shirai, Masahisa Toyohara, Akira Mitoro, Jun-ichi Yamao, Hisao Fujii, Hiroshi Fukui

Tatsuhiro Tsujimoto, Erika Shioyama, Kei Moriya, Hideto Kawaratani, Yasuyo Shirai, Masahisa Toyohara, Akira Mitoro, Jun-ichi Yamao, Hiroshi Fukui, Third Department of Internal Medicine, Nara Medical University, Kashihara, Nara 634-8522, Japan
Hisao Fujii, Department of Endoscopy and Ultrasound, Nara Medical University, Kashihara, Nara 634-8522, Japan
Author contributions: Tsujimoto T and Shioyama E designed the work; Tsujimoto T wrote the article; Shioyama E, Moriya K, Kawaratani H, Shirai Y, Toyohara M, Mitoro A and Yamao J followed up the patient; Fujii H and Fukui H reviewed the article.
Correspondence to: Tatsuhiro Tsujimoto, MD, PhD, Third Department of Internal Medicine, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan. tat-tyan@xa2.so-net.ne.jp
Telephone: +81-744-223051 Fax: +81-744-247122
Received: July 28, 2008 Revised: September 24, 2008
Accepted: October 1, 2008
Published online: October 21, 2008

Abstract

A 69-year-old man was diagnosed as having myasthenia gravis (MG) in September 2004, and treated with thymectomy and prednisolone. He was then diagnosed as having steroid-induced diabetes mellitus, and received sulfonylurea (SU) therapy in May 2005. An alpha-glucosidase inhibitor (αGI) was added in March 2006, resulting in good glycemic control. He experienced symptoms of abdominal distention, increased flatus, and constipation in October 2007, and was admitted into our hospital in late November with hematochezia. Plain abdominal radiography revealed small linear radiolucent clusters in the wall of the colon. Computed tomography (CT) showed intramural air in the sigmoid colon. Colonoscopy revealed multiple smooth surfaced hemispherical protrusions in the sigmoid colon. The diagnosis of pneumatosis cystoides intestinalis (PCI) was made on the basis of these findings. As the αGI voglibose was suspected as the cause of this patient's PCI, treatment was conservative, ceasing voglibose, with fasting and fluid supplementation. The patient progressed well, and was discharged 2 wk later. Recently, several reports of PCI associated with αGI therapy have been published, predominantly in Japan where αGIs are commonly used. If the use of αGIs becomes more widespread, we can expect more reports of this condition on a global scale. The possibility of PCI should be considered in diabetic patients complaining of gastrointestinal symptoms, and the gastrointestinal tract should be thoroughly investigated in these patients.

Key words: Alpha-glucosidase inhibitor; Colonoscopy; Diabetes mellitus; Pneumatosis cystoides intestinalis; Voglibose

Peer reviewer: Gary A Abrams, Associate Professor, Department of Medicine, University of Alabama at Birmingham, 1530 3rd Ave South, Birmingham 35294, United States

Tsujimoto T, Shioyama E, Moriya K, Kawaratani H, Shirai Y, Toyohara M, Mitoro A, Yamao J, Fujii H, Fukui H. Pneumatosis cystoides intestinalis following alpha-glucosidase inhibitor treatment: A case report and review of the literature. *World J Gastroenterol* 2008; 14(39): 6087-6092 Available from: URL: http://www.wjgnet.com/1007-9327/14/6087.asp DOI: http://dx.doi.org/10.3748/wjg.14.6087

INTRODUCTION

Pneumatosis cystoides intestinalis (PCI) is a rare condition in which multiple submucosal or subserosal pneumocystis develop in the submucosa or in subserosa of the colon[1,2]. The etiological mechanisms are unclear, although PCI has been reported to develop in association with raised intra-abdominal pressure due to ileus surgery[3-5], colonoscopy[6], pulmonary diseases such as chronic bronchitis and emphysema[7], trichloroethylene exposure[8], connective tissue disorders[9,10], the use of immunosuppressants[11], and ingestion of carbohydrates such as lactulose[12] and sorbitol[13]. Recently, the development of PCI during treatment with alpha-glucosidase inhibitors (αGIs), a new class of anti-diabetic agents, has been reported[14,15]. Our literature search yielded only 13 cases of PCI associated with αGI therapy[14-26]. Herein, we present a case depicting αGI as the probable cause of PCI, along with a review of the literature.

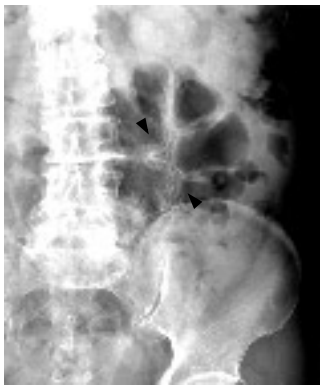


Figure 1 Plain radiography of the abdomen on admission revealing small linear and round radiolucent clusters in the wall of the colon (black arrows).

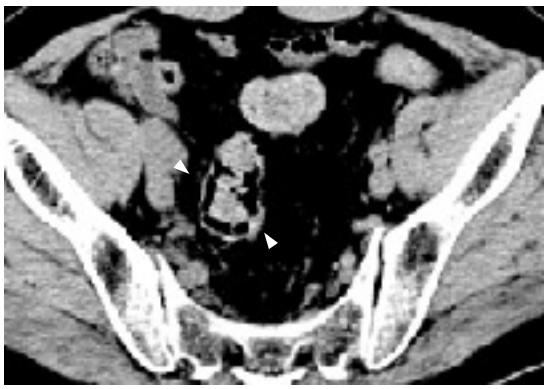


Figure 2 Computed tomography (CT) scanning of the abdomen on admission revealing intramural gas in the sigmoid colon (white arrows).

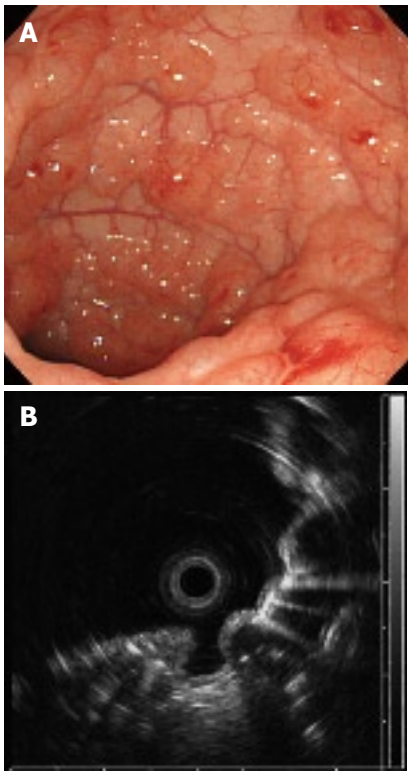


Figure 3 Colonoscopy on admission showing multiple round and smooth-surfaced elevated lesions like submucosal tumors in the sigmoid colon (**A**) and endoscopic ultrasonography (EUS) revealing hyperechoic lesions and acoustic shadows in the submucosal layer (**B**).

CASE REPORT

A 69-year-old man was diagnosed as having severe myasthenia gravis (MG) in September 2004, and treated with prednisolone (5 mg/d) from October of that year. He underwent thymectomy in March 2005. Hyperglycemia was detected in May 2005, leading to the diagnosis of steroid-induced diabetes mellitus, and sulfonylurea (SU) therapy was commenced immediately. As his blood sugar could not be controlled, αGI was prescribed in March 2006, resulting in good glycemic control. He claimed to have experienced abdominal distension, increased flatus and constipation, and noticed small amounts of bright rectal bleeding as early as mid-October 2007, but did nothing about it. The amount of rectal bleeding increased in late November that year, and he was referred to our hospital for investigation and treatment.

Laboratory investigations revealed no abnormalities in white blood cell (WBC) count, hemoglobin (Hb), or C-reactive protein, and HbA1c was slightly elevated to 6.0%. Plain abdominal radiography revealed small linear radiolucent gas collections along the wall of the colon (Figure 1). Unenhanced computed tomography (CT) of the abdomen showed intramural air in the sigmoid colon, and free gas in the peritoneal cavity around the sigmoid colon (Figure 2). Colonoscopy revealed multiple smooth surfaced small hemispherical protrusions in the sigmoid colon, and endoscopic ultrasonography (EUS) demonstrated highly echogenic submucosal lesions with acoustic shadows (Figure 3). The diagnosis of PCI was made on the basis of these findings.

As voglibose was suspected to be the cause of this patient's PCI, conservative treatment was administered, including ceasing the voglibose, along with fasting and fluid supplementation. The patient progressed well and plain abdominal radiography 2 wk later showed that the linear collections of gas along the wall of the colon were disappeared, and consequently the patient was discharged. Colonoscopy 3 mo later showed complete resorption of the pneumocystis throughout the sigmoid colon, leaving white scars. EUS confirmed disappearance of the submucosal acoustic shadows, indicating the resolution of PCI.

DISCUSSION

PCI, first reported by Du Vernoi[27] in 1730, is a rare condition in which multiple submucosal or subserosal pneumocystis develop in the submucosa or subserosa of the colon. It was previously thought to occur most frequently in the ileum, but with the recent increase in the number of barium enemas and colonoscopies performed, PCI now reportedly affects the colon more commonly.

There are some recent reports on PCI associated with αGI therapy[14-26]. The mechanism is thought to involve intestinal gas production through fermentation-by the intestinal flora-of carbohydrates whose absorption is inhibited by αGI. Along with peristaltic hypofunction

Table 1 A summary of previously reported cases of pneumatosis cystoides intestinalis (PCI) after an alpha-glucosidase inhibitor (αGI) treatment

Case No.	Author Reference number Yr	Age Sex	Chief complaint	The αGI agent Quantity of αGI Dosage period of αGI prior to PCI onset	Disease other than diabetes mellitus	Concomitant drug	Prescription of αGI after PCI onset	Treatment Outcome Duration to the outcome
1	Hayakawa *et al* 14 1999	64 F	Abdominal distention	Voglibose 0.6 mg/day 1 mo	Unknown	Insulin	Discontinuation	Conservative treatment Healing 4 d
2	Azami 15 2000	87 F	Abdominal distention Appetite loss	Acarbose 150 mg/day 1 yr	Hypothyroidism	SU	Discontinuation	Conservative treatment Healing 5 d
3	Maeda *et al* 16 2002	55 F	Abdominal distention	Acarbose 300 mg/day 42 d	Pemphigus vulgaris	Insuline steroid Immunosupressant	Continuation	Conservative treatment Healing 141 d
4	Tachibana *et al* 17 2002	73 F	Abdominal distention	Acarbose 150 mg/day 8 yr	Henoch-Schonlein purpura nephritis	SU Steroid	Discontinuation	Conservative treatment Healing 28 d
5	Yanaru *et al* 18 2002	61 M	Abdominal distention Constipation Hematochezia	Voglibose 0.6 mg/day 5 yr	Unknown	SU	Discontinuation	Conservative treatment Healing 28 d
6	Matsuda *et al* 19 2004	62 M	Abdominal pain	Voglibose Unknown Unknown	Lung cancer	Morphine sulfate	Unknown	Operation Remission 16 d
7	Nagahara *et al* 20 2006	66 M	Left abdominal pain	Acarbose Unknown 11 yr	Unknown	Unknown	Discontinuation	Conservative treatment Healing 21 d
8	Hisamoto *et al* 21 2006	56 M	No abdominal symptoms	Voglibose 0.6 mg/day 7 d	Interstitial pneumonia	Steroid	Discontinuation	Conservative treatment Healing 7 d
9	Furio *et al* 22 2006	64 F	Abdominal pain Diarrhea Tenesmus Weight loss	Acarbose Unknown 3 yr	Unknown	Insulin	Discontinuation	Conservative treatment Healing 15 d
10	Miyagawa *et al* 23 2006	65 M	Abdominal pain Diarrhea	Voglibose 0.6 mg/day 6 years	Gastric cancer	SU	Continuation→ Discontinuation	Conservative treatment Healing 120 d
11	Yasuoka *et al* 24 2007	75 M	Abdominal distention	Voglibose 0.6 mg/day 10 yr	Lung cancer Rectal carcinoid	SU	Discontinuation	Conservative treatment Healing 20 d
12	Maeda *et al* 25 2007	72 F	Rt lower abdominal pain	Voglibose 0.9 mg/day 3 yr	Minimal change disease	Insulin Steroid Immunosupressant	Discontinuation	Conservative treatment Healing 7 d
13	Saito *et al* 26 2007	53 F	Abdominal distention Nausea	Voglibose 0.6 mg/day 1 mo	Dermatomyositis	Steroid Immunosupressant	Discontinuation	Conservative treatment Healing 21 d
14	Our case 2008	69 M	Abdominal distention Hematochezia	Voglibose 0.6 mg/day 1 yr 8 mo	Myasthenia gravis	SU Steroid	Discontinuation	Conservative treatment Healing 14 d

associated with diabetes mellitus, this leads to raised intraluminal pressure, allowing the gas-producing bacteria to invade the colonic mucosa through breaks in the mucosal integrity, forming pneumocystis[14,15].

Our review of the medical literature between 1983 and 2008 yielded 7 cases of PCI associated with αGI therapy in PubMed [English language; 14, 15, 18, 21, 22, 25, 26], and 6 in Japana Centra Revuo Medicina [Japanese language; 16, 17, 19, 20, 23, 24]. The details of these cases, totaling 14 with the addition of our present case, are shown in Tables 1 and 2. All but 1 of the 14 cases was reported in Japan. About 30% of

Table 2 Imaging findings in previously reported cases of PCI after an αGI treatment

Case No.	Author Reference number Year	Plain radiography of the abdomen	Computed tomography of the abdomen	Barium enema	Colonoscopy
1	Hayakawa *et al* 14 1999	Distention of the ascending and proximal transverse colon with cystic radiolucencies, indicating intramural gas	Subserosal cystic areas of gas and distention of the involved segments	Translucent areas of gas clustered along the distorted contours of the ascending and transverse colon	ND
2	Azami 15 2000	Noticeable gaseous distension of the small intestine	Noticeable gaseous distention of the small intestine with pockets of small gas bubbles in the submucosal space	No constriction in the sigmoid or lower descending colon	ND
3	Maeda *et al* 16 2002	Multiple cystic radiolucencies in the abdomen	Pneumatosis intestinalis around the bowel wall and gas within the retroperitoneum	ND	ND
4	Tachibana *et al* 17 2002	Free gas of the right peritoneal cavity and pneumatosis intestinalis throughout the ascending colon	Free gas below the right diaphragm, and pneumatosis intestinalis throughout the ascending colon	ND	Polypoid lesions in the ascending and transverse colon
5	Yanaru *et al* 18 2002	Small round radiolucent clusters in the middle abdomen	ND	Numerous submucosal protrusions of sessile of semipedunculated configurations	Numerous submucosal protrusions of sessile of semipedunculated configurations
6	Matsuda *et al* 19 2004	Noticeable gaseous distention of the colon, and curvilinear radiolucency within the bowel wall	Free gas in the peritoneal cavity, pneumatosis intestinalis throughout the bowel wall	ND	ND
7	Nagahara *et al* 20 2006	Free air below the diaphragm	Pneumatosis intestinalis throughout the ascending colon	ND	ND
8	Hisamoto *et al* 21 2006	Free air below the diaphragm, and noticeable gaseous distention of the ascending and transverse colon	Slight dialatation, mesenteric edema, and diffuse pneumatosis intestinalis throughout the ascending colon	Many cystic areas of the ascending colon	Multiple sessile polypoid lesions covered with normal-appearing mucosa in the area from the ascending colon
9	Furio *et al* 22 2006	ND	The presence of numerous intraparietal cysts, of varying size, diffuse in the varied colic segments, compatible with wall pneumatosis of the colon	ND	Multiple polypoid formations of varying sizes in the sigmoid, descending, ascending and cecum
10	Miyagawa *et al* 23 2006	Cystic radiolucencies in the colon	ND	Multiple numerous round polypoid lesions from the ascending colon to the sigmoid colon	Numerous round polypoid lesions from the ascending colon to the sigmoid colon
11	Yasuoka *et al* 24 2007	Noticeable gaseous distension of the small intestine	Free gas in the peritoneal cavity	ND	ND
12	Maeda *et al* 25 2007	Diffuse air shadows along the intestine suggesting gas accumulation in the bowel wall	Circumferential collections of air adjacent to the bowel lumen that ran parallel to the bowel wall	ND	ND
13	Saito *et al* 26 2007	Pneumoperitoneum with free air under the diaphragm and curvilinear radiolucency within the bowel wall	Intramural air in the ascending colon, and gas collection in the mesentery	ND	ND
14	Our case 2008	Small linear radiolucent gas collections along the wall of the colon	Intramural air in the sigmoid colon, and free gas in the peritoneal cavity around the sigmoid colon	ND	Multiple smooth surfaced small hemispherical protrusions in the sigmoid colon

ND: Not done.

the Japanese diabetics are prescribed αGIs, which are rarely administered in Western countries where fats account for a larger proportion of the caloric intake than carbohydrates[28,29]. The preponderance of Japanese reports on αGI-associated PCI is not surprising as the Japanese market accounts for 98% of the total sales of voglibose, and 34% of those for acarbose.

The mean age of the 14 patients was 65.9 years, while 7 were male and 7 were female. The causative agent was voglibose in 9 cases and acarbose in 5, while none was caused by miglitol. The global market share in 2005 for voglibose and acarbose was in a ratio of

roughly 3:2. Miglitol was not released in Japan until 2006, and accordingly no reports are available on PCI associated with the newest agent. As future cases are reported, we expect that there will be no significant differences in the incidence of PCI between these agents. The mean prescribed dosages were 0.64 mg/d for voglibose and 200 mg/d for acarbose. The interval between commencement of αGI therapy and the onset of PCI varied greatly, ranging from 7 d to 11 years. The most common symptoms were abdominal distention (57%) and abdominal pain (36%), while only 2 cases had hematochezia (14%) as in the case described herein (Table 1).

Different radiological and endoscopic modalities are useful in the diagnosis of PCI. To summarize the imaging findings in the 14 reported cases (Table 2), linear or round radiolucent gas collections were seen along the wall of the colon in plain abdominal radiographs in most cases, and pneumatosis was seen within or along the wall of the colon on abdominal CT scanning. Subserous pneumocystis in particular are liable to rupture, releasing free gas into the peritoneal cavity, making it important to distinguish this condition from bowel perforation[30]. Multiple rounded protrusions are a common finding in barium enema examinations of patients with PCI. The colonoscopic findings may be similar to multiple polyposis or collections of submucosal tumors, but subserous pneumatosis may go undetected.

With cessation of αGI therapy, conservative treatment could lead to resolution of PCI within 28 d. In the 2 cases where αGI therapy was continued, resolution took more than 120 d. Therefore, ceasing the αGI therapy is the key to successful treatment of PCI. One case underwent emergency surgery due to the presence of free air in the peritoneal cavity, where bowel perforation could not be ruled out[19]. In our case, there were 2 possible causes, namely, the αGI voglibose and prednisolone. Since our patient claimed to have experienced abdominal distention, increased flatus and constipation prior to the onset of PCI, we considered voglibose the causative agent. We therefore ceased αGI and continued corticosteroid therapy, and kept our patient fasting with fluid supplementation, achieving resolution of PCI after 14 d (Table 1).

The symptoms of PCI include abdominal pain, diarrhea and abdominal distention, none of which is disease specific. Diabetic patients sometimes develop autonomic neuropathy, with gastrointestinal symptoms similar to those of PCI. As αGI therapy is commonly used in Japan, it is difficult to determine whether diabetic patients complaining of gastrointestinal symptoms are suffering only from diabetes mellitus or from PCI. If the clinical picture of diabetes mellitus is consistent with that of PCI, diabetes mellitus can be detected by plain abdominal radiography. The possibility of PCI should be considered in diabetic patients complaining of gastrointestinal symptoms, and appropriate investigations should be performed with this potential diagnosis in mind.

In this report, we presented a case of PCI associated with αGI therapy, and a review of the literature. Our patient recovered rapidly after conservative treatment, including ceasing of the voglibose, fasting, and fluid supplementation. Recently, several reports on PCI associated with αGI therapy have been published, predominantly from Japan where αGIs are commonly used[14-21,23,24]. If the use of αGIs becomes more widespread internationally, we can expect more reports of this condition globally. The possibility of PCI should be considered in diabetic patients complaining of gastrointestinal symptoms, and the gastrointestinal tract should be thoroughly investigated in these patients.

REFERENCES

1 **Christl SU**, Gibson GR, Murgatroyd PR, Scheppach W, Cummings JH. Impaired hydrogen metabolism in pneumatosis cystoides intestinalis. *Gastroenterology* 1993; **104**: 392-397

2 **Florin TH**, Hills BA. Does counterperfusion supersaturation cause gas cysts in pneumatosis cystoides coli, and can breathing heliox reduce them? *Lancet* 1995; **345**: 1220-1222

3 **Gruenberg JC**, Grodsinsky C, Ponka JL. Pneumatosis intestinalis: a clinical classification. *Dis Colon Rectum* 1979; **22**: 5-9

4 **Ho LM**, Mosca PJ, Thompson WM. Pneumatosis intestinalis after lung transplant. *Abdom Imaging* 2005; **30**: 598-600

5 **Horiuchi A**, Akamatsu T, Mukawa K, Ochi Y, Arakura N, Kiyosawa K. Case report: Pneumatosis cystoides intestinalis associated with post-surgical bowel anastomosis: a report of three cases and review of the Japanese literature. *J Gastroenterol Hepatol* 1998; **13**: 534-537

6 **McCollister DL**, Hammerman HJ. Air, air, everywhere: pneumatosis cystoides coli after colonoscopy. *Gastrointest Endosc* 1990; **36**: 75-76

7 **Keyting WS**, McCarver RR, Kovarik JL, Daywitt AL. Pneumatosis intestinalis: a new concept. *Radiology* 1961; **76**: 733-741

8 **Yamaguchi K**, Shirai T, Shimakura K, Akamatsu T, Nakama H, Kono K, Sakato M, Shigeno T, Furuta S, Nakajima T. Pneumatosis cystoides intestinalis and trichloroethylene exposure. *Am J Gastroenterol* 1985; **80**: 753-757

9 **Mueller CF**, Morehead R, Alter AJ, Michener W. Pneumatosis intestinalis in collagen disorders. *Am J Roentgenol Radium Ther Nucl Med* 1972; **115**: 300-305

10 **Quiroz ES**, Flannery MT, Martinez EJ, Warner EA. Pneumatosis cystoides intestinalis in progressive systemic sclerosis: a case report and literature review. *Am J Med Sci* 1995; **310**: 252-255

11 **Heng Y**, Schuffler MD, Haggitt RC, Rohrmann CA. Pneumatosis intestinalis: a review. *Am J Gastroenterol* 1995; **90**: 1747-1758

12 **Goodman RA**, Riley TR 3rd. Lactulose-induced pneumatosis intestinalis and pneumoperitoneum. *Dig Dis Sci* 2001; **46**: 2549-2553

13 **Duncan B**, Barton LL, Eicher ML, Chmielarczyk VT, Erdman SH, Hulett RL. Medication-induced pneumatosis intestinalis. *Pediatrics* 1997; **99**: 633-636

14 **Hayakawa T**, Yoneshima M, Abe T, Nomura G. Pneumatosis cystoides intestinalis after treatment with an alpha-glucosidase inhibitor. *Diabetes Care* 1999; **22**: 366-367

15 **Azami Y**. Paralytic ileus accompanied by pneumatosis cystoides intestinalis after acarbose treatment in an elderly diabetic patient with a history of heavy intake of maltitol. *Intern Med* 2000; **39**: 826-829

16 **Maeda A**, Yokoi S, Kunou T, Murata T. [A case of pneumatosis cystoides intestinalis assumed to be induced by acarbose administration for diabetes mellitus and pemphigus vulgaris] *Nippon Shokakibyo Gakkai Zasshi* 2002;

99: 1345-1349
17 **Tachibana Y**, Band H, Asai J, Notsumata K, Toya D, Tanaka N, Miura M. A case of pneumatosis cystoides intestinalis with abdominal free air. *Nihon Arukoru Yakubutsu Igakkai Zasshi* 2002; **22**: 1103-1106
18 **Yanaru R**, Hizawa K, Nakamura S, Yoshimura R, Watanabe K, Nakamura U, Yoshinari M, Matsumoto T. Regression of pneumatosis cystoides intestinalis after discontinuing of alpha-glucosidase inhibitor administration. *J Clin Gastroenterol* 2002; **35**: 204-205
19 **Matsuda Y**, Yoshida H, Sugimoto H, Tanaka K. A case of pneumatosis cystoides intestinalis with intraperitoneal free air in palliative care. *J Jpn Surg Assoc* 2004; **65**: 3288-3292
20 **Nagahara Y**, Hakoda T, Imada T, Kurose H, Okuno T, Yoshida T. Pneumatosis cystoides intestinalis after treatment with an alpha-glucosidase inhibitor. *Diabetes J* 2006; **34**: 104-107
21 **Hisamoto A**, Mizushima T, Sato K, Haruta Y, Tanimoto Y, Tanimoto M, Matsuo K. Pneumatosis cystoides intestinalis after alpha-glucosidase inhibitor treatment in a patient with interstitial pneumonitis. *Intern Med* 2006; **45**: 73-76
22 **Furio L**, Vergura M, Russo A, Bisceglia N, Talarico S, Gatta R, Tomaiuolo M, Tomaiuolo P. Pneumatosis coli induced by acarbose administration for diabetes mellitus. Case report and literature review. *Minerva Gastroenterol Dietol* 2006; **52**: 339-346
23 **Miyagawa M**, Kanemasa H, Nakagawa S, Nitan T, Matsumoto M, Tokita K, Kajita Y, Mitsufuji S, Okanoue T. A case of pneumatosis cystoides intestinalis after treatment with an α-glucosidase inhibitor. *Gastroenterol Endosc* 2006; **48**: 329-333
24 **Yasuoka R**, Sonoyama Y, Fujiki H, Morita S, Mitsuo M, Kadotani Y. A case of intestinal emphysema with pneumoperitoneum in which α-glucosidase inhibitor participated. *J Jpn Surg Assoc* 2007; **68**: 2014-2018
25 **Maeda Y**, Inaba N, Aoyagi M, Kanda E, Shiigai T. Fulminant pneumatosis intestinalis in a patient with diabetes mellitus and minimal change nephrotic syndrome. *Intern Med* 2007; **46**: 41-44
26 **Saito M**, Tanikawa A, Nakasute K, Tanaka M, Nishikawa T. Additive contribution of multiple factors in the development of pneumatosis intestinalis: a case report and review of the literature. *Clin Rheumatol* 2007; **26**: 601-603
27 **Du Vernoi GJ**. Aer intestinorum tam subextima guam intima tunica inclusus. Obsergatinonae Anatomicae Acad. *Scient Imp Petoropal* 1730; **5**: 213-215
28 **Wysowski DK**, Armstrong G, Governale L. Rapid increase in the use of oral antidiabetic drugs in the United States, 1990-2001. *Diabetes Care* 2003; **26**: 1852-1855
29 **Smyth S**, Heron A. Diabetes and obesity: the twin epidemics. *Nat Med* 2006; **12**: 75-80
30 **Caudill JL**, Rose BS. The role of computed tomography in the evaluation of pneumatosis intestinalis. *J Clin Gastroenterol* 1987; **9**: 223-226

S- Editor Zhong XY **L- Editor** Wang XL **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6093

World J Gastroenterol 2008 October 21; 14(39): 6093-6095
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Endoscopic fibrin glue injection for closure of pancreatocutaneous fistula following transgastric endoscopic necrosectomy

Ji Woong Jang, Do Hyun Park, Sung-Hoon Moon, Sang Soo Lee, Dong Wan Seo, Sung Koo Lee, Myung-Hwan Kim

Ji Woong Jang, Do Hyun Park, Sung-Hoon Moon, Sang Soo Lee, Dong Wan Seo, Sung Koo Lee, Myung-Hwan Kim, Division of Gastroenterology, Department of Internal Medicine, University of Ulsan College of Medicine, Asan Medical Center, Seoul 138-736, South Korea
Author contributions: Jang JW, and Park DH wrote the paper; Jang JW, Park DH, and Moon SH carried out the procedures; Park DH, Seo DW, Lee SK, and Kim MH advised and informed about the knowledge.
Correspondence to: Do Hyun Park, MD, PhD, Division of Gastroenterology, Department of Internal Medicine, University of Ulsan College of Medicine, Asan Medical Center, 388-1 pungnap-2dong, Songpagu, Seoul 138-736, South Korea. dhpark@amc.seoul.kr
Telephone: +82-2-30103194 Fax: +82-2-4760824
Received: June 10, 2008 Revised: August 30, 2008
Accepted: September 6, 2008
Published online: October 21, 2008

Abstract

Transgastric endoscopic necrosectomy has been recently introduced as the effective and alternative management of infected pancreatic necrosis and pancreatic abscess. However, up to 40% of patients who undergo endoscopic necrosectomy may need an additional percutaneous approach for subsequent peripancreatic fluid collection or non-resolution of pancreatic necrosis. This percutaneous approach may lead to persistent pancreatocutaneous fistula, which remains a serious problem and usually requires prolonged hospitalization, or even open-abdominal surgery. We describe the first case of pancreatocutaneous fistula and concomitant abdominal wall defect following transgastric endoscopic necrosectomy and percutaneous drainage, which were endoscopically closed with fibrin glue injection *via* the necrotic cavity.

Key words: Fibrin glue; Pancreatocutaneous fistula; Infected pancreatic necrosis; Pancreatic abscess; Endoscopic necrosectomy

Peer reviewer: Ibrahim A Al Mofleh, Professor, Department of Medicine, College of Medicine, King Saud University, PO Box 2925, Riyadh 11461, Saudi Arabia

Jang JW, Park DH, Moon SH, Lee SS, Seo DW, Lee SK, Kim MH. Endoscopic fibrin glue injection for closure of pancreatocutaneous fistula following transgastric endoscopic necrosectomy. *World J Gastroenterol* 2008; 14(39): 6093-6095 Available from: URL: http://www.wjgnet.com/1007-9327/14/6093.asp DOI: http://dx.doi.org/10.3748/wjg.14.6093

INTRODUCTION

Infected pancreatic necrosis and pancreatic abscess are serious complications of acute pancreatitis, and open necrosectomy is the mainstay of management for these complications[1]. However, operation-related morbidity and mortality, and longer hospitalization are not uncommon complications[1,2]. Transgastric endoscopic necrosectomy has been recently introduced as the effective and alternative management for infected pancreatic necrosis and pancreatic abscess[3-7]. However, up to 40% of patients who undergo endoscopic necrosectomy may need an additional percutaneous approach for subsequent peripancreatic fluid collection or non-resolution of pancreatic necrosis[8]. This percutaneous approach may lead to persistent pancreatocutaneous fistula, which remains a serious problem and usually requires prolonged hospitalization, or even open-abdominal surgery[9-12]. We describe the first case of pancreatocutaneous fistula and concomitant abdominal wall defect following transgastric endoscopic necrosectomy and percutaneous drainage, which were endoscopically closed with fibrin glue injection *via* the necrotic cavity.

CASE REPORT

A 63-year-old woman with acute biliary pancreatitis was admitted to our hospital. A computer tomography (CT) scan showed pancreatitis, peripancreatic fluid collection and gallbladder (GB) wall thickening with polypoid lesions. Endoscopic retrograde cholangiopancreatography (ERCP) showed a GB cancer with combined anomalous union of the pancreaticobiliary duct. After interdisciplinary consultation, although the patient suffered from acute biliary pancreatitis with peripancreatic fluid collection,

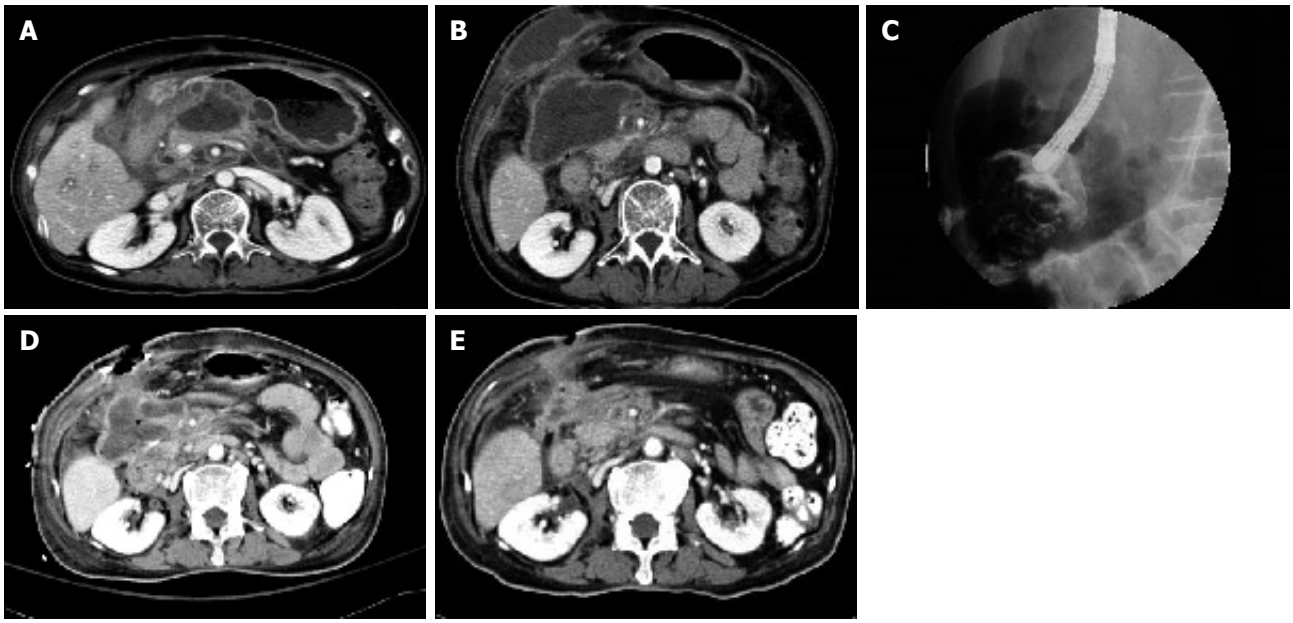


Figure 1 Multiple peripancreatic fluid collections on the body and head portions of the pancreas with a subcutaneous abscess on the right upper quadrant connected to theses peripancreatic fluid collections (**A**, **B**), fluoroscopy and follow-up CT showing a frank pancreatocutaneous fistula and abdominal wall defects alongside the percutaneous route (**C**, **D**), follow-up CT showing nearly resolved infected peripancreatic nerosis, pancreatocutaneous fistula, and abdominal wall defect after endoscopic necrosectomy and repair of the fistula with fibrin glue (**E**).

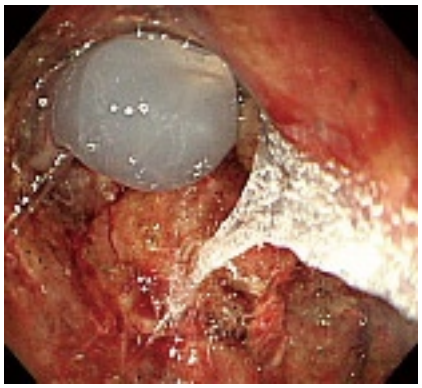


Figure 2 Complete obliteration of the fistula tract after injection of fibrin glue beyond and on the luminal side of the fistula.

we decided to perform an open cholecystectomy. Resected specimen revealed a stage Ⅱ GB cancer. The patient's postoperative recovery was uneventful.

Two months later, she was readmitted for fever and previous operation wound site bulging, redness, and heat. A CT scan revealed the aggravation of fluid collection in her abdominal wall's right upper quadrant, in addition to peripancreatic fluid collection bulging into the body portion of her stomach (Figure 1A and B). We inserted a percutaneous pig-tail catheter for subcutaneous fluid collection and performed an endoscopic transgastric necrosectomy because the patient refused a surgical intervention.

As our previous reports illustrate[13,14], a transgastric endoscopic necrosectomy was performed. During endoscopic necrosectomy and saline irrigation with a water-jet scope (GIF-Q 260J; Olympus Optical Co, Tokyo, Japan), skin wound dehiscence at the incision site of open cholecystectomy occurred alongside the percutaneous route (Figure 1C and D). The patient suffered from discharge (about 100 CC/d) of necrotic materials through the skin wound dehiscence, and extensive skin excoriation. During follow-up endoscopic examination, 50-100 CC of pus with a high concentration of amylase (23220 IU/L) was drained daily through the fistula tract and abdominal wall defect. Subsequent ERCP showed the long common channel of pancreatobiliary duct but no disruption of the main pancreatic duct. Because the surgeon recommended conservative management with additional insertion of a Penrose drain to resolve the wound dehiscence, rather than an operative wound revision, we decided to close the fistula endoscopically. On follow-up endoscopic evaluation, we identified a fistula tract between the necrotic cavity and skin connected to the subcutaneous abscess. The size of fistula was about 1 cm. We endoscopically injected fibrin glue as a sealing material *via* the necrotic cavity (Figure 2). A total of 8 mL of fibrin glue (Tisseel; Baxter, West-lake Village, Calif.) was injected into the fistula tract through a double lumen catheter. The patient showed no post-procedural complications, such as anaphylactic reaction. The day after endoscopic closure of the fistula with fibrin glue injection, no pus was discharged from the pancreatocutaneous fistula. On the fifth day after endoscopic closure of the fistula, the wound's dehiscence was also spontaneously closed, with no additional sutures. A follow-up CT scan also showed near resolution of the infected peripancreatic necrosis and sealing of the pancreatocutaneous fistula and abdominal wall defect (Figure 1E). During a 3-mo follow-up period, no relapse of the pancreatocutaneous fistula occurred.

DISCUSSION

During transgastric endoscoic necrosectomy with

a percutaneous approach, patients may develop a pancreatocutaneous fistula. Although small external fistulae with integrity of pancreatic duct can be spontaneously closed, management of large external fistulae or abdominal wall defects, as in our case, may be cumbersome[9-11]. During endoscopic necrosectomy, adequate endoscopic irrigation is essential for removal of necrotic tissue[7]. As in this case, however, saline irrigation with a water-jet scope during endoscopic necrosectomy may be a precipitating factor for frank pancreatocutaneous fistula and wound site dehiscence alongside the percutaneous catheter because the endoscope may block the outflow tract of the cavity during lavage and consequence pressure becomes too high and the wall is perforated[7]. For closure of this pancreatocutaneous fistula, we selected fibrin glue. Fibrin glue injection has been previously used during endoscopy for wound closure and fistula repair[15-18]. Fibrin glue is a biologic tissue adhesive based on a combination of fibrinogen and thrombin that forms a cell-free clot, and it has been shown to have tissue-healing properties and to be fully reabsorbed by macrophages and fibroblasts within 2 wk of application[15,16,19].

High-output (> 500 CC/d) external fistulae have a particularly poor prognosis, and are a potential cause of recurrence compared to low-output (< 200 CC/d) external fistulae[10,11]. Our patient showed a low-output external fistula and an intact main pancreatic duct on pancreatography. This is why endoscopic fibrin glue injection *via* the necrotic cavity alone is effective for rapid closure of fistula and abdominal wall defect, without further surgical management. Though there are few data about the fistula size, a small size of fistula might have contributed to our complete result and good prognosis. In addition, we did not consider using a hemoclip to close the fistula because its placement *via* the necrotic cavity would have been "in-body" and we were concerned with the difficulty of approximating, and bleeding of the friable necrotic cavity using a hemoclip. After the fistula was closed, no fluid recollection was observed, thus complete removal of necrotic tissue was achieved.

In summary, endoscopic fibrin glue injection *via* necrotic cavity is a safe, less invasive, rapid, and effective technique for closure of pancreatocutaneous fistulae and abdominal wall defects following transgastric necrosectomy with percutaneous drainage. We think that this procedure is proper for low-output (< 200 CC/d), small size (diameter < 1 cm) fistulae, and inner surface should be reached endoscopically. However, some established indications for selecting patients are lack as yet. Therefore, further studies are necessary.

REFERENCES

1 **Bassi C**, Butturini G, Falconi M, Salvia R, Frigerio I, Pederzoli P. Outcome of open necrosectomy in acute pancreatitis. *Pancreatology* 2003; **3**: 128-132

2 **Connor S**, Alexakis N, Raraty MG, Ghaneh P, Evans J, Hughes M, Garvey CJ, Sutton R, Neoptolemos JP. Early and late complications after pancreatic necrosectomy. *Surgery* 2005; **137**: 499-505

3 **Baron TH**, Thaggard WG, Morgan DE, Stanley RJ. Endoscopic therapy for organized pancreatic necrosis. *Gastroenterology* 1996; **111**: 755-764

4 **Charnley RM**, Lochan R, Gray H, O'Sullivan CB, Scott J, Oppong KE. Endoscopic necrosectomy as primary therapy in the management of infected pancreatic necrosis. *Endoscopy* 2006; **38**: 925-928

5 **Raczynski S**, Teich N, Borte G, Wittenburg H, Mossner J, Caca K. Percutaneous transgastric irrigation drainage in combination with endoscopic necrosectomy in necrotizing pancreatitis (with videos). *Gastrointest Endosc* 2006; **64**: 420-424

6 **Seewald S**, Groth S, Omar S, Imazu H, Seitz U, de Weerth A, Soetikno R, Zhong Y, Sriram PV, Ponnudurai R, Sikka S, Thonke F, Soehendra N. Aggressive endoscopic therapy for pancreatic necrosis and pancreatic abscess: a new safe and effective treatment algorithm (videos). *Gastrointest Endosc* 2005; **62**: 92-100

7 **Voermans RP**, Veldkamp MC, Rauws EA, Bruno MJ, Fockens P. Endoscopic transmural debridement of symptomatic organized pancreatic necrosis (with videos). *Gastrointest Endosc* 2007; **66**: 909-916

8 **Papachristou GI**, Takahashi N, Chahal P, Sarr MG, Baron TH. Peroral endoscopic drainage/debridement of walled-off pancreatic necrosis. *Ann Surg* 2007; **245**: 943-951

9 **Fotoohi M**, D'Agostino HB, Wollman B, Chon K, Shahrokni S, vanSonnenberg E. Persistent pancreatocutaneous fistula after percutaneous drainage of pancreatic fluid collections: role of cause and severity of pancreatitis. *Radiology* 1999; **213**: 573-578

10 **Morgan KA**, Adams DB. Management of internal and external pancreatic fistulas. *Surg Clin North Am* 2007; **87**: 1503-1513, x

11 **Sikora SS**, Khare R, Srikanth G, Kumar A, Saxena R, Kapoor VK. External pancreatic fistula as a sequel to management of acute severe necrotizing pancreatitis. *Dig Surg* 2005; **22**: 446-451; discussion 452

12 **Tajima Y**, Yamanouchi E, Fukuda K, Kitazato A, Kosaka T, Tsutsumi R, Kuroki T, Furui J, Kanematsu T. A secure and less-invasive new method for creation of an internal enteric fistula by using magnets as a therapeutic modality for pancreaticocystocutaneous fistula: a case report. *Gastrointest Endosc* 2004; **60**: 463-467

13 **Kang SG**, Park do H, Kwon TH, Park JY, Park SH, Park JH, Lee SH, Chung IK, Kim HS, Kim SJ. Transduodenal endoscopic necrosectomy via pancreaticoduodenal fistula for infected peripancreatic necrosis with left pararenal space extension (with videos). *Gastrointest Endosc* 2008; **67**: 380-383

14 **Lee TH**, Park do H, Lee SH, Park JH, Park JY, Kim HS, Park SH, Chung IK, Kim SJ. Simultaneous multitransgastric drainage for large and noncontagious pancreatic pseudocysts (with video). *Gastrointest Endosc* 2007; **66**: 1237-1238

15 **Mutignani M**, Iacopini F, Dokas S, Larghi A, Familiari P, Tringali A, Costamagna G. Successful endoscopic closure of a lateral duodenal perforation at ERCP with fibrin glue. *Gastrointest Endosc* 2006; **63**: 725-727

16 **Pescatore P**, Verbeke C, Harle M, Manegold BC. Fibrin sealing in peptic ulcer bleeding: the fate of the clot. *Endoscopy* 1998; **30**: 519-523

17 **Huang CS**, Hess DT, Lichtenstein DR. Successful endoscopic management of postoperative GI fistula with fibrin glue injection: Report of two cases. *Gastrointest Endosc* 2004; **60**: 460-463

18 **Papavramidis ST**, Eleftheriadis EE, Papavramidis TS, Kotzampassi KE, Gamvros OG. Endoscopic management of gastrocutaneous fistula after bariatric surgery by using a fibrin sealant. *Gastrointest Endosc* 2004; **59**: 296-300

19 **Petersen B**, Barkun A, Carpenter S, Chotiprasidhi P, Chuttani R, Silverman W, Hussain N, Liu J, Taitelbaum G, Ginsberg GG. Tissue adhesives and fibrin glues. *Gastrointest Endosc* 2004; **60**: 327-333

S- Editor Li DL **L- Editor** Wang XL **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6096

World J Gastroenterol 2008 October 21; 14(39): 6096-6099
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Perforation of the colon by invading recurrent gastrointestinal stromal tumors during sunitinib treatment

Hoon Hur, Ae Ryoung Park, Sung Bae Jee, Seung Eun Jung, Wook Kim, Hae Myung Jeon

Hoon Hur, Sung Bae Jee, Wook Kim, Hae Myung Jeon, Department of Surgery, College of Medicine, the Catholic University of Korea, Seoul 150-713, South Korea
Ae Ryoung Park, Department of Pharmacy, St. Mary's Hospital, Seoul 150-713, South Korea
Seung Eun Jung, Department of Radiology, College of Medicine, the Catholic University of Korea, Seoul 150-713, South Korea
Author contributions: Hur H wrote the paper; Jeon HM and Kim W designed this case report; Park AR and Jee SB contributed to the information of the drug; Jung SE contributed to the results of imaging studies.
Correspondence to: Hae Myung Jeon, MD, PhD, Department of Surgery, St. Mary's Hospital, College of Medicine, the Catholic University of Korea, 62 Youido-dong, Yeongdeungpo-gu, Seoul 150-713, South Korea. hmjeon@catholic.ac.kr
Telephone: +82-2-37791175 Fax: +82-2-7860802
Received: July 13, 2007 Revised: August 18, 2008
Accepted: August 25, 2008
Published online: October 21, 2008

Abstract

The molecular targets of sunitinib are receptor tyrosine kinases (RTKs), and this drug has also been known to exert blocking effects on the activation of KIT, which is similar to the mechanism of action of imatinib. Moreover, sunitinib has an additional anti-angiogenic effect through its inhibition of the vascular endothelial growth factor receptor activation. We report here a 70-year-old patient diagnosed with a recurrent gastrointestinal stromal tumor (GIST), which invaded the transverse colon and led to a perforation during sunitinib treatment. A computed tomography scan and 3-dimensional reconstruction showed necrosis of the recurrent hepatic mass and perforation of the invaded transverse colon. After percutaneous drainage of the intraperitoneal abscess, antibiotic treatment and restricted diet, the condition of the patient improved. The present case is the first to report that sunitinib, which is administered to treat GIST resistant to imatinib, can cause unexpected colon perforation and subsequent peritonitis.

Key words: Gastrointestinal stromal tumors; Recurrence; Sunitinib; Intestinal perforation

Peer reviewers: Damian Casadesus Rodriguez, MD, PhD, Calixto Garcia University Hospital, J and University, Vedado, Havana City, Cuba; Marc Basson, MD, PhD, MBA, Chief of Surgery, John D. Dingell VA Medical Center, 4646 John R. Street, Detroit, MI 48301, United States

Hur H, Park AR, Jee SB, Jung SE, Kim W, Jeon HM. Perforation of the colon by invading recurrent gastrointestinal stromal tumors during sunitinib treatment. *World J Gastroenterol* 2008; 14(39): 6096-6099 Available from: URL: http://www.wjgnet.com/1007-9327/14/6096.asp DOI: http://dx.doi.org/10.3748/wjg.14.6096

INTRODUCTION

Sutent (Pfizer, New York, NY) is the malate salt of sunitinib, which is a small molecule that inhibits multiple receptor tyrosine kinases (RTKs)[1]. This molecular target is the basis for the application of sunitinib in the treatment of gastrointestinal stromal tumors (GISTs), which result from the molecular abnormalities of tyrosine kinases such as KIT[2]. In addition, the targets of sunitinib involve vascular endothelial growth factor receptors (VEGFR1, VEGFR2 and VEGFR3), platelet-derived growth factor receptors (PDGFRα and PDGFRβ) and the like. Although this drug has been approved by the US Food and Drug Administration (FDA) for treatment of GIST patients following progression or resistance to imatinib (Gleevec; Novartis, Switzerland), randomized phase Ⅲ clinical trials have shown some common adverse effects such as diarrhea, mucositis, abnormal heart function and myelosuppression of imatinib[3]. However, there has never been a report focused on the relationship between bowel perforation and sunitinib treatment. In the present paer, we describe, for the first time, a case of unexpected colon perforation during sunitinib treatment.

CASE REPORT

Here we report a 70-year-old patient who was initially treated with proximal gastrectomy, distal pancreatectomy, splenectomy and transverse colectomy for a GIST of the stomach that was categorized as high risk in 1993. Eight years later, the GIST relapsed on the gastrohepatic ligament, which led to left hepatectomy

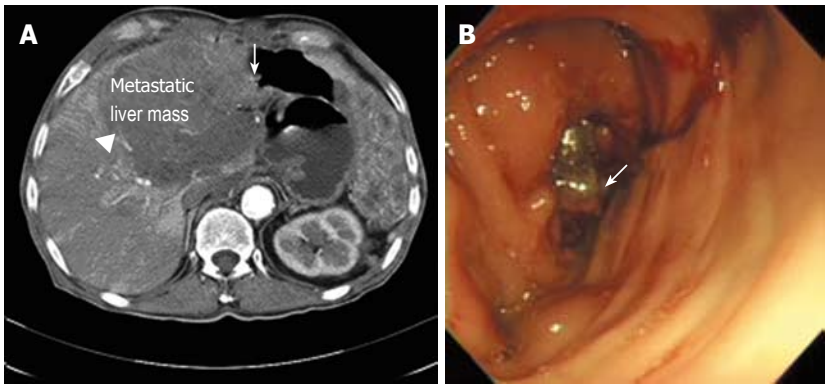


Figure 1 Diagnostic evaluations of recurrent gastrointestinal stromal tumors before starting sunitinib. **A**: In computed tomography, arrowhead and arrow show metastatic liver mass and colon invasion, respectively. **B**: In the colonoscopic finding, arrow shows a protruding mass invading from the external lumen.

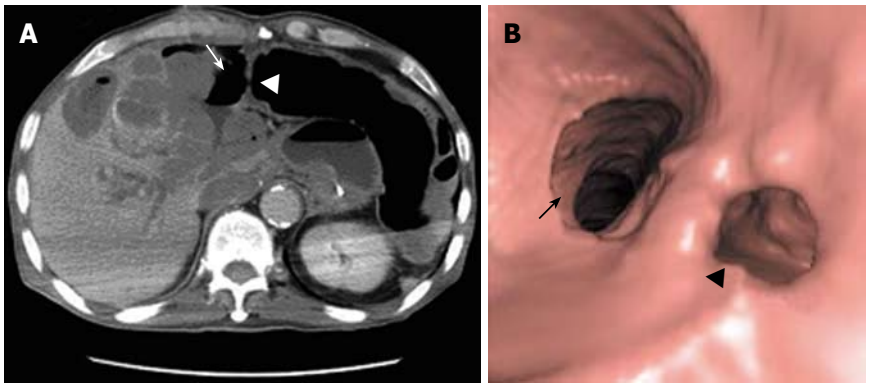


Figure 2 Diagnostic evaluation of perforating colon in present symptomatic patient during sunitinib treatment. **A**: In computed tomography, arrow and arrowhead show intraperitoneal free air and the site of colon perforation, respectively. **B**: In 3-dimensional reconstruction, arrow and arrowhead show the colon lumen and the site of perforation, respectively..

and cholecystectomy to excise the mass curatively. A year later, the tumor recurred in the liver, when imatinib (400 mg/d) was started as the initial treatment. Three years later, a follow-up computed tomography (CT) showed the progression of the hepatic metastasis, which resulted in an escalation in the imatinib dose to 600 mg/d. Unfortunately, two years later in April 2007, the hepatic mass grew so large that it invaded the transverse colon as evaluated by CT and colonoscopy (Figure 1). Consequently, we changed the therapy to oral sunitinib at 50 mg/d with a 4-wk-on and 2-wk-off regimen. He had no combined diseases but a history of smoking with two to thirty packs/year.

In June 2007, the patient was on the second day of the second cycle of sunitinib treatment and complained of diffuse abdominal pain and general prostration upon visiting our emergency room. The patient presented with symptoms and signs of localized peritonitis on the right side of the abdomen. The systolic and diastolic blood pressures were 90 mmHg and 60 mmHg, respectively, pulse rate was 120 per min and respiratory rate was 25 per min. The body temperature was 37.3℃. Laboratory studies were conducted immediately after the patient's arrival at the emergency room. He had anemia (Hb 98 g/L) (normal range: 140-180) and thrombocytopenia (83×10^9/L) (normal range: 150-450), but a WBC count was normal (7.25×10^3/mm^3) with 84% neutrophils. Other laboratory findings were presented as high serum levels of CRP (293.07 mg/L) (normal range: < 5), ALP (416 IU/L) (normal range: 96-254), slightly increased BUN (34 μmol/L) (normal range: 8-23) and creatinine (1.53 μmol/L) (normal range: 0.5-1.2), low level of sodium (127 mEq/L) (normal range: 135-148), potassium (2.8 mEq/L) (normal range: 3.5-5.1) and serum albumin (2.17 g/dL) (normal range: 3.8-5.3), and normal range of GOT (15 IU/L) (normal range: 13-36), GPT (8 IU/L) (normal range: 5-33). A CT scan showed necrosis of the recurrent hepatic mass and perforation of the invaded transverse colon, which led to intraperitoneal air and pus collection (Figure 2A). Three-dimensional reconstruction also revealed colonic perforation (Figure 2B). We could not perform the operation for a correction of colon perforation owing to the poor patient's condition. With percutaneous drainage of the intraperitoneal abscess under guided ultrasonography, the patient's diet was restricted to parenteral nutrition, intravenous fluid administration for correction of dehydration, and both cefoperazone (4 g/d, intravenous) and metronidazole (1500 mg/d, intravenous) were injected for 2 wk. After drainage procedure and conservative treatments, the patient's condition was fortunately improved without operation and laboratory findings 2 wk after treatment were decreased serum CRP level (70.43 mg/L), normal range of platelets (413×10^9/L), BUN (11.3 μmol/L), creatinine (0.72 μmol/L), albumin (3.23 g/dL), sodium (139 mEq/L) and potassium (3.6 mEq/L) (Figure 3). The patient was discharged from the hospital after his condition improved. After completion of the second cycle, the disease was stable according to the response evaluation criteria in the solid tumor group (RECIST) with a 23% decrease in the diameter of the mass.

DISCUSSION

GIST is the most common mesenchymal neoplasm

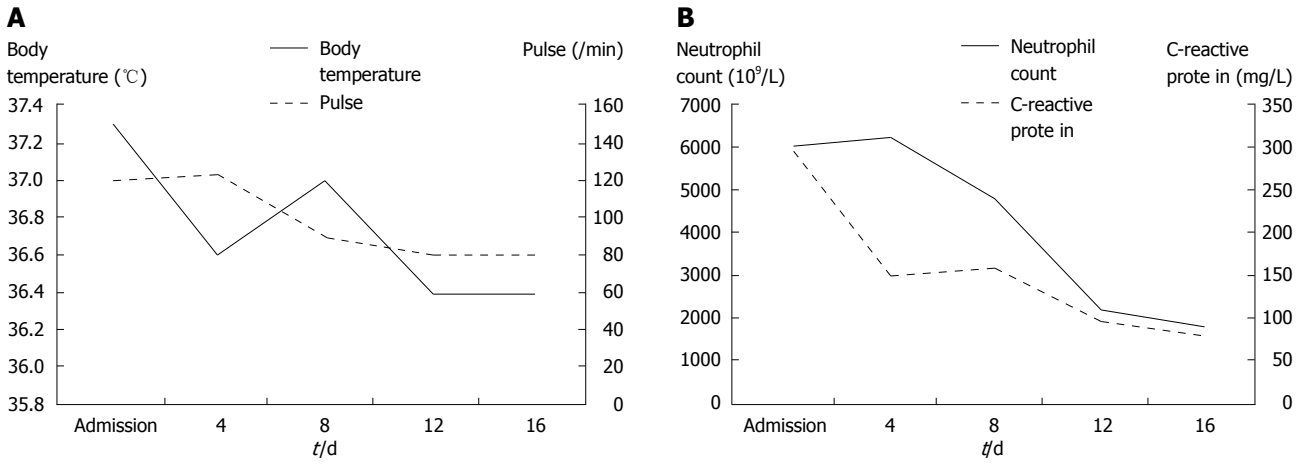


Figure 3 Changes in body temperature and pulse (**A**) and in neutrophil counts and C-reactive protein (**B**) of the patient.

of the gastrointestinal tract. Although this tumor has been known as a neoplasm that is highly resistant to conventional chemotherapy, several recent findings of molecular abnormalities could provide a rationale for treatment with targeted therapies[2]. Recently, applications of several molecularly targeted therapies like imatinib and sunitinib have been proven sufficient for systemic treatment of inoperable or metastatic GIST.

Imatinib is a small molecule tyrosine kinase inhibitor with actions on various transduction molecules such as ABL, BCR-ABL, and KIT[4]. Some clinical trials have shown that imatinib therapy affects prolonged disease-free survival of advanced GIST patients. Sunitinib has recently received approval from the FDA for patients with GIST who are resistant to imatinib[3]. In one randomized controlled trial, sunitinib resulted in a significantly longer time of tumor progression than placebo in patients with GIST after failure of imatinib treatment[5]. Thus, the drug has become a new treatment strategy for these patients, since there is no proven second-line therapy for GIST. The molecular targets of sunitinib are RTKs, and this drug has also been known to have blocking effects on the activation of KIT or PDGFR, which has a similar mechanism of action to imatinib[1]. Moreover, sunitinib exerts an additional anti-angiogenic effect by inhibiting the activation of VEGFR[6]. Because of this effect, sunitinib has been considered beneficial for GIST patients who are resistant to imatinib or cannot tolerate it as a second line treatment[7,8]. A series of clinical studies on sunitinib reported that sunitinib can result in various adverse events, including hypertension, abnormalities of the thyroid and adrenal function, gastrointestinal symptoms such as constipation or diarrhea, and dermatologic abnormalities such as hand-foot syndrome[5,9]. However, bowel perforation associated with the use of sunitinib has not been reported to date. In this case, physical examination of localized tenderness and 3-D reconstructed CT scan confirmed the colon perforation. Then, the perforation was completely treated by percutaneous drainage without operation. This treatment strategy was possible because there was a small amount of colon content localized in a particular area of the peritoneum due to the adhesions from previous operations.

Several cases of colon perforation related to anti-cancer therapy have been reported[10,11], particularly with the VEGFR inhibitor bevacizumab (Avastin; Genentech, South San Francisco, CA), which shares one of the mechanisms of action of sunitinib. A randomized controlled clinical trial showed that bevacizumab, a humanized monoclonal antibody that binds to and neutralizes VEGF, led to gastrointestinal perforation in six of 393 patients (1.5%) who had metastatic colon cancer[10]. Furthermore, another case of colon perforation was documented after treatment with bevacizumab in a patient with non-small cell lung cancer[11]. In addition, the mechanism by which bevacizumab causes bowel perforation is attributed, at least in part, to the increased risk of arterial thrombosis that is observed in the use of drugs targeting VEGF[12,13]. Therefore, this drug is likely to generate rapid tumor degeneration, which in turn leads to colon perforation in patients with bowel metastasis or invasion, due to this inhibitory effect. In addition, the patient's history of smoking might contribute to risk of colon perforation in our case. However, the mechanism of anti-angiogenic agents-induced colon perforation remains to be elucidated.

In conclusion, sunitinib, which is administered to treat GISTs resistant to imatinib, causes unexpected colon perforation and subsequent peritonitis. Hence, careful attention and appropriate clinical evaluation are required for patients presenting with gastrointestinal symptoms during sunitinib treatment.

REFERENCES

1 **Abrams TJ**, Lee LB, Murray LJ, Pryer NK, Cherrington JM. SU11248 inhibits KIT and platelet-derived growth factor receptor beta in preclinical models of human small cell lung cancer. *Mol Cancer Ther* 2003; **2**: 471-478

2 **Rubin BP**, Heinrich MC, Corless CL. Gastrointestinal stromal tumour. *Lancet* 2007; **369**: 1731-1741

3 **Rock EP**, Goodman V, Jiang JX, Mahjoob K, Verbois SL, Morse D, Dagher R, Justice R, Pazdur R. Food and Drug Administration drug approval summary: Sunitinib malate for the treatment of gastrointestinal stromal tumor and

advanced renal cell carcinoma. *Oncologist* 2007; **12**: 107-113
4 **Buchdunger E**, Cioffi CL, Law N, Stover D, Ohno-Jones S, Druker BJ, Lydon NB. Abl protein-tyrosine kinase inhibitor STI571 inhibits in vitro signal transduction mediated by c-kit and platelet-derived growth factor receptors. *J Pharmacol Exp Ther* 2000; **295**: 139-145
5 **Demetri GD**, van Oosterom AT, Garrett CR, Blackstein ME, Shah MH, Verweij J, McArthur G, Judson IR, Heinrich MC, Morgan JA, Desai J, Fletcher CD, George S, Bello CL, Huang X, Baum CM, Casali PG. Efficacy and safety of sunitinib in patients with advanced gastrointestinal stromal tumour after failure of imatinib: a randomised controlled trial. *Lancet* 2006; **368**: 1329-1338
6 **Mendel DB**, Laird AD, Xin X, Louie SG, Christensen JG, Li G, Schreck RE, Abrams TJ, Ngai TJ, Lee LB, Murray LJ, Carver J, Chan E, Moss KG, Haznedar JO, Sukbuntherng J, Blake RA, Sun L, Tang C, Miller T, Shirazian S, McMahon G, Cherrington JM. In vivo antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: determination of a pharmacokinetic/pharmacodynamic relationship. *Clin Cancer Res* 2003; **9**: 327-337
7 **Motzer RJ**, Michaelson MD, Redman BG, Hudes GR, Wilding G, Figlin RA, Ginsberg MS, Kim ST, Baum CM, DePrimo SE, Li JZ, Bello CL, Theuer CP, George DJ, Rini BI. Activity of SU11248, a multitargeted inhibitor of vascular endothelial growth factor receptor and platelet-derived growth factor receptor, in patients with metastatic renal cell carcinoma. *J Clin Oncol* 2006; **24**: 16-24
8 **Potapova O**, Laird AD, Nannini MA, Barone A, Li G, Moss KG, Cherrington JM, Mendel DB. Contribution of individual targets to the antitumor efficacy of the multitargeted receptor tyrosine kinase inhibitor SU11248. *Mol Cancer Ther* 2006; **5**: 1280-1289
9 **Desai J**, Yassa L, Marqusee E, George S, Frates MC, Chen MH, Morgan JA, Dychter SS, Larsen PR, Demetri GD, Alexander EK. Hypothyroidism after sunitinib treatment for patients with gastrointestinal stromal tumors. *Ann Intern Med* 2006; **145**: 660-664
10 **Hurwitz H**, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 2004; **350**: 2335-2342
11 **Gray J**, Murren J, Sharma A, Kelley S, Detterbeck F, Bepler G. Perforated viscus in a patient with non-small cell lung cancer receiving bevacizumab. *J Thorac Oncol* 2007; **2**: 571-573
12 **Ratner M**. Genentech discloses safety concerns over Avastin. *Nat Biotechnol* 2004; **22**: 1198
13 **Heinzerling JH**, Huerta S. Bowel perforation from bevacizumab for the treatment of metastatic colon cancer: incidence, etiology, and management. *Curr Surg* 2006; **63**: 334-337

S- Editor Li DL **L- Editor** Wang XL **E- Editor** Zhang WB

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6100

World J Gastroenterol 2008 October 21; 14(39): 6100-6103
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Concomitant gastric carcinoid and gastrointestinal stromal tumors: A case report

Ying-Lung Lin, Chang-Kou Wei, Jui-Kun Chiang, An-Liang Chou, Chih-Wei Chen, Chih-En Tseng

Ying-Lung Lin, Jui-Kun Chiang, Department of Family Medicine, Buddhist Dalin Tzu Chi General Hospital, Chiayi 622, Taiwan, China
Chang-Kou Wei, Department of General Surgery, Buddhist Dalin Tzu Chi General Hospital, Chiayi 622, Taiwan, China
An-Liang Chou, Department of Gastroenterology, Buddhist Dalin Tzu Chi General Hospital, Chiayi 622, Taiwan, China
Chih-Wei Chen, Department of Cardiology, Buddhist Dalin Tzu Chi General Hospital, Chiayi 622, Taiwan, China
Chih-En Tseng, Department of Anatomic Pathology, Buddhist Dalin Tzu Chi General Hospital, Chiayi 622, Taiwan, China; School of Medicine, Buddhist Tzu Chi University, Hualien, Taiwan, China
Author contributions: Lin YL performed the endoscopic examination of the tumor; Wei CK performed the operation of the tumor; Chiang JK, Chou AL, and Chen CW collected the data; Tseng CE made the pathological diagnosis of the tumor; Lin YL and Tseng CE wrote the paper.
Correspondence to: Chih-En Tseng, Department of Anatomic Pathology, Buddhist Dalin Tzu Chi General Hospital, 2 Min Sheng Road, Dalin, Chiayi 622, Taiwan, China. p121521@tzuchi.com.tw
Telephone: +886-5-2648000-5725 Fax: +886-5-2648999
Received: March 6, 2008 Revised: May 20, 2008
Accepted: May 27, 2008
Published online: October 21, 2008

Abstract

A gastric carcinoid tumor concomitant with gastrointestinal stromal tumor (GIST) is rarely encountered in clinical practice. We report a 65-year-old female who had a 0.8 cm gastric carcinoid tumor on the posterior wall of the upper gastric corpus detected during an esophagogastroduodenoscopy at a routine physical examination, and a concomitant 1.1 cm GIST on the anterior wall of the upper gastric corpus incidentally found during surgery of the gastric carcinoid tumor. Normal serum gastrin level and histological findings suggested that she had a type Ⅲ gastric carcinoid tumor and a GIST which were categorized a very low risk of malignancy, based on their small size and lack of mitosis. Both tumors were treated successfully by surgical excision. The patient had an uneventful recovery. Neither recurrence nor metastasis was found after a 28-mo follow-up.

Key words: Gastric carcinoid tumor; Gastrointestinal stromal tumor; Esophagogastroduodenoscopy; Digestive system

Peer reviewer: Xin Chen, Professor, University of California, 513 Parnassus Ave. S-816, Box 0912, San Francisco 94143, United States

Lin YL, Wei CK, Chiang JK, Chou AL, Chen CW, Tseng CE. Concomitant gastric carcinoid and gastrointestinal stromal tumors: A case report. *World J Gastroenterol* 2008; 14(39): 6100-6103 Available from: URL: http://www.wjgnet.com/1007-9327/14/6100.asp DOI: http://dx.doi.org/10.3748/wjg.14.6100

INTRODUCTION

Gastrointestinal neuroendocrine tumors are derived from the diffuse neuroendocrine system of the gastrointestinal (GI) tract, composed of amine- and acid-producing cells with different hormonal profiles, depending on their site of origin[1]. Gastrointestinal stromal tumors (GISTs) are mesenchymal tumors arising from interstitial Cajal cells of the wall of the GI tract[2,3]. GISTs can be distinguished from other mesenchymal tumors by optimal immunostaining for CD117, and a prognostic classification is based on tumor size, mitotic score, and MIB-1 grade[4]. Gain-of-function mutation of the *c-kit* gene, and immunoreactivity of the c-kit protein (CD117) in many GIST support the idea that GIST is a biologically distinct entity. Both carcinoid tumors and GISTs are malignant or potentially malignant tumors, and are considered to have a specific molecular pathogenesis. Herein we report a gastric carcinoid tumor concomitant with a gastric GIST, and also provide a review of the literature.

CASE REPORT

In October 2005, a 65-year-old Asian female came to our hospital for a routine physical examination. She had no history of peptic ulcer, epigastralgia, abdominal pain, diarrhea, flushing, or palpitations. Esophagogastroduodenoscopy showed an approximately 0.8 cm sessile polypoid lesion, with superficial reddish striation, on the posterior wall of the upper gastric corpus (Figure 1). A biopsy sample was taken and eight specimens were acquired. Histological studies showed a gastric mucosa tumor. The tumor demonstrated uniform ovoid cells with cordal and small nestic patterns within the lamina propria. After immunohistochemical

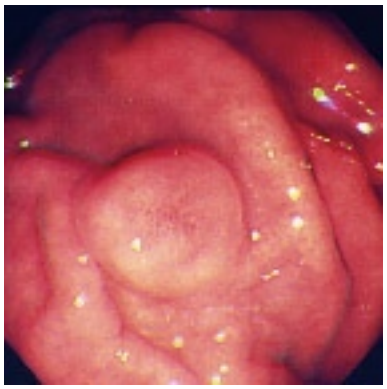


Figure 1 EGD endoscopy showing a protruding polypoid mass with superficial reddish striation on the posterior wall of the gastric upper corpus.

(IHC) staining, the tumor cells were positive for cytokeratin, synaptophysin, and chromogranin-A (Figure 2A-D). The Ki-67 index was < 1%. A gastric carcinoid tumor was diagnosed pathologically, and Helicobacter-like microorganisms were also found. A biopsy urease test (CLO test) for *Helicobacter pylori* (*H pylori*) infection also demonstrated a positive reaction. The serum gastrin level was 34.4 ng/L (normal range, 25-111 ng/L). Owing to the tumor's potentially malignant nature, surgery was performed one week later. During the surgical procedure, in addition to the 0.8 cm carcinoid tumor, a 1.1 cm tumor was found incidentally over the serosal side of the anterior wall of the upper gastric corpus. Both small tumors were locally resected simultaneously and separately. Grossly, a patch of gastric tissue, measuring 3.9 cm × 2.5 cm × 0.7 cm, from the posterior wall of the upper corpus disclosed a 0.8 cm × 0.6 cm × 0.3 cm carcinoid tumor. The other gastric tissue taken from the anterior wall of the upper gastric corpus, measuring 2.5 cm × 2.0 cm× 0.5 cm, demonstrated a 1.1 cm × 0.7 cm × 0.3 cm tumor within the muscularis propria of the gastric wall. Histologically, this 1.1 cm tumor displayed swirling bundles of spindle cells with focally palisading areas within the fibrotic stroma. Neither mitotic activity nor tumor necrosis was found. The spindle tumor cells were positive for CD117, CD34, neuron-specific enolase, and S-100 protein, but negative for actin-M851 and glial fibrillary acidic protein after IHC staining (Figure 2E-H). Finally, a gastric carcinoid tumor and a GIST were pathologically diagnosed. The patient had an uneventful recovery, and was discharged one week after surgery. After a 28-mo follow-up, there was no evidence of tumor recurrence or metastasis.

DISCUSSION

Gastric carcinoid tumor concomitant with gastric GIST is clinically rare. To the best of our knowledge, it has not been reported in the English literature. The pathogenesis of gastric carcinoid tumor concomitant with GIST is unclear. It was reported that *H pylori* are related to the pathogenesis of gastric carcinoma and mucosa-associated lymphoid tumor[5,6]. We previously reported a case of a 0.4-cm GIST concomitant with an early gastric cancer in 2005[7], and between 2003 and 2007, we intermittently detected one case of multiple 0.2-cm to 0.5-cm gastric neuroendocrine tumors and additional 5 cases of 0.4-15 cm gastric GISTs. All except for 2 of the cases yielded positive CLO tests. The present case also showed positive *H pylori* infection. However, this finding is more like an incidental event rather than a causal association. Whether the concomitant carcinoid tumor with GIST correlates to *H pylori* infection or not requires more collected cases and further studies.

Gastric carcinoids are classified into three subtypes, all of which originate from gastric enterochromaffin-like cells in the gastric mucosa. The first subtype is combined with chronic atrophic gastritis (type Ⅰ). The second subtype, Zollinger-Ellison syndrome, is nearly a part of the multiple endocrine neoplasia-1 (MEN-1) syndrome (type Ⅱ). Clinically, these two subtypes are linked to a hypergastrinemic state. The third sporadic subtype (type Ⅲ) occurs without hypergastrinemia but takes an aggressive course, with 54%-66% metastasis[1]. As stated by Shinohara and colleagues, even a 0.5-cm carcinoid tumor can present with metastasis[8]. On account of neither atrophic gastric mucosa nor elevated seral gastrin level in our case, a small type Ⅲ carcinoid tumor was favored. The potential for metastasis cannot be ignored and demands close follow-up.

Gastric carcinoids may have different clinical features in different locations of GI tract, including abdominal pain, vomiting, and anemia[9]. Carcinoid tumor associated with vascular malformation may cause massive gastric bleeding[10]. Carcinoid syndrome with symptoms of flushing, diarrhea, abdominal pain, cutaneous edema, and bronchoconstriction is uncommon. Due to a small nonfunctional carcinoid, our case never experienced any GI symptom or carcinoid syndrome.

Since 1999, GISTs have been considered to be a group of mesenchymal neoplasms arising from interstitial Cajal cells of the gastrointestinal walls[2,3]. GISTs are now preferentially defined as tumors with c-kit (CD117) positive mesenchymal spindle cells or epithelioid neoplasms, found primarily in the GI tract, omentum, and mesentery[11]. The most important manifestation of this tumor is its indolent, slow-growing nature. This tumor is generally found within the deeper stroma and the submucosa, and incidentally during an imaging study and surgery. In our case, a GIST protruding to the serosal side of the gastric wall was found incidentally during a surgical procedure. Histologically, it arose from the muscularis propria of the gastric wall.

Patients with GIST often present with nonspecific symptoms, such as nausea, vomiting, abdominal pain, GI bleeding, and may have metastatic disease. Bleeding is the most common symptom. The tumor size and mitotic score are considered important diagnostic criteria and prognostic predictive indicators[12]. Our case was asymptomatic and diagnosed as GIST with a very low risk of malignancy based on its small size and lack of mitosis and was positive for CD117 after IHC staining.

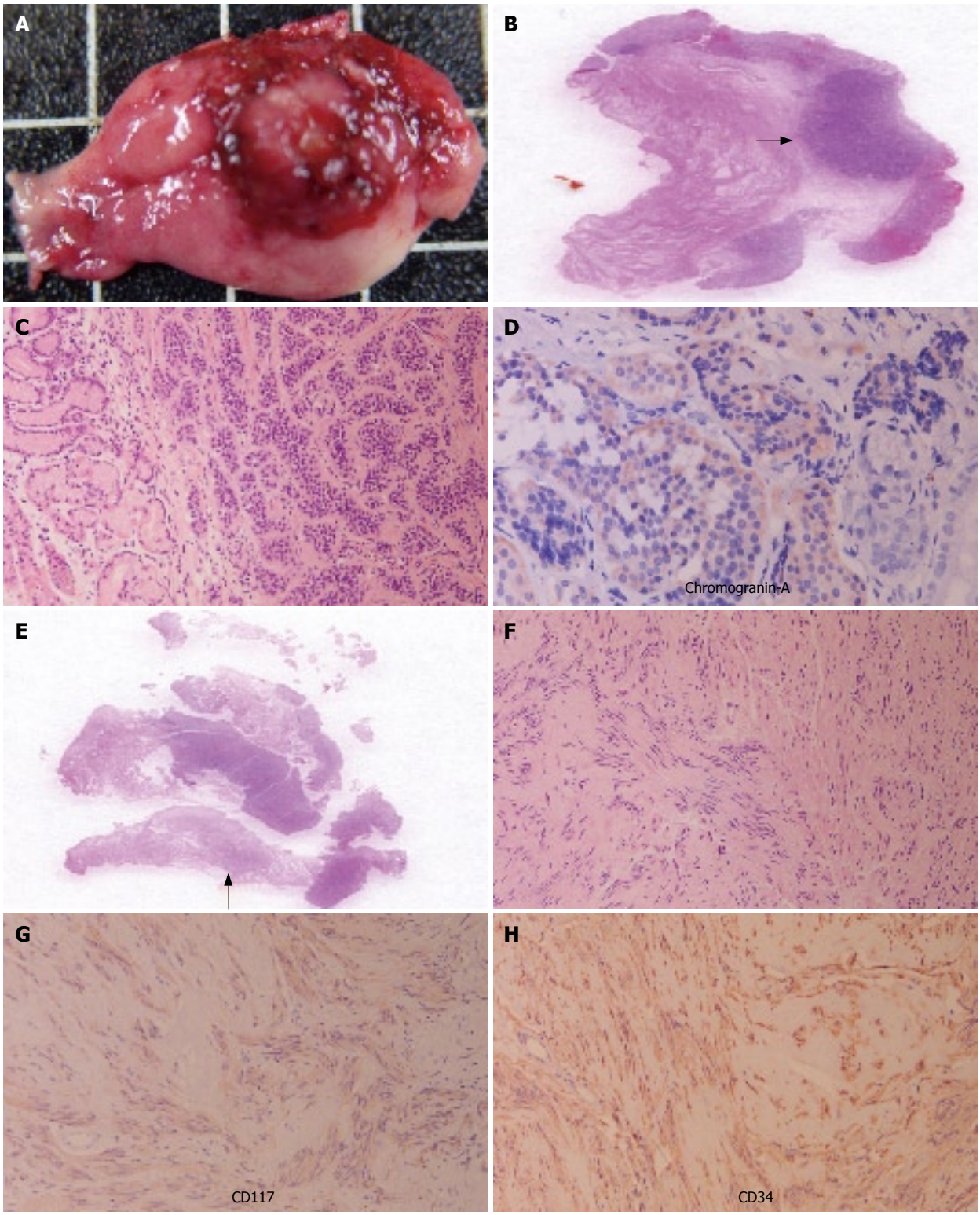


Figure 2 Postoperative wedge-shaped gastric tissue demonstrating a 0.8-cm centrally polypoid mass (**A**), scanning microscopic view disclosing a crescent ulcerative mass (arrow) involving gastric mucosa and submucosa (**B**), carcinoid tumor (right half) displaying uniform ovoid cells in chordal and small nestic patterns (hematoxylin and eosin stain, × 200) (**C**), chromogranin a positively stained tumor cells (IHC staining, × 400) (**D**), stromal tumor (arrow) within the gastric wall (scanning microscopic view) (**E**), tumor cells demonstrating whirling and palisading spindle cells interlaced with smooth muscle cells of gastric muscularis propria (hematoxylin and eosin stain, × 200) (**F**), CD117 (**G**) and CD34 (**H**) stained spindle tumor cells.

Treatment modalities for non-metastatic small carcinoid tumors include endoscopic mucosal resection, minimally invasive laparoscopic wedge resection, and surgery[13,14]. To date, surgery is the mainstay and the only potentially curative therapy for carcinoid tumors. Treatment modalities for metastatic carcinoid tumors include orthotopic liver transplant, hepatic artery embolization, and somatostatin analog, adjuvant indium-111 octreotide-receptor targeted therapy[9]. Therapeutic options for GISTs include surgery and

treatment with STI-571 (Gleevec). When inoperative, residual or recurrent tumor exists, STI-571 is the choice[15,16]. Owing to the treatment of two synchronized small gastric tumors in our case, local resections were performed simultaneously and separately. No evidence of tumor recurrence or metastasis was found after a 28-mo follow-up period.

In conclusion, we report a rare case of small gastric carcinoid tumor concomitant with a small gastric GIST with no clinical symptoms and positive *H pylori* infection. More studies are required for evaluating the relation between *H pylori* infections and tumorigenesis of concomitant gastric carcinoid and gastric GIST. A long term follow-up period of all carcinoids and GISTs is greatly needed, due to their potential for metastasis.

ACKNOWLEDGMENTS

The authors greatly appreciate the financial and technical support of Dalin Tzu Chi General Hospital, and the help of the President of Japan Jichi Medical University, Professor Fumimaro Takaku, who kindly revised the manuscript.

REFERENCES

1 **Jensen RT**. Neuroendocrine tumors of the gastrointestinal tract and pancreas. In: Kasper DL, Braunwald E, Fauci AS, Hauser SL, Longo DL, Jameson JL. Harrison's Principles of Internal Medicine. 16th ed. New York: McGraw-Hill, 2005: 2220-2226

2 **Sircar K**, Hewlett BR, Huizinga JD, Chorneyko K, Berezin I, Riddell RH. Interstitial cells of Cajal as precursors of gastrointestinal stromal tumors. *Am J Surg Pathol* 1999; **23**: 377-389

3 **Robinson TL**, Sircar K, Hewlett BR, Chorneyko K, Riddell RH, Huizinga JD. Gastrointestinal stromal tumors may originate from a subset of CD34-positive interstitial cells of Cajal. *Am J Pathol* 2000; **156**: 1157-1163

4 **Hasegawa T**, Matsuno Y, Shimoda T, Hirohashi S. Gastrointestinal stromal tumor: consistent CD117 immunostaining for diagnosis, and prognostic classification based on tumor size and MIB-1 grade. *Hum Pathol* 2002; **33**: 669-676

5 **Moss SF**, Malfertheiner P. Helicobacter and gastric malignancies. *Helicobacter* 2007; **12** Suppl 1: 23-30

6 **Wotherspoon AC**, Doglioni C, Diss TC, Pan L, Moschini A, de Boni M, Isaacson PG. Regression of primary low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of Helicobacter pylori. *Lancet* 1993; **342**: 575-577

7 **Lin YL**, Tzeng JE, Wei CK, Lin CW. Small gastrointestinal stromal tumor concomitant with early gastric cancer: a case report. *World J Gastroenterol* 2006; **12**: 815-817

8 **Shinohara T**, Ohyama S, Nagano H, Amaoka N, Ohta K, Matsubara T, Yamaguchi T, Yanagisawa A, Kato Y, Muto T. Minute gastric carcinoid tumor with regional lymph node metastasis. *Gastric Cancer* 2003; **6**: 262-266

9 **Leung LK**, Ng EKW, Sung JJY. Tumors of the Stomach. In: Yamada T, Alpers DH, Laine L, Kaplowitz N, Owyang C, Powell DW. Textbook of Gastroenterology. 4th ed. Philadephia: Lipincott, Williams & Wilkins, 2003: 1434-1435

10 **Roncoroni L**, Costi R, Canavese G, Violi V, Bordi C. Carcinoid tumor associated with vascular malformation as a cause of massive gastric bleeding. *Am J Gastroenterol* 1997; **92**: 2119-2121

11 **Miettinen M**, Lasota J. Gastrointestinal stromal tumors--definition, clinical, histological, immunohistochemical, and molecular genetic features and differential diagnosis. *Virchows Arch* 2001; **438**: 1-12

12 **Miettinen M**, Sarlomo-Rikala M, Sobin LH, Lasota J. Gastrointestinal stromal tumors and leiomyosarcomas in the colon: a clinicopathologic, immunohistochemical, and molecular genetic study of 44 cases. *Am J Surg Pathol* 2000; **24**: 1339-1352

13 **Kadikoylu G**, Yavasoglu I, Yukselen V, Ozkara E, Bolaman Z. Treatment of solitary gastric carcinoid tumor by endoscopic polypectomy in a patient with pernicious anemia. *World J Gastroenterol* 2006; **12**: 4267-4269

14 **Ishikawa K**, Etoh T, Shiromizu A, Inomata M, Shiraishi N, Kashima K, Kitano S. A case of sporadic gastric carcinoid tumor treated successfully by laparoscopy-assisted distal gastrectomy. *Surg Laparosc Endosc Percutan Tech* 2005; **15**: 348-350

15 **Nishida T**, Hirota S. Biological and clinical review of stromal tumors in the gastrointestinal tract. *Histol Histopathol* 2000; **15**: 1293-1301

16 **Duffaud F**, Blay JY. Gastrointestinal stromal tumors: biology and treatment. *Oncology* 2003; **65**: 187-197

S- Editor Zhong XY **L- Editor** Wang XL **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2008 October 21; 14(39): 6104
World Journal of Gastroenterology ISSN 1007-9327

ACKNOWLEDGMENTS

Acknowledgments to Reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

Marc Basson, MD, PhD, MBA, Chief of Surgery
John D. Dingell VA Medical Center, 4646 John R. Street, Detroit, MI 48301, United States

Carla W Brady, MD, MHS
Duke University Medical Center, Division of Gastroenterology, DUMC Box 3913, Durham, NC 27705, United States

Roberto de Franchis, Professor, Professor of Medicine (Gastroenterology)
Department of Medical Siences, University of Milan, Head, Gastroenterology 3 Unit, IRCCS Ospedale Maggiore Policlinico, Mangiagalli and Regina Elena Foundation, Via Pace 9, 20122 Milano, Italy

Giovanni D De Palma, Professor
Department of Surgery and Advanced Technologies, University of Naples Federico II, School of Medicine, Naples 80131, Italy

James E East, BSc, MBChB, MRCP
St. Mary's Hospital, Endoscopy Unit, Clarence Wing, 3rd Floor, Praed Street, London, W2 1NY, United Kingdom

Ronnie Fass MD, FACP, FACG, Professor
Internal Medicine, University of Arizona Head, Neuroenteric Clinical Research Group and Director of GI Motility Laboratories, University of Arizona Health Sciences Center and Southern Arizona VA Health Care System, Section of Gastroenterology (1-111-GI) 3601 S. 6th Ave., Tucson, AZ 85723, United States

Diego, MD Garcia-Compean, Professor
Faculty of Medicine, University Hospital, Department of Gastroenterology, Autonomous University of Nuevo Leon, Ave Madero y Gonzalitos, 64700 Monterrey, NL, México

Keiji Hirata, MD
Surgery 1, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan

Samuel B Ho, MD, Chief
Gastroenterology Section (111D), VA San Diego Healthcare System, 3350 La Jolla Village Drive, San Diego, CA 92161, United States

Michael Horowitz, Professor
Department of Medicine, University of Adelaide and Director, Endocrine and Metabolic Unit, Royal Adelaide Hospital, Level 6, Eleanor Harrald Bullding, North Terrace, Adelaide 5000, Australia

Anders E Lehmann, PhD
Associate Professor, Senior Principal Scientist, Bioscience, AstraZeneca R&D Mölndal, Mölndal, Sweden

Ton Lisman, PhD
Thrombosis and Haemostasis Laboratory, Department of Haematology G.03.550, University Medical Centre, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands

Wendy M Mars, PhD
Department of Pathology, University of Pittsburgh, S-411B South Biomedical Science Tower Pittsburgh, PA 15261, United States

Emiko Mizoguchi, MD, PhD
Department of Medicine, Gastrointestinal Unit, GRJ 702, Massachusetts General Hospital, Boston, MA 02114, United States

Peter L Moses, MD, FACG, AGAF, Professor
University of Vermont College of Medicine Section of Gastroenterology & Hepatology, 111 Colchester Avenue, Smith 237B, MCHV, Burlington, VT 05401, United States

Shotaro Nakamura, MD
Department of Medicine and Clinical Science, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan

Hans Seifert, MD
Gastroenterology and Hepatology, Klinikum Oldenburg, MD Eden-Str.10, Oldenburg 26133, Germany

Dr. Marco Senzolo
Liver Transplantation and Hepatobiliary Unit, Royal Free Hospital, London, United Kingdom

Shanthi V Sitaraman, MD
Division of Digestive Diseases, Room 201-F, 615, Michael Street, Whitehead Research Building, Emory University, Atlanta, Georgia 30322, United States

Ned Snyder, MD, FACP, AGAF, Professor of Medicine, Chief of Clinical Gastroenterology and Hepatology
Department of Internal Medicine, The University of Texas Medical Branch, 301 University Blvd., Galveston, Texas77555-0764, United States

Bruno Stieger, Professor
Department of Medicine, Division of Clinical Pharmacology and Toxicology, University Hospital, Zurich 8091, Switzerland

Christian D Stone, MD, MPH, Director
Inflammatory Bowel Disease Program, Assistant Professor of Medicine, Division of Gastroenterology, Washington University School of Medicine, 660 South Euclid Avenue, Campus Box 8124, Saint Louis, MO 63110, United States

Wing-Kin Syn, MD
Division of Gastroenterology, GSRB-1, Suite 1073, DUMC 3256, 595 LaSalle Street, Durham, NC27710, United States

Frank I Tovey, OBE, ChM, FRCS, Honorary Research Felllow
Department of Surgery, University College London, London, United Kingdom

Dr. Stefan Wirth, Professor
Children's Hospital, Heusnerstt. 40, Wuppertal 42349, Germany

Harry HX Xia, PhD, MD
Novartis Pharmaceuticals Corporation, One Health Plaza, East Hanover, NJ 07936-1080, United States

Yuan Yuan, Professor
Cancer Institute of China Medical University, 155 North Nanjing Street, Heping District, Shenyang 110001, Liaoning Province, China

World J Gastroenterol 2008 October 21; 14(39): 6105
World Journal of Gastroenterology ISSN 1007-9327

Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
January 24-25, Frankfurt, Germany
Falk Workshop: Perspectives in Liver
Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
E-mail: education@asge.org

June 4-7, Helsinki, Finland
The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
ESGAR 2008 19th Annual Meeting and Postgraduate Course
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden
16th International Congress of the European Association for Endoscopic Surgery
E-mail: info@eaes-eur.org

June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
10th World Congress on Gastrointestinal Cancer
Imedex and ESMO
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland
Joint Meeting of the European Pancreatic Club (EPC)
and the International Association of Pancreatology (IAP)
E-mail: office@epc-iap2008.org
www.e-p-c.org
www.pancreatology.org

June 26-28, Bratislava, Slovakia
5th Central European Gastroenterology Meeting
www.ceurgem2008.cz

July 9-12, Paris, France
ILTS 14th Annual International Congress
www.ilts.org

September 10-13, Budapest, Hungary
11th World Congress of the International Society for Diseases of the Esophagus
E-mail: isde@isde.net

September 13-16, New Delhi, India
Asia Pacific Digestive Week
E-mail: apdw@apdw2008.net

APDW 2008
September 13-16, New Delhi, Indian
Organized: Indian Society of Gastroenterology

III FALK GASTRO-CONFERENCE
September 17, Mainz, Germany
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
Falk Symposium 166:
GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
Falk Symposium 167:
Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
Third Annual Meeting
European Society of Coloproctology
www.escp.eu.com

October 8-11, Istanbul, Turkey
18th World Congress of the International Association of Surgeons,
Gastroenterologists and Oncologists
E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria
16th United European Gastroenterology Week
www.negf.org
www.acv.at

October 22-25, Minnesota, USA
Anstralian Gastroenterology Week 2008
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia
71st Annual Colon and Rectal Surgery Conference
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA
59th AASLD Annual Meeting and Postgraduate Course
The Liver Meeting
Information: www.aasld.org

November 6-9, Lucerne, Switzerland
Neurogastroenterology & Motility Joint International Meeting 2008
E-mail: ngm2008@mci-group.com
www.ngm2008.com

November 12, Santiago de Chile, Chile
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt
1st Hepatology and Gastroenterology Post Graduate Course
www.egyptgastrohep.com

December 7-9, Seoul, Korea
6th International Meeting
Hepatocellular Carcinoma: Eastern and Western Experiences
E-mail: sglee@amc.seoul.kr

INFORMATION FOR ALL
FALK FOUNDATION e.V.
E-mail: symposia@falkfoundation.de
www.falkfoundation.de

Advanced Courses - European Institute of Telesurgery EITS - 2008
Strasbourg, France
January 18-19, March 28-29, June 6-7, October 3-4

N.O.T.E.S
April 3-5, November 27-29
Laparoscopic Digestive Surgery

June 27-28, November 7-8
Laparoscopic Colorectal Surgery

July 3-5
Interventional GI Endoscopy Techniques
Contact address for all courses:
E-mail: info@eits.fr

International Gastroenterological Congresses 2009
March 23-26, Glasgow, Scotland
Meeting of the British Society of Gastroenterology (BSG)
E-mail: bsg@mailbox.ulcc.ac.uk

May 17-20, Denver, Colorado, USA
Digestive Disease Week 2009

November 21-25, London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2008 October 21; 14(39): 6106-6108
World Journal of Gastroenterology ISSN 1007-9327

Instructions to authors

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol* ISSN 1007-9327 CN 14-1219/R) is a weekly open access peer-reviewed journal supported by an editorial board consisting of 1208 experts in gastroenterology and hepatology from 60 countries. The aim of the journal is to deliver the most clinically relevant original and commentary articles to readers, and to make the full text publicly available to all clinicians, scientists, patients and biomedical students on an unrestricted platform, so that they can access and learn about the most recent key advances in the field.

In addition to the open access nature, another key characteristic of *WJG* is its reading guidance for each article which includes background, research frontier, related reports, breakthroughs, applications, terminology, and comments of peer reviewers for the general readers.

WJG publishes articles on esophageal, gastrointestinal, hepatobiliary and pancreatic tumors, and other esophageal, gastrointestinal, hepatic-biliary and pancreatic diseases in relation to epidermiology, immunology, microbiology, motility & nerve-gut interaction, endocrinology, nutrition & obesity, endoscopy, imaging and advanced hi-technology.

The main goal of *WJG* is to publish high quality commentary articles contributed by leading experts in gastroenterology and hepatology and original articles that combine the clinical practice and advanced basic research, to provide an interactive platform for clinicians and researchers in internal medicine, surgery, infectious diseases, traditional Chinese medicine, oncology, integrated Chinese and Western medicine, imaging, endoscopy, interventional therapy, pathology and other basic medical specialities, and thus eventually improving the clinical practice and healthcare for patients.

Indexed and abstracted in

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, Index Medicus, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology* and *Hepatology*, CAB Abstracts and Global Health. ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

Published by

The WJG Press

SUBMISSION OF MANUSCRIPTS

Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of The WJG Press, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the International Committee of Medical Journal Editors to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now available, to our knowledge, is http://www. clinicaltrials.gov sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

Authors should retain one copy of the text, tables, photographs and illustrations because rejected manuscripts will not be returned to the author(s) and the editors will not be responsible for loss or damage to photographs and illustrations sustained during mailing.

Online submissions

Manuscripts should be submitted through the Online Submission System at: http://wjg.wjgnet.com/wjg. Authors are highly recommended to consult the ONLINE INSTRUCTIONS TO AUTHORS (http://www.wjgnet.com/wjg/help/instructions.jsp) before attempting to submit online. For assistance, authors encountering problems with the Online Submission System may send an email describing the problem to submission@wjgnet.com, or by telephone: +86-10-85381892. If you submit your manuscript online, do not make a postal contribution. Repeated online submission for the same manuscript is strictly prohibited.

MANUSCRIPT PREPARATION

All contributions should be written in English. All articles must be submitted using word-processing software. All submissions must be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Style should conform to our house format. Required information for each of the manuscript sections is as follows:

Title page

Full manuscript title, running title, all author(s) name(s), affiliations, institution(s) and/or department(s) where the work was carried out; author contributions; disclosure of any financial support for the research; and the name, full address, telephone and fax numbers and email address of the corresponding author should be included. Titles should be concise and informative (remove all unnecessary words), emphasize what is new, and avoid abbreviations. A short running title of less than 40 letters should be provided. List the author(s)' name(s) as follows: initial and/or first name, middle name or initial(s), and full family name.

Author controbutions: The format of this section should be like this: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed research; Wang CL, Zou CC, Hong F and Wu XM performed research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed data; and Wang CL, Liang L and Fu JF wrote the paper.

Peer reviewers: All articles received are subject to peer review. Normally, three experts are invited for each article. Decision for acceptance is made only when at least two experts recommend an article for publication. Reviewers for accepted manuscripts are acknowledged in each manuscript, and reviewers of articles which were not accepted will be acknowledged at the end of each issue. To ensure the quality of the articles published in *WJG*, reviewers of accepted manuscripts will be announced by publishing the name, title/position and institution of the reviewer in the footnote accompanying the printed article. For example, reviewers: Professor Jing-Yuan Fang, Shanghai Institute of Digestive Disease, Shanghai, Affiliated Renji Hospital, Medical Faculty, Shanghai Jiaotong University, Shanghai, China; Professor Xin-Wei Han, Department of Radiology, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan Province, China; and Professor Anren Kuang, Department of Nuclear Medicine, Huaxi Hospital, Sichuan University, Chengdu, Sichuan Province, China.

Abstract

An informative, structured abstract of no more than 350 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections: AIM: Only the purpose should be included. METHODS: The materials, techniques, instruments and equipment, and the experimental procedures should be included. RESULTS: The observed and experimental results, including data, effects, outcome, *etc.* should be included. Authors should present *P* value where necessary, and also include any significant data. CONCLUSION: Accurate view and the value of the results should be included.

The format for structured abstracts can be found at: http://www.wjgnet.com/wjg/help/11.doc.

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles, rapid communication

and case reports, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the body text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, should be found at: http://www.wjgnet.com/wjg/help/instructions.jsp.

Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: …*etc.* It is our principle to publish high resolution-figures for the printed and E-versions.

Tables

Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of P values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscripts and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

Coding system

The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability[1,2]". If references are cited directly in the text, they should be put together within the text, for example, "From references[19,22-24], we know that..."

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

PMID requirement

PMID roots in the abstract serial number indexed by PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed). The author should supply the PMID for journal citation. For those references that have not been indexed by PubMed, a printed copy of the first page of the full reference should be submitted.

The accuracy of the information for journal citations is very important. Using the reference testing system, the authors and editor should check the authors name, title, journal title, publication date, volume number, start page, and end page. We will interlink all references with PubMed in an ASP file so that the readers can immediately access the abstract of the citations online.

DOI requirement

A CrossRef DOI® (Digital Object Identifier) name is a unique string created to identify a piece of scholarly content in the online environment. The author should supply the DOIs for journal citation(doi:10.3748/wjg.13.6458). This link (http://www.crossref.org/SimpleTextQuery/) allows you to retrieve Digital Object Identifiers (DOIs) for journal articles, books, and chapters by simply cutting and pasting the reference list into the box. You may use the form with any reference style, although the tool works most reliably if references are formatted in a standard style such as shown in this example: Assimakopoulos SF, Scopa CD, Vagianos CE. Pathophysiology of increased intestinal permeability in obstructive jaundice. *World J Gastroenterol* 2007; 13(48): 6458-6464

The accuracy of the information of journal citations is very important. We will interlink all references with DOI in ASP file so that readers can access the abstracts of cited articles online immediately.

Style for journal references

Authors: the name of the first author should be typed in bold-faced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

Style for book references

Authors: the name of the first author should be typed in bold-faced letters. The surname of all authors should be typed with the initial letter capitalized, followed by their abbreviated middle and first initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR) Book title. Publication number. Publication place: Publication press, Year: start page and end page.

Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Inappropriate references

Authors should always cite references that are relevant to their article, and avoid any inappropriate references. Inappropriate references include those linked with a hyphen when the difference between the two numbers is greater than five. For example, [1-6], [2-14] and [1, 3, 4-10, 22] are all considered inappropriate references. Authors should not cite their own unrelated published articles.

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/wjg/help/15.doc.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H pylori*, *E coli*, *etc.*

SUBMISSION OF THE REVISED MANUSCRIPTS AFTER ACCEPTED

Please revise your article according to the revision policies of *WJG*. The revised version including manuscript and high-resolution image figures (if any) should be copied on a floppy or compact disk. The author should send the revised manuscript, along with printed high-resolution color or black and white photos, copyright transfer letter, and responses to the reviewers by courier (such as EMS/DHL).

Editorial Office

World Journal of Gastroenterology
Editorial Department: Room 903
Ocean International Center, Building D
No. 62 Dongsihuan Zhonglu
Chaoyang District, Beijing 100025, China
E-mail: wjg@wjgnet.com
http://www.wjgnet.com
Telephone: +86-10-59080039
Fax: +86-10-85381893

Language evaluation

The language of a manuscript will be graded before it is sent for revision. (1) Grade A: priority publishing; (2) Grade B: minor language polishing; (3) Grade C: a great deal of language polishing needed; (4) Grade D: rejected. Revised articles should reach Grade A or B.

Copyright assignment form

Please download a Copyright assignment form from http://www.wjgnet.com/wjg/help/9.doc.

Responses to reviewers

Please revise your article according to the comments/suggestions provided by the reviewers. The format for responses to the reviewers' comments can be found at: http://www.wjgnet.com/wjg/help/10.doc.

Proof of financial support

For paper supported by a foundation, authors should provide a copy of the document and serial number of the foundation.

Links to documents related to the manuscript

WJG will be initiating a platform to promote dynamic interactions between the editors, peer reviewers, readers and authors. After a manuscript is published online, links to the PDF version of the submitted manuscript, the peer-reviewers' report and the revised manuscript will be put on-line. Readers can make comments on the peer reviewer's report, authors' responses to peer reviewers, and the revised manuscript. We hope that authors will benefit from this feedback and be able to revise the manuscript accordingly in a timely manner.

Science news releases

Authors of accepted manuscripts are suggested to write a science news item to promote their articles. The news will be released rapidly at EurekAlert/AAAS (http://www.eurekalert.org). The title for news items should be less than 90 characters; the summary should be less than 75 words; and main body less than 500 words. Science news items should be lawful, ethical, and strictly based on your original content with an attractive title and interesting pictures.

Publication fee

Authors of accepted articles must pay a publication fee.
EDITORIAL, TOPIC HIGHLIGHTS, BOOK REVIEWS and LETTERS TO THE EDITOR are published free of charge.

ISSN 1007-9327
CN 14-1219/R

World Journal of Gastroenterology®

Indexed and Abstracted in:

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, *Index Medicus*, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology and Hepatology*, CAB Abstracts and Global Health.
ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

Volume 14 Number 40
October 28, 2008
World J Gastroenterol
2008 October 28; 14(40): 6109-6272

Online Submissions
wjg.wjgnet.com
www.wjgnet.com
Printed on Acid-free Paper
世界胃肠病学杂志

A Weekly Journal of Gastroenterology and Hepatology

World Journal of Gastroenterology®

Editorial Board

2007-2009

Published by The WJG Press and Baishideng
Room 903, Ocean International Center, Building D
No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Fax: +86-10-8538-1893 E-mail: wjg@wjgnet.com http://www.wjgnet.com

The World Journal of Gastroenterology Editorial Board consists of 1208 members, representing a team of worldwide experts in gastroenterology and hepatology. They are from 60 countries, including Albania (1), Argentina (4), Australia (39), Austria (10), Belarus (1), Belgium (15), Brazil (2), Bulgaria (1), Canada (28), Chile (1), China (60), Croatia (2), Cuba (1), Czech (2), Denmark (7), Egypt (4), Estonia (1), Finland (4), France (44), Germany (108), Greece (9), Hungary (2), Iceland (1), India (12), Iran (3), Ireland (4), Israel (8), Italy (96), Japan (176), Lebanon (3), Lithuania (1), Macedonia (1), Malaysia (3), Mexico (6), Monaco (1), Morocco (1), The Netherlands (26), New Zealand (1), Nigeria (1), Norway (3), Pakistan (2), Peru (1), Poland (6), Portugal (1), Russia (3), Saudi Arabia (2), Serbia (1), Singapore (4), Slovakia (2), Slovenia (1), South Africa (2), South Korea (14), Spain (38), Sweden (15), Switzerland (13), Turkey (8), United Arab Emirates (1), United Kingdom (83), United States (316) and Uruguay (2).

Andrew V Biankin, *Sydney*
Filip Braet, *Sydney*
Andrew D Clouston, *Sydney*
Graham Cooksley, *Queensland*
Darrell HG Crawford, *Brisbane*
Adrian G Cummins, *Woodville South*
Guy D Eslick, *Sydney*
Michael A Fink, *Melbourne*
Robert JL Fraser, *Daw Park*
Peter Raymond Gibson, *Victoria*
Jacob George, *Westmead*
Mark D Gorrell, *Sydney*
Yik-Hong Ho, *Townsville*
Gerald J Holtmann, *Adelaide*
Michael Horowitz, *Adelaide*
John E Kellow, *Sydney*
Rupert Leong, *Concord*
Geoffrey W McCaughan, *Sydney*
Finlay A Macrae, *Victoria*
Daniel Markovich, *Brisbane*
Phillip S Oates, *Perth*
Jacqui Richmond, *Victoria*
Stephen M Riordan, *Sydney*
Ian C Roberts-Thomson, *Adelaide*
Devanshi Seth, *Camperdown*
Arthur Shulkes, *Melbourne*
Ross C Smith, *Sydney*
Kevin J Spring, *Brisbane*
Huy A Tran, *New South Wales*
Debbie Trinder, *Fremantle*
Martin J Veysey, *Gosford*
Daniel L Worthley, *Bedford*

Austria

Peter Ferenci, *Vienna*
Valentin Fuhrmann, *Vienna*
Alfred Gangl, *Vienna*
Christoph Gasche, *Vienna*
Kurt Lenz, *Linz*
Markus Peck-Radosavljevic, *Vienna*
Rudolf E Stauber, *Auenbruggerplatz*
Herbert Tilg, *Innsbruck*
Michael Trauner, *Graz*
Harald Vogelsang, *Vienna*
Guenter Weiss, *Innsbruck*

Belarus

Yury K Marakhouski, *Minsk*

Belgium

Rudi Beyaert, *Gent*
Bart Rik De Geest, *Leuven*
Inge I Depoortere, *Leuven*
Olivier Detry, *Liège*
Benedicte Y De Winter, *Antwerp*
Karel Geboes, *Leuven*
Thierry Gustot, *Brussels*
Yves J Horsmans, *Brussels*
Geert G Leroux-Roels, *Ghent*
Louis Libbrecht, *Leuven*
Etienne M Sokal, *Brussels*
Marc Peeters, *De Pintelaan*
Gert A Van Assche, *Leuven*
Yvan Vandenplas, *Brussels*
Eddie Wisse, *Keerbergen*

Brazil

Heitor Rosa, *Goiania*
Ana Cristina Simões e Silva, *Belo Horizonte*

Bulgaria

Zahariy Krastev, *Sofia*

Canada

Fernando Alvarez, *Québec*
David Armstrong, *Ontario*
Jeffrey P Baker, *Toronto*
Olivier Barbier, *Québec*
Nancy Baxter, *Toronto*
Matthew Bjerknes, *Toronto*
Frank J Burczynski, *Manitoba*
Michael F Byrne, *Vancouver*
Wang-Xue Chen, *Ottawa*
Chantal Guillemette, *Québec*
Samuel S Lee, *Calgary*
Gary A Levy, *Toronto*
Andrew L Mason, *Alberta*
John K Marshall, *Ontario*
Donna-Marie McCafferty, *Calgary*
Thomas I Michalak, *St. John's*
Gerald Y Minuk, *Manitoba*
Paul Moayyedi, *Hamilton*
Kostas Pantopoulos, *Québec*
William G Paterson, *Kingston*
Eldon Shaffer, *Calgary*
Morris Sherman, *Toronto*
Martin Storr, *Calgary*
Elena F Verdu, *Ontario*
John L Wallace, *Calgary*
Eric M Yoshida, *Vancouver*

Chile

Silvana Zanlungo, *Santiago*

China

Henry LY Chan, *Hongkong*
Xiao-Ping Chen, *Wuhan*
Zong-Jie Cui, *Beijing*
Da-Jun Deng, *Beijing*
Er-Dan Dong, *Beijing*
Sheung-Tat Fan, *Hong Kong*
Jin Gu, *Beijing*
Xin-Yuan Guan, *Pokfulam*
De-Wu Han, *Taiyuan*
Ming-Liang He, *Hong Kong*
Wayne HC Hu, *Hong Kong*
Chee-Kin Hui, *Hong Kong*
Ching-Lung Lai, *Hong Kong*
Kam Chuen Lai, *Hong Kong*
James YW Lau, *Hong Kong*
Yuk-Tong Lee, *Hong Kong*
Suet-Yi Leung, *Hong Kong*
Wai-Keung Leung, *Hong Kong*
John M Luk, *Pokfulam*
Chung-Mau Lo, *Hong Kong*
Jing-Yun Ma, *Beijing*
Ronnie Tung Ping Poon, *Hong Kong*
Lun-Xiu Qin, *Shanghai*
Yu-Gang Song, *Guangzhou*
Qin Su, *Beijing*
Wai-Man Wong, *Hong Kong*
Hong Xiao, *Shanghai*
Dong-Liang Yang, *Wuhan*
Winnie Yeo, *Hong Kong*
Yuan Yuan, *Shenyang*
Man-Fung Yuen, *Hong Kong*
Jian-Zhong Zhang, *Beijing*
Xin-Xin Zhang, *Shanghai*
Bo-Jian Zheng, *Hong Kong*
Shu Zheng, *Hangzhou*

Croatia

Tamara Cacev, *Zagreb*
Marko Duvnjak, *Zagreb*

Cuba

Damian C Rodriguez, *Havana*

Czech

Milan Jirsa, *Praha*
Pavel Trunečka, *Prague*

Denmark

Peter Bytzer, *Copenhagen*
Asbjørn M Drewes, *Aalborg*
Hans Gregersen, *Aalborg*
Jens H Henriksen, *Hvidovre*
Claus P Hovendal, *Odense*
Fin S Larsen, *Copenhagen*
Søren Møller, *Hvidovre*

Egypt

Abdel-Rahman El-Zayadi, *Giza*
Amr M Helmy, *Cairo*
Ayman Yosry, *Cairo*

Estonia

Riina Salupere, *Tartu*

Finland

Irma E Jarvela, *Helsinki*
Katri M Kaukinen, *Tampere*
Minna Nyström, *Helsinki*
Pentti Sipponen, *Espoo*

France

Bettaieb Ali, *Dijon*
Corlu Anne, *Rennes*
Denis Ardid, *Clermont-Ferrand*
Charles P Balabaud, *Bordeaux*
Soumeya Bekri, *Rouen*
Jacques Belghiti, *Clichy*
Jacques Bernuau, *Clichy Cedex*
Pierre Brissot, *Rennes*
Patrice P Cacoub, *Paris*
Franck Carbonnel, *Besancon*
Laurent Castera, *Pessac*
Bruno Clément, *Rennes*
Benoit Coffin, *Colombes*
Jacques Cosnes, *Paris*
Thomas Decaens, *Cedex*

Francoise L Fabiani, *Angers*
Gérard Feldmann, *Paris*
Jean Fioramonti, *Toulouse*
Jean-Noël Freund, *Strasbourg*
Jean-Paul Galmiche, *Nantes*
Catherine Guettier, *Villejuif*
Chantal Housset, *Paris*
Juan L Iovanna, *Marseille*
Rene Lambert, *Lyon*
Patrick Marcellin, *Paris*
Philippe Mathurin, *Lille*
Tamara Matysiak–Budnik, *Paris*
Francis Mégraud, *Bordeaux*
Richard Moreau, *Clichy*
Thierry Piche, *Nice*
Raoul Poupon, *Paris*
Jean Rosenbaum, *Bordeaux*
Dominique Marie Roulot, *Bobigny*
Thierry Poynard, *Paris*
Jean-Philippe Salier, *Rouen*
Didier Samuel, *Villejuif*
Jean-Yves Scoazec, *Lyon*
Khalid A Tazi, *Clichy*
Emmanuel Tiret, *Paris*
Baumert F Thomas, *Strasbourg*
Marie-Catherine Vozenin-brotons, *Villejuif*
Jean-Pierre H Zarski, *Grenoble*
Jessica Zucman-Rossi, *Paris*

Germany

Hans-Dieter Allescher, *G-Partenkirchen*
Martin Anlauf, *Kiel*
Rudolf Arnold, *Marburg*
Max G Bachem, *Ulm*
Thomas F Baumert, *Freiburg*
Daniel C Baumgart, *Berlin*
Hubert Blum, *Freiburg*
Thomas Bock, *Tuebingen*
Katja Breitkopf, *Mannheim*
Dunja Bruder, *Braunschweig*
Markus W Büchler, *Heidelberg*
Christa Buechler, *Regensburg*
Reinhard Buettner, *Bonn*
Elke Cario, *Essen*
Uta Dahmen, *Essen*
Christoph F Dietrich, *Bad Mergentheim*
Arno J Dormann, *Koeln*
Rainer J Duchmann, *Berlin*
Volker F Eckardt, *Wiesbaden*
Paul Enck, *Tuebingen*
Fred Fändrich, *Kiel*
Ulrich R Fölsch, *Kiel*
Helmut Friess, *Heidelberg*
Peter R Galle, *Mainz*
Nikolaus Gassler, *Aachen*
Andreas Geier, *Aachen*
Markus Gerhard, *Munich*
Wolfram H Gerlich, *Giessen*
Dieter Glebe, *Giessen*
Burkhard Göke, *Munich*
Florian Graepler, *Tuebingen*
Axel M Gressner, *Aachen*
Veit Gülberg, *Munich*
Rainer Haas, *Munich*
Eckhart G Hahn, *Erlangen*
Stephan Hellmig, *Kiel*
Martin Hennenberg, *Bonn*
Johannes Herkel, *Hamburg*
Klaus R Herrlinger, *Stuttgart*
Eva Herrmann, *Homburg/Saar*
Eberhard Hildt, *Berlin*
Joerg C Hoffmann, *Berlin*
Ferdinand Hofstaedter, *Regensburg*
Werner Hohenberger, *Erlangen*
Jörg C Kalff, *Bonn*
Ralf Jakobs, *Ludwigshafen*
Jutta Keller, *Hamburg*
Andrej Khandoga, *Munich*
Sibylle Koletzko, *München*
Stefan Kubicka, *Hannover*
Joachim Labenz, *Siegen*
Frank Lammert, *Bonn*
Thomas Langmann, *Regensburg*
Christian Liedtke, *Aachen*
Matthias Löhr, *Mannheim*
Christian Maaser, *Muenster*
Ahmed Madisch, *Dresden*
Peter Malfertheiner, *Magdeburg*
Michael P Manns, *Hannover*
Helmut Messmann, *Augsburg*
Stephan Miehlke, *Dresden*
Sabine Mihm, *Göttingen*
Silvio Nadalin, *Essen*
Markus F Neurath, *Mainz*
Johann Ockenga, *Berlin*
Florian Obermeier, *Regensburg*
Gustav Paumgartner, *Munich*
Ulrich KS Peitz, *Magdeburg*
Markus Reiser, *Bochum*
Emil C Reisinger, *Rostock*
Steffen Rickes, *Magdeburg*
Tilman Sauerbruch, *Bonn*
Dieter Saur, *Munich*
Hans Scherubl, *Berlin*
Joerg Schirra, *Munich*
Roland M Schmid, *München*
Volker Schmitz, *Bonn*
Andreas G Schreyer, *Regensburg*
Tobias Schroeder, *Essen*
Henning Schulze-Bergkamen, *Mainz*
Hans Seifert, *Oldenburg*
Norbert Senninger, *Muenster*
Manfred V Singer, *Mannheim*
Gisela Sparmann, *Rostock*
Christian J Steib, *München*
Jurgen M Stein, *Frankfurt*
Ulrike S Stein, *Berlin*
Manfred Stolte, *Bayreuth*
Christian P Strassburg, *Hannover*
Wolfgang R Stremmel, *Heidelberg*
Harald F Teutsch, *Ulm*
Robert Thimme, *Freiburg*
Hans L Tillmann, *Leipzig*
Tung-Yu Tsui, *Regensburg*
Axel Ulsenheimer, *Munich*
Patrick Veit-Haibach, *Essen*
Claudia Veltkamp, *Heidelberg*
Siegfried Wagner, *Deggendorf*
Henning Walczak, *Heidelberg*
Heiner Wedemeyer, *Hannover*
Fritz von Weizsacker, *Berlin*
Jens Werner, *Heidelberg*
Bertram Wiedenmann, *Berlin*
Reiner Wiest, *Regensburg*
Stefan Wirth, *Wuppertal*
Stefan JP Zeuzem, *Homburg*

Greece

Alexandra A Alexopoulou, *Athens*
George N Dalekos, *Larissa*
Christos Dervenis, *Athens*
Melanie Maria Deutsch, *Athens*
Tsianos Epameinondas, *Ioannina*
Elias A Kouroumalis, *Heraklion*
George Papatheodoridis, *Athens*
Spiros Sgouros, *Athens*

Hungary

Peter L Lakatos, *Budapest*
Zsuzsa Szondy, *Debrecen*

Iceland

Hallgrimur Gudjonsson, *Reykjavik*

India

Philip Abraham, *Mumbai*
Rakesh Aggarwal, *Lucknow*
Kunissery A Balasubramanian, *Vellore*
Deepak Kumar Bhasin, *Chandigarh*
Sujit K Bhattacharya, *Kolkata*
Yogesh K Chawla, *Chandigarh*
Radha K Dhiman, *Chandigarh*
Sri Prakash Misra, *Allahabad*
Ramesh Roop Rai, *Jaipur*
Nageshwar D Reddy, *Hyderabad*
Rakesh Kumar Tandon, *New Delhi*

Iran

Seyed-Moayed Alavian, *Tehran*
Reza Malekzadeh, *Tehran*
Seyed A Taghavi, *Shiraz*

Ireland

Billy Bourke, *Dublin*
Ronan A Cahill, *Cork*
Anthony P Moran, *Galway*

Israel

Simon Bar-Meir, *Hashomer*
Abraham R Eliakim, *Haifa*
Zvi Fireman, *Hadera*
Yaron Ilan, *Jerusalem*
Avidan U Neumann, *Ramat-Gan*
Yaron Niv, *Pardesia*
Ran Oren, *Tel Aviv*
Ami D Sperber, *Beer-Sheva*

Italy

Giovanni Addolorato, *Roma*
Luigi E Adinolfi, *Naples*
Domenico Alvaro, *Rome*
Mario Angelico, *Rome*
Vito Annese, *San Giovanni Rotond*
Filippo Ansaldi, *Genoa*
Adolfo F Attili, *Roma*
Giovanni Barbara, *Bologna*
Claudio Bassi, *Verona*
Gabrio Bassotti, *Perugia*
Pier M Battezzati, *Milan*
Stefano Bellentani, *Carpi*
Antomio Benedetti, *Ancona*
Mauro Bernardi, *Bologna*
Livia Biancone, *Rome*
Luigi Bonavina, *Milano*
Flavia Bortolotti, *Padova*
Giuseppe Brisinda, *Rome*
Elisabetta Buscarini, *Crema*
Giovanni Cammarota, *Roma*

Antonino Cavallari, *Bologna*
Giuseppe Chiarioni, *Valeggio*
Michele Cicala, *Rome*
Massimo Colombo, *Milan*
Amedeo Columbano, *Cagliari*
Massimo Conio, *Sanremo*
Dario Conte, *Milano*
Gino R Corazza, *Pavia*
Francesco Costa, *Pisa*
Antonio Craxi, *Palermo*
Silvio Danese, *Milan*
Roberto de Franchis, *Milano*
Roberto De Giorgio, *Bologna*
Maria Stella De Mitri, *Bologna*
Giovanni D De Palma, *Naples*
Fabio Farinati, *Padua*
Giammarco Fava, *Ancona*
Francesco Feo, *Sassari*
Fiorucci Stefano, *Perugia*
Andrea Galli, *Firenze*
Valeria Ghisett, *Turin*
Gianluigi Giannelli, *Bari*
Edoardo G Giannini, *Genoa*
Paolo Gionchetti, *Bologna*
Fabio Grizzi, *Milan*
Salvatore Gruttadauria, *Palermo*
Mario Guslandi, *Milano*
Pietro Invernizzi, *Milan*
Ezio Laconi, *Cagliari*
Giacomo Laffi, *Firenze*
Giovanni Maconi, *Milan*
Lucia Malaguarnera, *Catania*
Emanuele D Mangoni, *Napoli*
Paolo Manzoni, *Torino*
Giulio Marchesini, *Bologna*
Fabio Marra, *Florence*
Marco Marzioni, *Ancona*
Giuseppe Mazzella, *Bologna*
Mario U Mondelli, *Pavia*
Giuseppe Montalto, *Palermo*
Giovanni Monteleone, *Rome*
Giovanni Musso, *Torino*
Gerardo Nardone, *Napoli*
Valerio Nobili, *Rome*
Fabio Pace, *Milano*
Luisi Pagliaro, *Palermo*
Francesco Pallone, *Rome*
Fabrizio R Parente, *Milan*
Maurizio Parola, *Torino*
Francesco Perri, *San Giovanni Rotondo*
Raffaele Pezzilli, *Bologna*
Alberto Pilotto, *San Giovanni Rotondo*
Alberto Piperno, *Monza*
Mario Pirisi, *Novara*
Anna C Piscaglia, *Roma*
Paolo Del Poggio, *Treviglio*
Gabriele B Porro, *Milano*
Piero Portincasa, *Bari*
Cosimo Prantera, *Roma*
Bernardino Rampone, *Siena*
Oliviero Riggio, *Rome*
Claudio Romano, *Messina*
Marco Romano, *Napoli*
Gerardo Rosati, *Potenza*
Mario Del Tacca, *Pisa*
Gloria Taliani, *Rome*
Pier A Testoni, *Milan*
Enrico Roda, *Bologna*
Domenico Sansonno, *Bari*
Vincenzo Savarino, *Genova*
Vincenzo Stanghellini, *Bologna*
Giovanni Tarantino, *Naples*
Roberto Testa, *Genoa*
Dino Vaira, *Bologna*
Anna Linda Zignego, *Florence*

Japan

Kyoichi Adachi, *Izumo*
Yasushi Adachi, *Sapporo*
Taiji Akamatsu, *Matsumoto*
Sk Md Fazle Akbar, *Ehime*
Takafumi Ando, *Nagoya*
Akira Andoh, *Otsu*
Taku Aoki, *Tokyo*
Masahiro Arai, *Tokyo*
Tetsuo Arakawa, *Osaka*
Yasuji Arase, *Tokyo*
Masahiro Asaka, *Sapporo*
Hitoshi Asakura, *Tokyo*
Takeshi Azuma, *Fukui*
Yoichi Chida, *Fukuoka*
Takahiro Fujimori, *Tochigi*
Jiro Fujimoto, *Hyogo*
Kazuma Fujimoto, *Saga*
Mitsuhiro Fujishiro, *Tokyo*
Yoshihide Fujiyama, *Otsu*
Hiroyuki Fukui, *Tochigi*
Hiroyuki Hanai, *Hamamatsu*
Naohiko Harada, *Fukuoka*
Makoto Hashizume, *Fukuoka*
Tetsuo Hayakawa, *Nagoya*
Toru Hiyama, *Higashihiroshima*
Kazuhide Higuchi, *Osaka*
Keisuke Hino, *Ube*
Keiji Hirata, *Kitakyushu*
Yuji Iimuro, *Nishinomiya*
Kenji Ikeda, *Tokyo*
Toru Ikegami, *Fukuoka*
Kenichi Ikejima, *Bunkyo-ku*
Fumio Imazeki, *Chiba*
Yutaka Inagaki, *Kanagawa*
Yasuhiro Inokuchi, *Yokohama*
Haruhiro Inoue, *Yokohama*
Masayasu Inoue, *Osaka*
Hiromi Ishibashi, *Nagasaki*
Shunji Ishihara, *Izumo*
Toru Ishikawa, *Niigata*
Kei Ito, *Sendai*
Masayoshi Ito, *Tokyo*
Hiroaki Itoh, *Akita*
Ryuichi Iwakiri, *Saga*
Yoshiaki Iwasaki, *Okayama*
Terumi Kamisawa, *Tokyo*
Hiroshi Kaneko, *Aichi-Gun*
Shuichi Kaneko, *Kanazawa*
Takashi Kanematsu, *Nagasaki*
Mitsuo Katano, *Fukuoka*
Junji Kato, *Sapporo*
Mototsugu Kato, *Sapporo*
Shinzo Kato, *Tokyo*
Norifumi Kawada, *Osaka*
Sunao Kawano, *Osaka*
Mitsuhiro Kida, *Kanagawa*
Yoshikazu Kinoshita, *Izumo*
Tsuneo Kitamura, *Chiba*
Seigo Kitano, *Oita*
Kazuhiko Koike, *Tokyo*
Norihiro Kokudo, *Tokyo*
Satoshi Kondo, *Sapporo*
Shoji Kubo, *Osaka*
Shigeki Kuriyama, *Kagawa*[2]
Katsunori Iijima, *Sendai*
Masato Kusunoki, *Tsu Mie*
Shin Maeda, *Tokyo*
Shigeru Marubashi, *Suita*
Masatoshi Makuuchi, *Tokyo*
Osamu Matsui, *Kanazawa*
Yasuhiro Matsumura, *Chiba*
Yasushi Matsuzaki, *Tsukuba*
Kiyoshi Migita, *Omura*
Kenji Miki, *Tokyo*
Tetsuya Mine, *Kanagawa*
Hiroto Miwa, *Hyogo*
Masashi Mizokami, *Nagoya*
Yoshiaki Mizuguchi, *Tokyo*
Motowo Mizuno, *Hiroshima*
Morito Monden, *Suita*
Hisataka S Moriwaki, *Gifu*
Yasuaki Motomura, *Iizuka*
Yoshiharu Motoo, *Kanazawa*
Naofumi Mukaida, *Kanazawa*
Kazunari Murakami, *Oita*
Kunihiko Murase, *Tusima*
Hiroaki Nagano, *Suita*
Masahito Nagaki, *Gifu*
Masaki Nagaya, *Kawasaki*
Yujl Naito, *Kyoto*
Atsushi Nakajima, *Yokohama*
Hisato Nakajima, *Tokyo*
Hiroki Nakamura, *Yamaguchi*
Shotaro Nakamura, *Fukuoka*
Mikio Nishioka, *Niihama*
Shuji Nomoto, *Nagoya*
Susumu Ohmada, *Maebashi*
Hirohide Ohnishi, *Akita*
Masayuki Ohta, *Oita*
Tetsuo Ohta, *Kanazawa*
Kazuichi Okazaki, *Osaka*
Katsuhisa Omagari, *Nagasaki*
Saburo Onishi, *Nankoku*
Morikazu Onji, *Ehime*
Satoshi Osawa, *Hamamatsu*
Masanobu Oshima, *Kanazawa*
Hiromitsu Saisho, *Chiba*
Hidetsugu Saito, *Tokyo*
Yutaka Saito, *Tokyo*
Isao Sakaida, *Yamaguchi*
Michiie Sakamoto, *Tokyo*
Yasushi Sano, *Chiba*
Hiroki Sasaki, *Tokyo*
Iwao Sasaki, *Sendai*
Motoko Sasaki, *Kanazawa*
Chifumi Sato, *Tokyo*
Shuichi Seki, *Osaka*
Hiroshi Shimada, *Yokohama*
Mitsuo Shimada, *Tokushima*
Tomohiko Shimatan, *Hiroshima*
Hiroaki Shimizu, *Chiba*
Ichiro Shimizu, *Tokushima*
Yukihiro Shimizu, *Kyoto*
Shinji Shimoda, *Fukuoka*
Tooru Shimosegawa, *Sendai*
Tadashi Shimoyama, *Hirosaki*
Ken Shirabe, *Iizuka City*
Yoshio Shirai, *Niigata*
Katsuya Shiraki, *Mie*
Yasushi Shiratori, *Okayama*
Masayuki Sho, *Nara*
Yasuhiko Sugawara, *Tokyo*
Hidekazu Suzuki, *Tokyo*
Minoru Tada, *Tokyo*
Tadatoshi Takayama, *Tokyo*
Tadashi Takeda, *Osaka*
Koji Takeuchi, *Kyoto*
Kiichi Tamada, *Tochigi*
Akira Tanaka, *Kyoto*
Eiji Tanaka, *Matsumoto*
Noriaki Tanaka, *Okayama*
Shinji Tanaka, *Hiroshima*
Hideki Taniguchi, *Yokohama*
Kyuichi Tanikawa, *Kurume*
Akira Terano, *Shimotsugagun*
Hitoshi Togash, *Yamagata*
Shinji Togo, *Yokohama*
Kazunari Tominaga, *Osaka*
Takuji Torimura, *Fukuoka*

Minoru Toyota, *Sapporo*
Akihito Tsubota, *Chiba*
Takato Ueno, *Kurume*
Naomi Uemura, *Tokyo*
Shinichi Wada, *Tochigi*
Hiroyuki Watanabe, *Kanazawa*
Toshio Watanabe, *Osaka*
Yuji Watanabe, *Ehime*
Toshiaki Watanabe, *Tokyo*
Chun-Yang Wen, *Nagasaki*
Satoshi Yamagiwa, *Niigata*
Koji Yamaguchi, *Fukuoka*
Takayuki Yamamoto, *Yokkaichi*
Takashi Yao, *Fukuoka*
Masashi Yoneda, *Tochigi*
Hiroshi Yoshida, *Tokyo*
Masashi Yoshida, *Tokyo*
Norimasa Yoshida, *Kyoto*
Hitoshi Yoshiji, *Nara*
Kentaro Yoshika, *Toyoake*
Yasunobu Yoshikai, *Fukuoka*
Masahide Yoshikawa, *Kashihara*
Katsutoshi Yoshizato, *Higashihiroshima*

Lebanon

Bassam N Abboud, *Beirut*
Ala I Sharara, *Beirut*
Joseph D Boujaoude, *Beirut*

Lithuania

Limas Kupcinskas, *Kaunas*

Macedonia

Vladimir C Serafimoski, *Skopje*

Malaysia

Andrew Seng Boon Chua, *Ipoh*
Khean-Lee Goh, Kuala *Lumpur*
Jayaram Menon, *Sabah*

Mexico

Diego Garcia-Compean, *Monterrey*
Eduardo R Marin-Lopez, *Jesús García*
Nahum Méndez-Sánchez, *Mexico*
Saúl Villa-Treviño, *México*

Monaco

Patrick Rampal, *Monaco*

Morocco

Abdellah Essaid, *Rabat*

The Netherlands

Ulrich Beuers, *Amsterdam*
Gerd Bouma, *Amsterdam*
Lee Bouwman, *Leiden*
J Bart A Crusius, *Amsterdam*
NKH de Boer, *Amsterdam*
Koert P de Jong, *Groningen*
Henrike Hamer, *Maastricht*
Frank Hoentjen, *Haarlem*
Janine K Kruit, *Groningen*
Ernst J Kuipers, *Rotterdam*
CBHW Lamers, *Leiden*
Ton Lisman, *Utrecht*
Yi Liu, *Amsterdam*
Jeroen Maljaars, *Maastricht*
Servaas Morré, *Amsterdam*
Chris JJ Mulder, *Amsterdam*
Michael Müller, *Wageningen*
Amado S Peña, *Amsterdam*
Robert J Porte, *Groningen*
Ingrid B Renes, *Rotterdam*
Andreas Smout, *Utrecht*
Paul E Sijens, *Groningen*
Reinhold W Stockbrugger, *Maastricht*
Luc JW van der Laan, *Rotterdam*
Karel van Erpecum, *Utrecht*
Gerard P VanBerge-Henegouwen,*Utrecht*

New Zealand

Ian D Wallace, *Auckland*

Nigeria

Samuel B Olaleye, *Ibadan*

Norway

Trond Berg, *Oslo*
Tom H Karlsen, *Oslo*
Helge L Waldum, *Trondheim*

Pakistan

Muhammad S Khokhar, *Lahore*
Syed MW Jafri, *Karachi*

Peru

Hector H Garcia, *Lima*

Poland

Tomasz Brzozowski, *Cracow*
Robert Flisiak, *Bialystok*
Hanna Gregorek, *Warsaw*
Dariusz M Lebensztejn, *Bialystok*
Wojciech G Polak, *Wroclaw*
Marek Hartleb, *Katowice*

Portugal

Miguel C De Moura, *Lisbon*

Russia

Vladimir T Ivashkin, *Moscow*
Leonid Lazebnik, *Moscow*
Vasiliy I Reshetnyak, *Moscow*

Saudi Arabia

Ibrahim A Al Mofleh, *Riyadh*
Ahmed Helmy, *Riyadh*

Serbia

Dusan M Jovanovic, *Sremska Kamenica*

Singapore

Bow Ho, *Singapore*
Khek-Yu Ho, *Singapore*
Fock Kwong Ming, *Singapore*
Francis Seow-Choen, *Singapore*

Slovakia

Silvia Pastorekova, *Bratislava*
Anton Vavrecka, *Bratislava*

Slovenia

Sasa Markovic, *Ljubljana*

South Africa

Rosemar Joyce Burnett, *Pretoria*
Michael C Kew, *Parktown*

South Korea

Byung Ihn Choi, *Seoul*
Ho Soon Choi, *Seoul*
Marie Yeo, *Suwon*
Sun Pyo Hong, *Gyeonggi-do*
Jae J Kim, *Seoul*
Jin-Hong Kim, *Suwon*
Myung-Hwan Kim, *Seoul*
Chang Hong Lee, *Seoul*
Jong Kyun Lee, *Seoul*
Eun-Yi Moon, *Seoul*
Jae-Gahb Park, *Seoul*
Dong Wan Seo, *Seoul*
Dong Jin Suh, *Seoul*
Byung Chul Yoo, *Seoul*

Spain

Juan G Abraldes, *Barcelona*
Agustin Albillos, *Madrid*
Raul J Andrade, *Málaga*
Luis Aparisi, *Valencia*
Fernando Azpiroz, *Barcelona*
Ramon Bataller, *Barcelona*
Josep M Bordas, *Barcelona*
Xavier Calvet, *Sabadell*
Jordi Camps, *Catalunya*
Andres Cardenas, *Barcelona*
Vicente Carreño, *Madrid*
Jose Castellote, *Barcelona*
Antoni Castells, *Barcelona*
Vicente Felipo, *Valencia*
Juan C Garcia-Pagán, *Barcelona*
Jaime B Genover, *Barcelona*
Javier P Gisbert, *Madrid*
Jaime Guardia, *Barcelona*
Isabel Fabregat, *Barcelona*
Mercedes Fernandez, *Barcelona*
Angel Lanas, *Zaragoza*
Juan-Ramón Larrubia, *Guadalajara*
Laura Lladóa, *Barcelona*
María IT López, *Jaén*
Juan R Malagelada, *Barcelona*
José M Mato, *Derio*
Juan F Medina, *Pamplona*
Miguel A Muñoz-Navas, *Pamplona*
Julian Panes, *Barcelona*
Miguel M Perez, *Valencia*
Miguel Perez-Mateo, *Alicante*

Josep M Pique, *Barcelona*
Jesús M Prieto, *Pamplona*
Sabino Riestra, *Pola De Siero*
Luis Rodrigo, *Oviedo*
Manuel Romero-Gómez, *Sevilla*
Joan Roselló-Catafau, *Barcelona*

Sweden

Einar S Björnsson, *Gothenburg*
Curt Einarsson, *Huddinge*
Per M Hellström, *Stockholm*
Ulf Hindorf, *Lund*
Elisabeth Hultgren-Hörnquist, *Örebro*
Anders E Lehmann, *Mölndal*
Hanns-Ulrich Marschall, *Stockholm*
Lars C Olbe, *Molndal*
Lars A Pahlman, *Uppsala*
Matti Sallberg, *Stockholm*
Magnus Simrén, *Göteborg*
Xiao-Feng Sun, *Linköping*
Ervin Tóth, *Malmö*
Weimin Ye, *Stockholm*
Christer S von Holstein, *Lund*

Switzerland

Chrish Beglinger, *Basel*
Pierre A Clavien, *Zurich*
Jean-Francois Dufour, *Bern*
Franco Fortunato, *Zürich*
Jean L Frossard, *Geneva*
Gerd A Kullak-Ublick, *Zurich*
Pierre Michetti, *Lausanne*
Francesco Negro, *Genève*
Bruno Stieger, *Zurich*
Radu Tutuian, *Zurich*
Stephan R Vavricka, *Zurich*
Gerhard Rogler, *Zurich*
Arthur Zimmermann, *Berne*

Turkey

Yusuf Bayraktar, *Ankara*
Figen Gurakan, *Ankara*
Aydin Karabacakoglu, *Konya*
Serdar Karakose, *Konya*
Hizir Kurtel, *Istanbul*
Osman C Ozdogan, *Istanbul*
Özlem Yilmaz, *Izmir*
Cihan Yurdaydin, *Ankara*

United Arab Emirates

Sherif M Karam, *Al-Ain*

United Kingdom

David H Adams, *Birmingham*
Simon Afford, *Birmingham*
Navneet K Ahluwalia, *Stockport*
Ahmed Alzaraa, *Manchester*
Lesley A Anderson, *Belfast*
Charalambos G Antoniades, *London*
Anthony TR Axon, *Leeds*
Qasim Aziz, *Manchester*
Nicholas M Barnes, *Birmingham*
Jim D Bell, *London*
Mairi Brittan, *London*
Alastair D Burt, *Newcastle*
Simon S Campbell, *Manchester*
Simon R Carding, *Leeds*
Paul J Ciclitira, *London*
Eithne Costello, *Liverpool*
Tatjana Crnogorac-Jurcevic, *London*
Harry Dalton, *Truro*
Amar P Dhillon, *London*
William Dickey, *Londonderry*
James E East, *London*
Emad M El-Omar, *Aberdeen*
Ahmed M Elsharkawy, *Newcastle Upon Tyne*
Annette Fristscher-Ravens, *London*
Elizabeth Furrie, *Dundee*
Daniel R Gaya, *Edinburgh*
Subrata Ghosh, *London*
William Greenhalf, *Liverpool*
Indra N Guha, *Southampton*
Peter C Hayes, *Edinburgh*
Gwo-Tzer Ho, *Edinburgh*
Anthony R Hobson, *Salford*
Lesley A Houghton, *Manchester*
Stefan G Hübscher, *Birmingham*
Robin Hughes, *London*
Pali Hungin, *Stockton*
David P Hurlstone, *Sheffield*
Rajiv Jalan, *London*
Janusz AZ Jankowski, *Oxford*
Brian T Johnston, *Belfast*
David EJ Jones, *Newcastle*
Roger Jones, *London*
Michael A Kamm, *Harrow*
Peter Karayiannis, *London*
Laurens Kruidenier, *Harlow*
Patricia F Lalor, *Birmingham*
Chee Hooi Lim, *Midlands*
Hong-Xiang Liu, *Cambridge*
Yun Ma, *London*
Kenneth E L McColl, *Glasgow*
Stuart AC McDonald, *London*
Dermot P Mcgovern, *Oxford*
Giorgina Mieli-Vergani, *London*
Nikolai V Naoumov, *London*
John P Neoptolemos, *Liverpool*
James Neuberger, *Birmingham*
Philip Noel Newsome, *Birmingham*
Mark S Pearce, *Newcastle Upon Tyne*
Stephen P Pereira, *London*
D Mark Pritchard, *Liverpool*
Sakhawat Rahman, *London*
Stephen E Roberts, *Swansea*
Marco Senzolo, *Padova*
Soraya Shirazi-Beechey, *Liverpool*
Robert Sutton, *Liverpool*
Simon D Taylor-Robinson, *London*
Paris P Tekkis, *London*
Ulrich Thalheimer, *London*
David G Thompson, *Salford*
Nick P Thompson, *Newcastle*
David Tosh, *Bath*
Frank I Tovey, *London*
Chris Tselepis, *Birmingham*
Diego Vergani, *London*
Geoffrey Warhurst, *Salford*
Alastair John Watson, *Liverpool*
Peter J Whorwell, *Manchester*
Roger Williams, *London*
Karen L Wright, *Bath*
Min Zhao, *Foresterhill*

United States

Manal F Abdelmalek, *Durham*
Gary A Abrams, *Birmingham*
Maria T Abreu, *New York*
Reid B Adams, *Virginia*
Golo Ahlenstiel, *Bethesda*
BS Anand, *Houston*
Frank A Anania, *Atlanta*
M Ananthanarayanan, *New York*
Gavin E Arteel, *Louisville*
Jasmohan S Bajaj, *Milwaukee*
Subhas Banerjee, *Palo Alto*
Peter A Banks, *Boston*
Jamie S Barkin, *Miami Beach*
Kim E Barrett, *San Diego*
Marc D Basson, *Detroit*
Anthony J Bauer, *Pittsburgh*
Wallace F Berman, *Durham*
Timothy R Billiar, *Pittsburgh*
Edmund J Bini, *New York*
David G Binion, *Milwaukee*
Jennifer D Black, *Buffalo*
Herbert L Bonkovsky, *Charlotte*
Carla W Brady, *Durham*
Andrea D Branch, *New York*
Robert S Bresalier, *Houston*
Alan L Buchman, *Chicago*
Ronald W Busuttil, *Los Angeles*
Alan Cahill, *Philadelphia*
John M Carethers, *San Diego*
David L Carr-Locke, *Boston*
Maurice A Cerulli, *New York*
Ravi S Chari, *Nashville*
Jiande Chen, *Galveston*
Xian-Ming Chen, *Omaha*
Xin Chen, *San Francisco*
Ramsey Chi-man Cheung, *Palo Alto*
William D Chey, *Ann Arbor*
John Y Chiang, *Rootstown*
Parimal Chowdhury, *Arkansas*
Raymond T Chung, *Boston*
James M Church, *Cleveland*
Ram Chuttani, *Boston*
Mark G Clemens, *Charlotte*
Ana J Coito, *Los Angeles*
Vincent Coghlan, *Beaverton*
David Cronin II, *New Haven*
John Cuppoletti, *Cincinnati*
Mark J Czaja, *New York*
Peter V Danenberg, *Los Angeles*
Kiron M Das, New *Brunswick*
Conor P Delaney, *Cleveland*
Jose L del Pozo, *Rochester*
Sharon DeMorrow, *Temple*
Deborah L Diamond, *Seattle*
Douglas A Drossman, *Chapel Hill*
Katerina Dvorak, *Tucson*
Bijan Eghtesad, *Cleveland*
Hala El-Zimaity, *Houston*
Michelle Embree-Ku, *Providence*
Sukru Emre, New *Haven*
Douglas G Farmer, *Los Angeles*
Alessio Fasano, *Baltimore*
Mark A Feitelson, *Philadelphia*
Ariel E Feldstein, *Cleveland*
Alessandro Fichera, *Chicago*
Robert L Fine, *New York*
Magali Fontaine, *Stanford*
Chris E Forsmark, *Gainesville*
Glenn T Furuta, *Aurora*
Chandrashekhar R Gandhi, *Pittsburgh*
Susan L Gearhart, *Baltimore*
Xupeng Ge, *Boston*
Xin Geng, *New Brunswick*
M Eric Gershwin, *Suite*
Jean-Francois Geschwind, *Baltimore*
Ignacio Gil-Bazo, *New York*
Shannon S Glaser, *Temple*
Ajay Goel, *Dallas*
Richard M Green, *Chicago*
Julia B Greer, *Pittsburgh*

James H Grendell, *New York*
David R Gretch, *Seattle*
Stefano Guandalini, *Chicago*
Anna S Gukovskaya, *Los Angeles*
Sanjeev Gupta, *Bronx*
David J Hackam, *Pittsburgh*
Stephen B Hanauer, *Chicago*
Gavin Harewood, *Rochester*
Margaret M Heitkemper, *Washington*
Alan W Hemming, *Gainesville*
Samuel B Ho, *San Diego*
Peter R Holt, *New York*
Colin W Howden, *Chicago*
Hongjin Huang, *Alameda*
Jamal A Ibdah, *Columbia*
Atif Iqbal, *Omaha*
Hajime Isomoto, *Rochester*
Hartmut Jaeschke, *Tucson*
Dennis M Jensen, *Los Angeles*
Cheng Ji, *Los Angeles*
Leonard R Johnson, *Memphis*
Michael P Jones, *Chicago*
Peter J Kahrilas, *Chicago*
Anthony N Kalloo, *Baltimore*
Marshall M Kaplan, *Boston*
Neil Kaplowitz, *Los Angeles*
Serhan Karvar, *Los Angeles*
Rashmi Kaul, *Tulsa*
Jonathan D Kaunitz, *Los Angeles*
Ali Keshavarzian, *Chicago*
Miran Kim, *Providence*
Joseph B Kirsner, *Chicago*
Leonidas G Koniaris, *Miami*
Burton I Korelitz, *New York*
Robert J Korst, *New York*
Richard A Kozarek, *Seattle*
Alyssa M Krasinskas, *Pittsburgh*
Michael Kremer, *Chapel Hill*
Shiu-Ming Kuo, *Buffalo*
Paul Y Kwo, *Indianapolis*
Daryl Tan Yeung Lau, *Galvesto*
Stephen J Lanspa, *Omaha*
Joel E Lavine, *San Diego*
Bret Lashner, *Cleveland*
Dirk J van Leeuwen, *Lebanon*
Glen A Lehman, *Indianapolis*
Alex B Lentsch, *Cincinnati*
Andreas Leodolter, *La Jolla*
Gene LeSage, *Houston*
Josh Levitsky, *Chicago*
Cynthia Levy, *Gainesville*
Ming Li, *New Orleans*
Zhiping Li, *Baltimore*
Zhe-Xiong Lian, *Davis*
Lenard M Lichtenberger, *Houston*
Gary R Lichtenstein, *Philadelphia*
Otto Schiueh-Tzang Lin, *Seattle*
Martin Lipkin, *New York*
Chen Liu, *Gainesville*
Edward V Loftus, *Rocheste*
Robin G Lorenz, *Birmingham*
Michael R Lucey, *Madison*
James D Luketich, *Pittsburgh*
Guangbin Luo, *Cheveland*
Henry T Lynch, *Omaha*
Patrick M Lynch, *Houston*
John S Macdonald, *New York*
Bruce V MacFadyen, *Augusta*
Willis C Maddrey, *Dallas*
Ashok Malani, *Los Angeles*
Mercedes Susan Mandell, *Aurora*
Peter J Mannon, *Bethesda*
Charles M Mansbach, *Tennessee*
John F Di Mari, *Texas*
John M Mariadason, *Bronx*
Jorge A Marrero, *Ann Arbor*
Paul Martin, *New York*
Paulo Ney Aguiar Martins, *Boston*
Wendy M Mars, *Pittsburgh*
Laura E Matarese, *Pittsburgh*
Richard W McCallum, *Kansas*
Beth A McCormick, *Charlestown*
Lynne V McFarland, *Washington*
Kevin McGrath, *Pittsburgh*
Harihara Mehendale, *Monroe*
Ali Mencin, *New York*
Fanyin Meng, *Ohio*
Stephan Menne, *New York*
Didier Merlin, *Atlanta*
Howard Mertz, *Nashville*
George W Meyer, *Sacramento*
George Michalopoulos, *Pittsburgh*
James M Millis, *Chicago*
Fabrizio Michelassi, *New York*
Albert D Min, *New York*
Pramod K Mistry, *New Haven*
Emiko Mizoguchi, *Boston*
Smruti R Mohanty, *Chicago*
Satdarshan S Monga, *Pittsburgh*
Timothy H Moran, *Baltimore*
Peter L Moses, *Burlington*
Steven F Moss, *Providence*
Andrew J Muir, *Durham*
Milton G Mutchnick, *Detroit*
Masaki Nagaya, *Boston*
Victor Navarro, *Philadelphia*
Laura E Nagy, *Cleveland*
Hiroshi Nakagawa, *Philadelphia*
Douglas B Nelson, *Minneapolis*
Justin H Nguyen, *Florida*
Patrick G Northup, *Charlottesville*
Christopher O'Brien, *Miami*
Robert D Odze, *Boston*
Brant K Oelschlager, *Washington*
Curtis T Okamoto, *Los Angeles*
Stephen JD O'Keefe, *Pittsburgh*
Dimitry Oleynikov, *Omaha*
Stephen J Pandol, *Los Angeles*
Georgios Papachristou, *Pittsburgh*
Pankaj J Pasricha, *Galveston*
Zhiheng Pei, *New York*
Michael A Pezzone, *Pittsburgh*
CS Pitchumoni, *New Brunswiuc*
Paul J Pockros, *La Jolla*
Jay Pravda, *Gainesville*
Massimo Raimondo, *Jacksonville*
GS Raju, *Galveston*
Raymund R Razonable, *Minnesota*
Murray B Resnick, *Providence*
Adrian Reuben, *Charleston*
Douglas K Rex, *Indianapolis*
Victor E Reyes, *Galveston*
Basil Rigas, *New York*
Yehuda Ringel, *Chapel Hill*
Richard A Rippe, *Chapel Hill*
Maribel Rodriguez-Torres, *Santurce*
Marcos Rojkind, *Washington*
Philip Rosenthal, *San Francisco*
Barry Rosser, *Jacksonville Florida*
Hemant K Roy, *Evanston*
Sammy Saab, *Los Angeles*
Shawn D Safford, *Norfolk*
Dushyant V Sahani, *Boston*
Bruce E Sands, *Boston*
James M Scheiman, *Ann Arbor*
Eugene R Schiff, *Miami*
Nicholas J Shaheen, *Chapel Hill*
Vanessa M Shami, *Charlottesville*
Prateek Sharma, *Kansas City*
Harvey L Sharp, *Minneapolis*
Stuart Sherman, *Indianapolis*
Shivendra Shukla, *Columbia*
Alphonse E Sirica, *Virginia*
Shanthi V Sitaraman, *Atlanta*
Stuart J Spechler, *Dallas*
Shanthi Srinivasan, *Atlanta*
Michael Steer, *Boston*
Peter D Stevens, *New York*
Charmaine A Stewart, *Rochester*
Christian D Stone, *Saint Louis*
Gary D Stoner, *Columbus*
R Todd Stravitz, *Richmond*
Liping Su, *Chicago*
Christina Surawicz, *Seattle*
Robert W Summers, *Iowa City*
Wing-Kin Syn, *Durham*
Gyongyi Szabo, *Worcester*
Yvette Taché, *Los Angeles*
Seng-Lai Tan, *Seattle*
Andrzej S Tarnawski, *Orange*
K-M Tchou-Wong, *New York*
Jonathan P Terdiman, *San Francisco*
Neil D Theise, *New York*
Christopher C Thompson, *Boston*
Swan N Thung, *New York*
Michael Torbenson, *Baltimore*
Natalie J Torok, *Sacramento*
RA Travagli, *Baton Rouge*
George Triadafilopoulos, *Stanford*
Chung-Jyi Tsai, *Lexington*
Janet Elizabeth Tuttle-Newhall, *Durham*
Andrew Ukleja, *Florida*
Michael F Vaezi, *Nashville*
Hugo E Vargas, *Scottsdale*
Arnold Wald, *Wisconsin*
Scott A Waldman, *Philadelphia*
Jian-Ying Wang, *Baltimore*
Timothy C Wang, *New York*
Irving Waxman, *Chicago*
Steven A Weinman, *Galveston*
Steven D Wexner, *Weston*
Keith T Wilson, *Baltimore*
Jacqueline L Wolf, *Boston*
Jackie Wood, *Ohio*
George Y Wu, *Farmington*
Jian Wu, *Sacramento*
Samuel Wyllie, *Houston*
Wen Xie, *Pittsburgh*
Vijay Yajnik, *Boston*
Vincent W Yang, *Atlanta*
Francis Y Yao, *San Francisco*
Hal F Yee, *San Francisco*
Xiao-Ming Yin, *Pittsburgh*
Min You, *Tampa*
Zobair M Younossi, *Virginia*
Liqing Yu, *Winston-Salem*
David Yule, *Rochester*
Ruben Zamora, *Pittsburgh*
Michael E Zenilman, *New York*
Zhi Zhong, *Chapel Hill*
Michael A Zimmerman, *Colorado*
Stephen D Zucker, *Cincinnati*

Uruguay

Henry Cohen, *Montevideo*

[1]Passed away on October 20, 2007
[2]Passed away on June 11, 2007

National Journal Award 2005

World Journal of Gastroenterology®

Weekly Established in October 1995

Volume 14 Number 40
October 28, 2008

Contents

Contents

World Journal of Gastroenterology
Volume 14 **Number** 40 **October** 28, 2008

World Journal of Gastroenterology
Volume 14 **Number** 40 **October** 28, 2008

Contents

NAME OF JOURNAL
World Journal of Gastroenterology

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

EDITING
Editorial Board of *World Journal of Gastroenterology,* Room 903, Ocean International Center, Building D, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

PUBLISHING
The WJG Press and Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Ocean International Center, Building D, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

PRINTING
Beijing Kexin Printing House

OVERSEAS DISTRIBUTOR
Beijing Bureau for Distribution of Newspapers and Journals
(Code No. 82-261)
China International Book Trading Corporation PO Box 399, Beijing, China (Code No. M4481)

PUBLICATION DATE
October 28, 2008

EDITOR-IN-CHIEF
Lian-Sheng Ma, *Beijing*

SUBSCRIPTION
RMB 50 Yuan for each issue, RMB 2400 Yuan for one year

CSSN
ISSN 1007-9327
CN 14-1219/R

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6109

World J Gastroenterol 2008 October 28; 14(40): 6109-6114
World Journal of Gastroenterology ISSN 1007-9327

EDITORIAL

Role of cannabinoids in chronic liver diseases

Anna Parfieniuk, Robert Flisiak

Anna Parfieniuk, Robert Flisiak, Department of Infectious Diseases and Hepatology, Medical University of Bialystok, Zurawia Str 14, Bialystok 15-540, Poland
Author contributions: Parfieniuk A was responsible for the review of the literature and initial preparation of the paper, Flisiak R prepared final version of the manuscript.
Correspondence to: Robert Flisiak, Department of Infectious Diseases and Hepatology, Medical University of Bialystok, Zurawia Str 14, Bialystok 15-540, Poland. flisiakr@poczta.onet.pl
Telephone: +48-85-7416921 Fax: +48-85-7416921
Received: April 2, 2008 Revised: June 30, 2008
Accepted: July 7, 2008
Published online: October 28, 2008

Abstract

Cannabinoids are a group of compounds acting primarily *via* CB1 and CB2 receptors. The expression of cannabinoid receptors in normal liver is low or absent. However, many reports have proven up-regulation of the expression of CB1 and CB2 receptors in hepatic myofibroblasts and vascular endothelial cells, as well as increased concentration of endocannabinoids in liver in the course of chronic progressive liver diseases. It has been shown that CB1 receptor signalling exerts profibrogenic and proinflammatory effects in liver tissue, primarily due to the stimulation of hepatic stellate cells, whereas the activation of CB2 receptors inhibits or even reverses liver fibrogenesis. Similarly, CB1 receptor stimulation contributes to progression of liver steatosis. In end-stage liver disease, the endocannabinoid system has been shown to contribute to hepatic encephalopathy and vascular effects, such as portal hypertension, splanchnic vasodilatation, relative peripheral hypotension and probably cirrhotic cardiomyopathy. So far, available evidence is based on cellular cultures or animal models. Clinical data on the effects of cannabinoids in chronic liver diseases are limited. However, recent studies have shown the contribution of cannabis smoking to the progression of liver fibrosis and steatosis. Moreover, controlling CB1 or CB2 signalling appears to be an attractive target in managing liver diseases.

Key words: Hepatic fibrosis; Endocannabinoids; Endocannabinoid receptors; CB1; CB2

Peer reviewer: Dr. Steven Alan Weinman, Professor, Internal Medicine and Neuroscience and Cell Biology, University of Texas Medical Branch, 301 University Blvd, Galveston, 77555-0620, United States

Parfieniuk A, Flisiak R. Role of cannabinoids in chronic liver diseases. *World J Gastroenterol* 2008; 14(40): 6109-6114 Available from: URL: http://www.wjgnet.com/1007-9327/14/6109.asp DOI: http://dx.doi.org/10.3748/wjg.14.6109

INTRODUCTION

Hepatic fibrosis is a dynamic process resulting from liver tissue injury. Previously, it was believed that hepatic fibrosis is irreversible. However, current knowledge allows us to consider fibrosis as an active, potentially reversible process originating from wound-healing responses to chronic liver injury of various etiology. The continuous influence of injuring stimuli leads to an imbalance between the accumulation and degradation of extracellular matrix (ECM) components, which include mainly deposits of fibrillar collagens, proteoglycans and glycoproteins. The major sources of ECM elements are hepatic stellate cells (HSCs), which physiologically constitute about 5%-8% of liver cells. In the course of chronic liver injury, HSCs are activated and transformed from fat-storing cells (Ito cells) to myofibroblast-like cells. Along with this transformation, they undergo morphological and functional changes into contractile, smooth muscle α-actin-positive cells expressing profibrogenic and proinflammatory properties[1,2]. Among mediators activating HSCs, transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) are of special concern. PDGF stimulates proliferation and migration of HSCs, whereas TGF-β, which is the most potent profibrogenic cytokine, acting through its receptor, induces downstream signalling involving Smad family mediators. Thus, it regulates transcription of TGF-β target genes. The results of the above-mentioned signalling are complex; these include increased synthesis of fibrillar collagens, especially collagen type 1, and other ECM components, reduced expression of matrix metalloproteinase, along with augmented production of tissue inhibitor of metalloproteinase-1 (TIMP-1). So far, the perfect non-invasive biomarkers of hepatic fibrosis are under investigation include TGF-β, ECM components and TIMPs[3-5]. The overproduction of ECM components and imbalanced processes of synthesis-degradation eventually lead to progressive liver fibrosis[2]. Thus, reduction of fibrosis

can be obtained by either reduced liver myofibroblast activity, resulting from inhibition of ECM components synthesis, or enhanced degradation of ECM. The clinical data demonstrated that even advanced liver fibrosis can be inhibited and reversed[6]. However, the compounds with antifibrotic activities, potentially useful in clinical practice, are still under investigation.

CANNABINOIDS AND ENDOCANNABINOID SYSTEM IN PHYSIOLOGY AND PATHOLOGY

Cannabinoids are a group of compounds acting primarily *via* CB1 and CB2 receptors. The first cannabinoid discovered, in 1964, was a plant-derived Δ^9-tetrahydrocannabinol (THC), the psychoactive component of *Cannabis sativa*[7]. Following this finding was the discovery and determination of receptors for cannabinoids in nervous tissue. The first results were obtained by Matsuda *et al*[8] and presented the effects of cloning cDNA of G protein-coupled receptor found in neural cells, recognized then as CB1 receptor. However, this receptor was shown to be responsible primarily for psychoactive and neuronal effects of cannabinoids, which did not explain the other effects exerted by THC. Hence, the research for other receptors led to the discovery of CB2 receptor, expressed in macrophages of the spleen[9]. The presence of cannabinoid receptors in mammalian tissues prompted the research for its endogenous ligands and resulted in isolation of anandamide (AEA) and 2-arachidonoyl-glycerol (2-AG)[10,11]. So far, among several endogenous discovered ligands for cannabinoid receptors, AEA and 2-AG are the best characterized. The interesting feature of endocannabinoid mediators is that they are not stored in cells, instead, they are synthesized from lipid precursors in cellular membranes and released in response to specific stimuli[12].

The endocannabinoid system is comprised of at least two types of receptors for THC (CB1 and CB2), endogenous lipid compounds acting as ligands, and molecules regulating the synthesis and degradation of endocannabinoids. The expression of CB1 receptors was initially associated with the nervous system, as they were shown to control perception, cognitive, motor and behavioral functions. Nevertheless, the CB1 receptors are present peripherally in endothelial cells, adipocytes, gut and liver cells[13-16]. It has been shown that cannabinoid CB1 signalling regulates intake of high-energy-containing food and alcohol, energy homeostasis and hepatic lipogenesis. As to CB2 receptors, they are largely expressed in several lines of peripheral blood immune cells, tonsils, spleen and testis[17]. Moreover, their presence at low levels has been confirmed in various other tissues and cells, like hepatic myofibroblasts[18].

The imbalance in endocannabinoid system signaling is observed in various pathological conditions, including nervous system disorders, metabolic disturbances, impaired immunological responses (both allergy and hypersensivity), cardiovascular and gastrointestinal diseases, and carcinogenesis[19].

CANNABINOIDS IN HEPATIC FIBROSIS

The expression of cannabinoid receptors in normal liver is very low, partially because they are not expressed in hepatocytes. However, many studies have demonstrated the up-regulation of the expression of CB1 and CB2 receptors in hepatic myofibroblasts and vascular endothelial cells, as well as increased concentration of endocannabinoids, especially AEA, in liver in the course of chronic progressive liver diseases[18,20,21]. Teixeira-Clerc *et al*[20] have provided evidence for the involvement of CB1 receptor in regulation of hepatic fibrosis and the profibrogenic effect of CB1 signaling. Increased expression of CB1 receptors has been observed in human cirrhotic liver samples, primarily in HSCs during their transformation into myofibroblasts during the course of chronic liver injury. Moreover, the effect of CB1 inactivation was demonstrated in three experimental models of liver injury induced by CCl_4, thioacetamide or biliary cholestasis. The favorable antifibrogenic results were obtained either by pharmacological inactivation with rimonabant (SR141716), a selective antagonist of CB1 receptor, and *via* genetic inactivation in homozygous CB1-deficient mice. Decreased progression of fibrosis was accompanied by reduced hepatic TGF-β expression, and growth inhibition and increased apoptosis of myofibroblasts. These effects seemed to result from reduced phosphorylation of protein kinase B (PKB/Akt) and extracellular signal-regulated kinase (ERK), thus affecting the pathways responsible for cell proliferation and survival.

Although CB1 receptor is believed to have profibrogenic effects, studies on the CB2 receptor have proven its antifibrogenic activity in liver, as CB2 knockout mice developed augmented cirrhosis when exposed to CCl_4, compared to wild type[18]. It has been demonstrated in human cirrhotic liver samples that the expression of CB2 receptor is limited primarily to the cells positive for smooth muscle α-actin located within fibrotic septa; however, it is also detected in non-parenchymal cells, inflammatory cells and bile duct epithelial cells adjacent to fibrotic septa. The supporting results were obtained in separate research on cultured human hepatic myofibroblasts and activated rat HSCs, which were shown to express CB2 receptor. The final effects of the stimulation of CB2 receptor with THC or selective agonist JWH-015 are dose-dependent and expressed as growth inhibition or apoptosis. Interestingly, these two endpoints result from two distinct pathways, the induction of cyclooxygenase-2 (COX-2) in growth inhibition and intracellular oxidative stress for apoptosis, as they are diminished by the selective COX-2 inhibitor and two potent antioxidants, respectively.

Additionally, apart from receptor-dependent mechanisms of endocannabinoid actions on HSCs, the direct mechanism exerted by AEA leading to cell death have been observed in the research of Siegmund *et al*[22]. Stimulation of cultured human HSCs with AEA induces cell death in the necrotic pathway. This event is preceded by reactive oxygen species (ROS) formation and an increase in intracellular Ca^{2+} in HSCs. The pharmacologi-

cal inactivation of CB1, CB2 and vanilloid receptor-1 (VR1) does not prevent AEA-triggered cell death, which appears to be mediated by membrane cholesterol. Furthermore, the distinction between cholesterol content in the cellular membrane of HSCs and hepatocytes results in selective elimination of HSCs that are richer in cholesterol[22]. The conclusion of their analysis is that AEA exerts a potential antifibrogenic effect by inhibition of HSC proliferation and induction of necrotic death. The elevated levels of circulating AEA in cirrhotic patients might reflect the regulatory antifibrotic response to progression of fibrosis[22,23]. However, due to disadvantageous properties, such as triggering a local inflammatory response to necrosis and tissue damage, as well as systemic vasodilatation, the usefulness of AEA in the treatment of liver fibrosis is limited.

CANNABINOIDS IN LIVER STEATOSIS

Metabolic syndrome, leading to liver steatosis, has emerged as an important and frequent cause of chronic liver injury, ranging from simple steatosis to steatohepatitis, which is accompanied by inflammatory reaction and progressive fibrosis of liver tissue. The involvement of the endocannabinoid system in the pathogenesis of fatty liver disease has been shown recently. Since endocannabinoids are essential in regulation of energy balance, food intake and lipogenesis, impairment of this homeostasis results in various metabolic disturbances. Apart from central control of energy homeostasis *via* CB1 receptors localized in the brain, endocannabinoids seem to exert, as well CB1-receptor-dependent, peripheral effects on lipid metabolism in adipocytes, liver tissue and skeletal muscle[14]. This could be partially explained by increased expression of lipogenic transcription factor and activation of downstream enzymes, which result in increased fatty acid synthesis. Moreover, fat-rich diet has been shown to contribute to enhanced hepatic expression of CB1 in liver tissue and increased levels of endocannabinoids, thus increasing the metabolic imbalance[15].

ROLE OF ENDOCANNABINOID SYSTEM IN CONDITIONS ACCOMPANYING END-STAGE LIVER DISEASE

The role of the endocannabinoid system in liver diseases is complex. It has been particularly examined in end-stage liver disease and shown to contribute to hepatic encephalopathy and vascular effects such as portal hypertension, splanchnic vasodilatation, relative peripheral hypotension and probably cirrhotic cardiomyopathy.

There is limited, but reliable data on the neuroprotective role of the endocannabinoid system in hepatic encephalopathy. It has been demonstrated in a murine model that during fulminant hepatic failure, the levels of 2-AG in the brain are elevated, probably as a response to liver damage. The administration of CB2 endogenous ligand 2-AG, an antagonist of CB1 receptor, SR141716A, or an agonist of CB2 receptor, HU308, accomplished a marked improvement in neurological score. Hence, influencing the endocannabinoid system with exogenous cannabinoid derivates specific for the CB2 or CB1 receptor might have a beneficial therapeutic effect on neurological dysfunction in liver diseases[24]. Further research has indicated the impact of CB2 signaling on the activity of cerebral AMP-activated protein kinase (AMPK) in conditions of liver failure. It has been shown in wild type mice that administration of THC leads to increased activity of AMPK in the brain and neurological improvement, possibly *via* stimulation of CB2 receptors, as this effect is absent in CB2 knock-out mice[25].

Numerous hemodynamic vascular effects contributing to the poor prognosis of disease outcome accompany end-stage liver disease. The cirrhotic rebuilding of hepatic tissue results in increased resistance in portal circulation and eventually in elevated portal pressure. Additionally, the arterial vasodilatation in splanchnic and systemic circulations contributes to hyperdynamic state, arterial hypotension and increased blood inflow from mesenteric arteries, which augments the unfavorable effect of portal hypertension. There are many reports linking these vascular effects with the improper activity of the endocannabinoid system, particularly with stimulation of CB1 receptor in vascular endothelial cells with endogenous cannabinoids. The cirrhotic state is often accompanied by the endotoxemia caused by release of bacterial lipopolysaccharide (LPS) synthesized by the intestinal flora into the systemic circulation, while its hepatic elimination is insufficient[26]. The effects of blood LPS on systemic circulation correspond to the hemodynamic changes observed in cirrhosis[27]. Batkai *et al*[21] have provided evidence that explains the association between the endocannabinoid system and its influence on circulatory changes in cirrhosis. It was demonstrated in an animal model of cirrhosis complicated by hemodynamic alterations, that treatment with CB1 receptor antagonist (SR141716A) sufficiently improved hemodynamic state, which manifested in elevation of arterial pressure and reduction of mesenteric blood flow and portal pressure. It was shown that the intravenous injection of the monocyte fraction isolated from the blood of both cirrhotic rats and a patient with cirrhosis, was able to induce hypotension. This was reversible by treatment with SR141716A, whereas the injection of monocytes from controls did not exert such an effect. Moreover, the examination of monocytes from cirrhotic and control individuals and animals demonstrated increased levels of AEA in the monocytes in cirrhotic state, which may have reflected the stimulation of endocannabinoid synthesis by bacterial LPS shown in previous studies[21]. Additionally, upregulation of CB1 receptors in hepatic arterial endothelial cells isolated from cirrhotic livers was observed, thus, indicating its increased sensitivity to vasodilatory stimuli, such as endocannabinoids secreted by the monocytes and platelets adhering to endothelium[21].

Vascular effects exerted by endocannabinoids are divergent and complex. It is postulated that endocannabinoids might contribute to potency disorders in cirrhosis, as was observed in animal models. AEA was shown to

augment the relaxation of samples of corpus cavernosum from biliary cirrhotic rats, probably through CB1 and VR1 signalling[28].

Recently, the role of endocannabinoid signaling in the development of cardiomyopathy during liver cirrhosis has been investigated. It is characterized by decreased β-adrenergic responsiveness, impaired cardiac conduction and insufficient heart muscle contraction to excitation stimuli, whereas cardiac output remains increased compared to baseline. Gaskari *et al*[29] have confirmed in an animal model the role of CB1 signaling in the pathogenesis of cirrhotic cardiomyopathy. It has been shown that, when the cardiac muscle probes from cirrhotic rats are pre-incubated with the CB1 antagonist, AM251, their contractility is similar to the controls[29]. The significance of CB1 signaling has been demonstrated *in vivo* in cirrhotic rats presenting late symptoms of decreased cardiac contractility, hypotension and tachycardia. These symptoms were ameliorated in cirrhotic rats, by the bolus injection of AM251, whereas its administration in controls had no effect. Hence, the authors concluded that the above-mentioned cardiac effects might have resulted from increased concentration of AEA in cardiac tissue in liver cirrhosis, as the cardiac expression of CB1 receptors was similar in cirrhotic and non-cirrhotic control rats[30]. These observations are consistent with the study of Bonz *et al*[31] assessed the influence of AEA on the contractility of human heart atrial muscle upon electrical stimulation. The inotropic negative effect exerted by AEA and other examined CB1 agonists was predictably abolished by pre-incubation with CB1 antagonist ation[31]. Thus, blocking the CB1 signaling might have advantageous therapeutic effects on various clinical aspects of cirrhotic cardiomyopathy and other related conditions.

CLINICAL ASPECTS

There are limited clinical data on the effects of cannabinoids in chronic liver diseases. According to clinical research of Hezode *et al*[32], daily cannabis smoking appears to be an independent factor of fibrosis progression in chronic hepatitis C (CHC) patients. The research was performed on a group of 270 CHC patients, divided into non-cannabis users (52.2%), occasional cannabis users (14.8%) and daily cannabis users (33.0%). The collected data on epidemiological, demographic, metabolic and virological aspects, and history of cannabis, alcohol and tobacco abuse, allowed them to specify the factors for fibrosis progression. This study confirmed the well-recognized independent fibrosis predictors such as necroinflammatory activity ⩾ A2 (METAVIR score), age > 40 years at the time of exposure, steatosis and serious alcohol abuse, but also rated the daily cannabis use as a distinct factor that influenced alone the progression of liver fibrosis. This could result from profibrogenic activity of CB1 signaling, thus implying the beneficial therapeutic potential of CB1 antagonists.

Moreover, it has been proven that regular daily cannabis use has a significant impact on the severity of steatosis, which may eventually contribute to fibrosis progression in the course of CHC[33]. It has been shown that high fat dietary supply increases the hepatic levels of AEA, expression of CB1 receptor and augments fatty acid synthesis, thus contributing to obesity and other metabolic disorders proceeding to liver steatosis[15,34]. The mechanisms, in which endocannabinoids lead to obesity-associated fatty liver, or even steatohepatitis, are CB1-receptor-dependent and include increase in fatty acids intake, induction of lipolysis in adipocytes, stimulation of hepatic lipogenesis and downregulation of adiponectin in adipose tissue. Interestingly, CB1-knockout mice are resistant to obesity induced by high-energy-containing food intake[35]. Similarly, the pharmacological inactivation of CB1 with rimonabant (SR141716) results in reduction of obesity and hepatic steatosis in rodents[36,37].

ENDOCANNABINOID SYSTEM AS A THERAPEUTIC TARGET

The beneficial effect of the regulation of endocannabinoid signaling is postulated in management of various pathological conditions, including obesity and metabolic syndrome; addiction to alcohol, tobacco and opiates; Alzheimer's disease, Parkinson's disease, schizophrenia, memory loss, chronic pain, liver fibrosis, and numerous inflammatory conditions and allergies[38].

Due to their regulatory functions in chronic hepatic disorders, especially fibrosis, influencing the endocannabinoid receptors seems to be an advantageous therapeutic target. It seems that treatment with CB1 antagonist, CB2 agonist or both, may offer clinical benefits, resulting in at least deceleration of disease progression. It has also been shown that blocking CB1 signaling is favorable in maintaining the proper blood pressure in hypotensive cirrhotic rats[21,39]. Moreover, in clinical studies, rimonabant has exerted additional beneficial actions influencing the profile of blood lipids and glycemia control in obesity, metabolic syndrome and type 2 diabetes mellitus. It also had an impact on lifestyle modification, for instance, cigarette smoking cessation rates were significantly higher during treatment with rimonabant[40]. Interestingly, Wang *et al*[41] have suggested a possible link between CB1 signaling and ethanol preference in immature mice, and this effect was diminished after administration of rimonabant. This observation might be particularly useful in patients with alcoholic liver disease who persist in drinking.

CONCLUSION

The role of the endocannabinoid system in hepatic physiology and pathologic conditions has been studied recently. Unquestionably, influencing endocannabinoid signaling may have a beneficial effect on delaying or even reversing hepatic fibrosis. It is particularly important due to the lack of antifibrotic drugs with established advantageous profiles of activity, despite years of investigations into this subject. Thus, further research may provide the valuable means of management in hepatic fibrosis in the future.

REFERENCES

1 **Geerts A**. History, heterogeneity, developmental biology, and functions of quiescent hepatic stellate cells. *Semin Liver Dis* 2001; **21**: 311-335
2 **Gressner AM**, Weiskirchen R. Modern pathogenetic concepts of liver fibrosis suggest stellate cells and TGF-beta as major players and therapeutic targets. *J Cell Mol Med* 2006; **10**: 76-99
3 **Flisiak R**, Maxwell P, Prokopowicz D, Timms PM, Panasiuk A. Plasma tissue inhibitor of metalloproteinases-1 and transforming growth factor beta 1--possible non-invasive biomarkers of hepatic fibrosis in patients with chronic B and C hepatitis. *Hepatogastroenterology* 2002; **49**: 1369-1372
4 **Flisiak R**, Pytel-Krolczuk B, Prokopowicz D. Circulating transforming growth factor beta(1) as an indicator of hepatic function impairment in liver cirrhosis. *Cytokine* 2000; **12**: 677-681
5 **Gressner OA**, Weiskirchen R, Gressner AM. Biomarkers of hepatic fibrosis, fibrogenesis and genetic pre-disposition pending between fiction and reality. *J Cell Mol Med* 2007; **11**: 1031-1051
6 **Kershenobich D**, Uribe M, Suarez GI, Mata JM, Perez-Tamayo R, Rojkind M. Treatment of cirrhosis with colchicine. A double-blind randomized trial. *Gastroenterology* 1979; **77**: 532-536
7 **Gaoni Y**, Mechoulam R. Isolation, structure and partial synthesis of an active constituent of hashish. *J Am Chem Soc* 1964; **86**: 1646-1647
8 **Matsuda LA**, Lolait SJ, Brownstein MJ, Young AC, Bonner TI. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 1990; **346**: 561-564
9 **Munro S**, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 1993; **365**: 61-65
10 **Devane WA**, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 1992; **258**: 1946-1949
11 **Mechoulam R**, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, Gopher A, Almog S, Martin BR, Compton DR. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol* 1995; **50**: 83-90
12 **Sugiura T**, Kobayashi Y, Oka S, Waku K. Biosynthesis and degradation of anandamide and 2-arachidonoylglycerol and their possible physiological significance. *Prostaglandins Leukot Essent Fatty Acids* 2002; **66**: 173-192
13 **Liu J**, Gao B, Mirshahi F, Sanyal AJ, Khanolkar AD, Makriyannis A, Kunos G. Functional CB1 cannabinoid receptors in human vascular endothelial cells. *Biochem J* 2000; **346** Pt 3: 835-840
14 **Cota D**, Marsicano G, Tschop M, Grubler Y, Flachskamm C, Schubert M, Auer D, Yassouridis A, Thone-Reineke C, Ortmann S, Tomassoni F, Cervino C, Nisoli E, Linthorst AC, Pasquali R, Lutz B, Stalla GK, Pagotto U. The endogenous cannabinoid system affects energy balance via central orexigenic drive and peripheral lipogenesis. *J Clin Invest* 2003; **112**: 423-431
15 **Osei-Hyiaman D**, DePetrillo M, Pacher P, Liu J, Radaeva S, Batkai S, Harvey-White J, Mackie K, Offertaler L, Wang L, Kunos G. Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. *J Clin Invest* 2005; **115**: 1298-1305
16 **Croci T**, Manara L, Aureggi G, Guagnini F, Rinaldi-Carmona M, Maffrand JP, Le Fur G, Mukenge S, Ferla G. In vitro functional evidence of neuronal cannabinoid CB1 receptors in human ileum. *Br J Pharmacol* 1998; **125**: 1393-1395
17 **Bouaboula M**, Rinaldi M, Carayon P, Carillon C, Delpech B, Shire D, Le Fur G, Casellas P. Cannabinoid-receptor expression in human leukocytes. *Eur J Biochem* 1993; **214**: 173-180
18 **Julien B**, Grenard P, Teixeira-Clerc F, Van Nhieu JT, Li L, Karsak M, Zimmer A, Mallat A, Lotersztajn S. Antifibrogenic role of the cannabinoid receptor CB2 in the liver. *Gastroenterology* 2005; **128**: 742-755
19 **Sugiura T**, Kishimoto S, Oka S, Gokoh M. Biochemistry, pharmacology and physiology of 2-arachidonoylglycerol, an endogenous cannabinoid receptor ligand. *Prog Lipid Res* 2006; **45**: 405-446
20 **Teixeira-Clerc F**, Julien B, Grenard P, Tran Van Nhieu J, Deveaux V, Li L, Serriere-Lanneau V, Ledent C, Mallat A, Lotersztajn S. CB1 cannabinoid receptor antagonism: a new strategy for the treatment of liver fibrosis. *Nat Med* 2006; **12**: 671-676
21 **Batkai S**, Jarai Z, Wagner JA, Goparaju SK, Varga K, Liu J, Wang L, Mirshahi F, Khanolkar AD, Makriyannis A, Urbaschek R, Garcia N Jr, Sanyal AJ, Kunos G. Endocannabinoids acting at vascular CB1 receptors mediate the vasodilated state in advanced liver cirrhosis. *Nat Med* 2001; **7**: 827-832
22 **Siegmund SV**, Uchinami H, Osawa Y, Brenner DA, Schwabe RF. Anandamide induces necrosis in primary hepatic stellate cells. *Hepatology* 2005; **41**: 1085-1095
23 **Fernandez-Rodriguez CM**, Romero J, Petros TJ, Bradshaw H, Gasalla JM, Gutierrez ML, Lledo JL, Santander C, Fernandez TP, Tomas E, Cacho G, Walker JM. Circulating endogenous cannabinoid anandamide and portal, systemic and renal hemodynamics in cirrhosis. *Liver Int* 2004; **24**: 477-483
24 **Avraham Y**, Israeli E, Gabbay E, Okun A, Zolotarev O, Silberman I, Ganzburg V, Dagon Y, Magen I, Vorobia L, Pappo O, Mechoulam R, Ilan Y, Berry EM. Endocannabinoids affect neurological and cognitive function in thioacetamide-induced hepatic encephalopathy in mice. *Neurobiol Dis* 2006; **21**: 237-245
25 **Dagon Y**, Avraham Y, Ilan Y, Mechoulam R, Berry EM. Cannabinoids ameliorate cerebral dysfunction following liver failure via AMP-activated protein kinase. *FASEB J* 2007; **21**: 2431-2441
26 **Lumsden AB**, Henderson JM, Kutner MH. Endotoxin levels measured by a chromogenic assay in portal, hepatic and peripheral venous blood in patients with cirrhosis. *Hepatology* 1988; **8**: 232-236
27 **Varga K**, Wagner JA, Bridgen DT, Kunos G. Platelet- and macrophage-derived endogenous cannabinoids are involved in endotoxin-induced hypotension. *FASEB J* 1998; **12**: 1035-1044
28 **Ghasemi M**, Sadeghipour H, Shafaroodi H, Nezami BG, Gholipour T, Hajrasouliha AR, Tavakoli S, Nobakht M, Moore KP, Mani AR, Dehpour AR. Role of the nitric oxide pathway and the endocannabinoid system in neurogenic relaxation of corpus cavernosum from biliary cirrhotic rats. *Br J Pharmacol* 2007; **151**: 591-601
29 **Gaskari SA**, Liu H, Moezi L, Li Y, Baik SK, Lee SS. Role of endocannabinoids in the pathogenesis of cirrhotic cardiomyopathy in bile duct-ligated rats. *Br J Pharmacol* 2005; **146**: 315-323
30 **Batkai S**, Mukhopadhyay P, Harvey-White J, Kechrid R, Pacher P, Kunos G. Endocannabinoids acting at CB1 receptors mediate the cardiac contractile dysfunction in vivo in cirrhotic rats. *Am J Physiol Heart Circ Physiol* 2007; **293**: H1689-H1695
31 **Bonz A**, Laser M, Kullmer S, Kniesch S, Babin-Ebell J, Popp V, Ertl G, Wagner JA. Cannabinoids acting on CB1 receptors decrease contractile performance in human atrial muscle. *J Cardiovasc Pharmacol* 2003; **41**: 657-664
32 **Hezode C**, Roudot-Thoraval F, Nguyen S, Grenard P, Julien B, Zafrani ES, Pawlotsky JM, Dhumeaux D, Lotersztajn S, Mallat A. Daily cannabis smoking as a risk factor for progression of fibrosis in chronic hepatitis C. *Hepatology* 2005; **42**: 63-71
33 **Hezode C**, Zafrani ES, Roudot-Thoraval F, Costentin C, Hessami A, Bouvier-Alias M, Medkour F, Pawlostky JM,

Lotersztajn S, Mallat A. Daily cannabis use: a novel risk factor of steatosis severity in patients with chronic hepatitis C. *Gastroenterology* 2008; **134**: 432-439

34 **Osei-Hyiaman D**, Harvey-White J, Batkai S, Kunos G. The role of the endocannabinoid system in the control of energy homeostasis. *Int J Obes* (Lond) 2006; **30** Suppl 1: S33-S38

35 **Ravinet Trillou C**, Delgorge C, Menet C, Arnone M, Soubrie P. CB1 cannabinoid receptor knockout in mice leads to leanness, resistance to diet-induced obesity and enhanced leptin sensitivity. *Int J Obes Relat Metab Disord* 2004; **28**: 640-648

36 **Gary-Bobo M**, Elachouri G, Gallas JF, Janiak P, Marini P, Ravinet-Trillou C, Chabbert M, Cruccioli N, Pfersdorff C, Roque C, Arnone M, Croci T, Soubrie P, Oury-Donat F, Maffrand JP, Scatton B, Lacheretz F, Le Fur G, Herbert JM, Bensaid M. Rimonabant reduces obesity-associated hepatic steatosis and features of metabolic syndrome in obese Zucker fa/fa rats. *Hepatology* 2007; **46**: 122-129

37 **Ravinet Trillou C**, Arnone M, Delgorge C, Gonalons N, Keane P, Maffrand JP, Soubrie P. Anti-obesity effect of SR141716, a CB1 receptor antagonist, in diet-induced obese mice. *Am J Physiol Regul Integr Comp Physiol* 2003; **284**: R345-R353

38 **Muccioli GG**. Blocking the cannabinoid receptors: drug candidates and therapeutic promises. *Chem Biodivers* 2007; **4**: 1805-1827

39 **Ros J**, Claria J, To-Figueras J, Planaguma A, Cejudo-Martin P, Fernandez-Varo G, Martin-Ruiz R, Arroyo V, Rivera F, Rodes J, Jimenez W. Endogenous cannabinoids: a new system involved in the homeostasis of arterial pressure in experimental cirrhosis in the rat. *Gastroenterology* 2002; **122**: 85-93

40 **Gelfand EV**, Cannon CP. Rimonabant: a selective blocker of the cannabinoid CB1 receptors for the management of obesity, smoking cessation and cardiometabolic risk factors. *Expert Opin Investig Drugs* 2006; **15**: 307-315

41 **Wang L**, Liu J, Harvey-White J, Zimmer A, Kunos G. Endocannabinoid signaling via cannabinoid receptor 1 is involved in ethanol preference and its age-dependent decline in mice. *Proc Natl Acad Sci USA* 2003; **100**: 1393-1398

S- Editor Zhong XY **L- Editor** Rippe RA **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6115

World J Gastroenterol 2008 October 28; 14(40): 6115-6121
World Journal of Gastroenterology ISSN 1007-9327

REVIEW

Ste20-related proline/alanine-rich kinase: A novel regulator of intestinal inflammation

Yutao Yan, Didier Merlin

Yutao Yan, Didier Merlin, Department of Medicine, Division of Digestive Diseases, Emory University School of Medicine, 615 Michael Street, Atlanta, GA 30322, United States
Author contributions: Yan Y and Merlin D contributed equally to this work.
Correspondence to: Didier Merlin, PhD, Associate Professor, Emory University Department of Medicine Division of Digestive Diseases 615 Michael Street Atlanta GA 30322, United States. dmerlin@emory.edu
Telephone: +01-404-7276454 Fax: +01-404-727-5767
Received: May 14, 2008 Revised: July 28, 2008
Accepted: August 3, 2008
Published online: October 28, 2008

Abstract

Recently, inflammatory bowel disease (IBD) has been the subject of considerable research, with increasing attention being paid to the loss of intestinal epithelial cell barrier function as a mechanism of pathogenesis. Ste20-related proline/alanine-rich kinase (SPAK) is involved in regulating barrier function. SPAK is known to interact with inflammation-related kinases (such as p38, JNK, NKCC1, PKCθ, WNK and MLCK), and with transcription factor AP-1, resulting in diverse biological phenomena, including cell differentiation, cell transformation and proliferation, cytoskeleton rearrangement, and regulation of chloride transport. This review examines the involvement of Ste20-like kinases and downstream mitogen-activated protein kinases (MAPKs) pathways in the pathogenesis and control of intestinal inflammation. The primary focus will be on the molecular features of intestinal inflammation, with an emphasis on the interaction between SPAK and other molecules, and the effect of these interactions on homeostatic maintenance, cell volume regulation and increased cell permeability in intestinal inflammation.

Key words: Inflammatory bowel diseases; WNK; NKCC1; Barrier function; Ste20-related proline/alanine-rich kinase

Peer reviewer: Alessandro Fichera, MD, FACS, FASCRS, Assistant Professor, Department of Surgery-University of Chicago, 5841 S. Maryland Ave, MC 5031, Chicago, IL 60637, United States

Yan Y, Merlin D. Ste20-related proline/alanine-rich kinase: A novel regulator of intestinal inflammation. *World J Gastroenterol* 2008; 14(40): 6115-6121 Available from: URL: http://www.wjgnet.com/1007-9327/14/6115.asp DOI: http://dx.doi.org/10.3748/wjg.14.6115

INTRODUCTION

Inflammatory bowel diseases (IBD), primarily ulcerative colitis (UC) and Crohn's disease (CD), are chronic idiopathic inflammatory disorders of the gastrointestinal tract that are thought to arise as a result of an interplay of genetic and environmental factors. The mechanisms implicated in the pathogenesis of IBD (Figure 1) include: (1) inappropriate regulation of the innate immune response at the level of the intestinal mucosa; (2) deregulation of the adaptive immune system stemming from an imbalance between regulatory and effector-cell immune responses to luminal antigens; and (3) increased permeability across the mucosal epithelial barrier due to loss of structural integrity and/or abnormal transepithelial transport[1,2]. The loss of barrier function, in particular, has gained increasing support as an IBD pathogenic mechanism because the epithelium represents a potential intersection of both genetic and environmental influences. The intestinal mucosa is composed of a single layer of polarized intestinal epithelial cells (IECs) that protects against direct contact with enteric antigens, bacteria and other pathogens (Figure 1). The integrity of the epithelium is maintained primarily through a combination of intercellular adhesion structures and specialized junctions. In addition, other factors such as the presence of mucins, rapid turnover of epithelial cells, and peristaltic movement of the gastrointestinal tract, all help to protect against colonization and invasion of the intestinal mucosa by pathogens[3]. Moreover, epidemiological and genetic linkage studies have confirmed a strong link between modulation of the barrier function and IBD; these include, for example, the loci IBD1-9, corresponding to regions on chromosomes 16, 12, 6, 14, 5, 19, 1, 16 and 3, respectively[4-13], and a new IBD locus on chromosome 2[14].

MITOGEN-ACTIVATED PROTEIN KINASES (MAPKs) ARE INVOLVED IN INTESTINAL INFLAMMATION

Intracellular signaling cascades are the main route of communication between the plasma membrane and

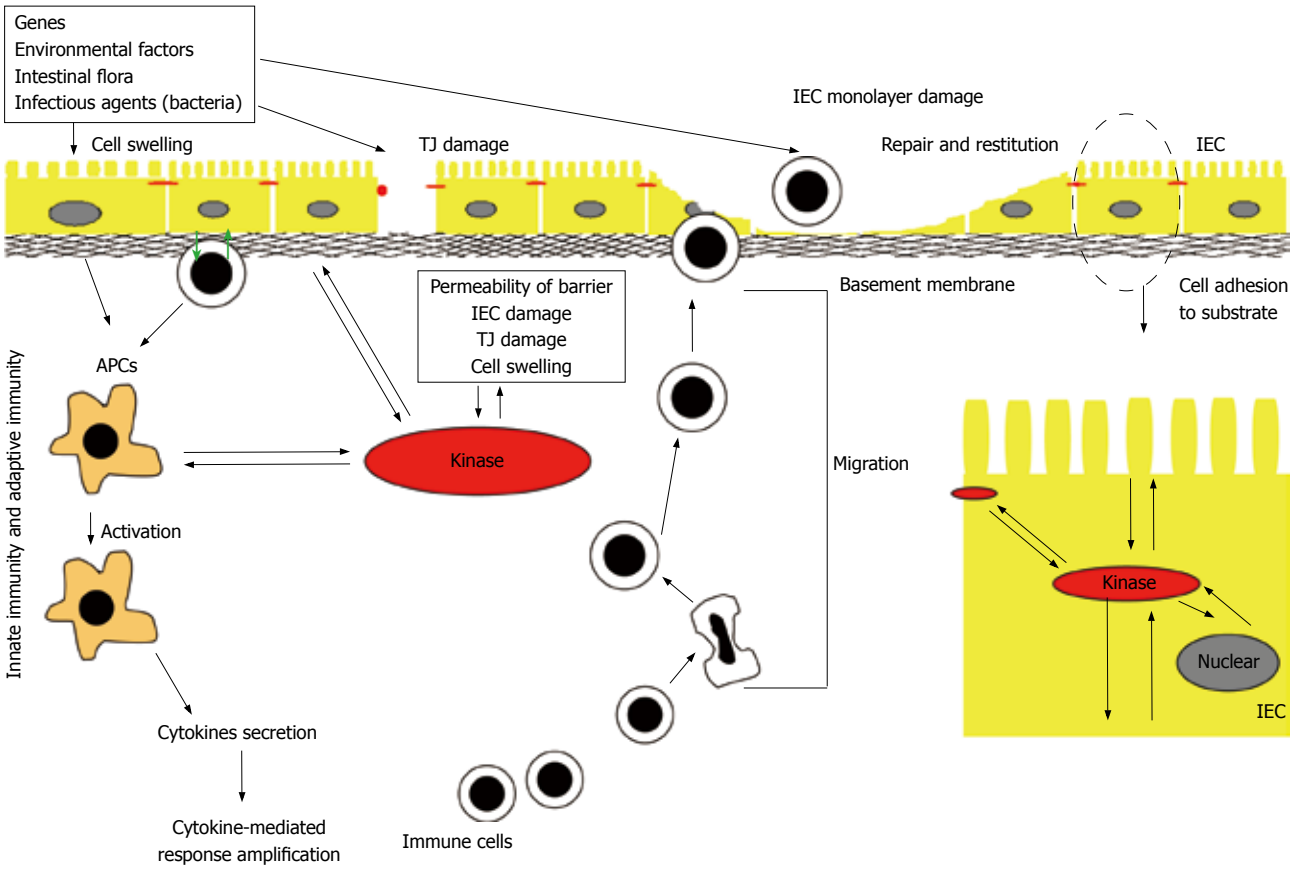


Figure 1 Pathogenesis of IBD. Many different factors, such as genetic factors, environmental factors, and intestinal non-pathogenic or pathogenic bacteria can damage the mucus, epithelium, or the tight junction, to initiate the inappropriate regulation or deregulation of the immune response, leading to the secretion of pro-inflammatory cytokines, decrease in epithelial barrier function and initiation of the inflammation-related signaling pathways. IEC: Intestinal epithelial cell; APC: Antigen presenting cell; TJ: Tight junction.

regulatory targets in various intracellular compartments. The evolutionarily conserved MAPK signaling pathway plays an important role in transducing signals from diverse extra-cellular stimuli (including growth factors, cytokines and environmental stresses) to the nucleus in order to affect a wide range of cellular processes, such as proliferation, differentiation, development, stress responses and apoptosis. MAPK signaling cascades, which comprise up to five levels of protein kinases that are sequentially activated by phosphorylation, are also involved in intestinal inflammation[15-17] (Figure 2).

MAPK signaling pathways are involved in regulating crucial inflammatory mediators and could thus serve as molecular targets for anti-inflammatory therapy. At least six distinct MAPK pathways have been identified in multicellular organisms, of which three, the extra-cellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK) and p38 cascades, are significantly activated and directly involved in inflammatory diseases such as IBD (Figure 2). In this context, cross-talk between these pathways and other inflammatory signaling pathways, including the NF-κB and Janus kinase/signal transducers, and activation of transcription (STAT) cascades[18-20], is also relevant to the action of MAPK pathways.

The involvement of some MAPK members in IBD is suggested by linkage studies. For example, the ERK1 gene is located in a major IBD susceptibility region on chromosome 16[4], and the p38α gene is located in a major IBD susceptibility region on chromosome 6[9]. Activation of p38 MAPK is also known to induce the production and secretion of pro-inflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor-α (TNF-α)[21], and increased activity of p38 MAPK has been observed in patients with IBD[18,22]. Inhibition of p38 has been well documented to suppress IBD[17], and the guanylhydrazone compound, CNI-1493, which inhibits both JNK and p38, strongly reduces clinical disease activity in CD patients. In addition, inhibition of either ERK or p38 kinase pathway decreases lipopolysaccharide (LPS)-induced production of the cytokines, IL-6 and TNF-α[23]. The involvement of JNK pathways in intestinal inflammation has been intensively studied both in patients with IBD and in an experimental colitis model[18,24,25]. JNK inhibitors, which affect either JNK signaling pathway indirectly (e.g. CEP1347) or block the catalytic domain of JNK (e.g. SP 600125), have been tested for their potential value in treating IBD. Collectively, these observations demonstrate a very important role for MAPK pathways in the control and therapy of IBD.

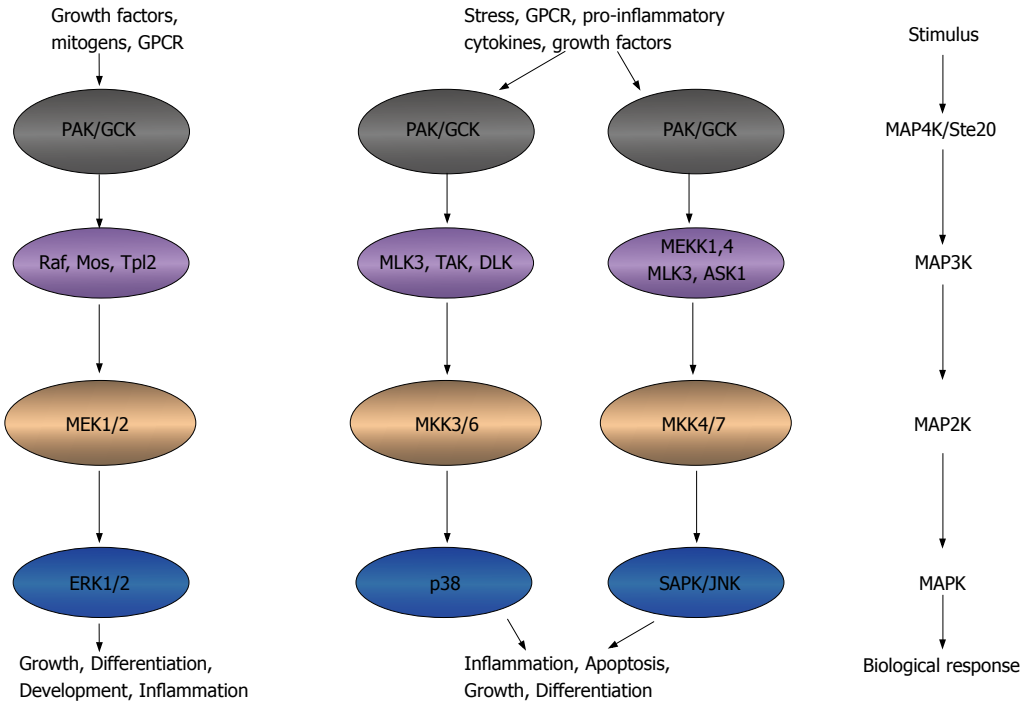


Figure 2 Ste20 kinases participate in inflammation. Ste20 kinases that function as an MAP4K can activate MAP3K, MAP2K and MAPK, leading to the inflammatory functions. This model adapted from the model presented in http://www.cellsignal.com/pathways/map-kinase.jsp. MAPK: Mitogen-activated protein kinase. GPCR: G-protein coupled receptor; PAK: p21 activated kinase; GCK: Germinal central kinase; MLK: Multiple lineage kinase; TAK: Tat-associated kinase; DLK: Dual leucine zipper-bearing kinase; MEK: MAPK/Erk kinase; MEKK: MEK kinase; ASK: Aspartate kinase; MKK: MAPK kinase; Erk: Extracellular signal-regulated kinase; SAPK: Stress-activated protein kinase; JNK: Jun-amino-terminal kinase.

STE20-LIKE KINASES ACT UPSTREAM OF MAPK PATHWAYS

The various MAPK pathways share a common family of upstream mediators: the Ste20 kinases. Ste20 was originally identified as a component of the pheromone-response pathway in budding yeast, and has also been shown to participate in the signaling pathways that regulate osmotic responses, including those to high osmolarity glycerol (HOG)[26]. Several mammalian Ste20 homologs have been identified. The Ste20 family includes two subfamilies that share basic structural and functional properties. The first subfamily includes the p21-activated kinases (PAKs), which are characterized by a C-terminal catalytic domain and an N-terminal binding site for the small G proteins, Rac1 and Cdc42. The second family comprises of the germinal center kinases (GCKs), which contain an N-terminal kinase domain and a C-terminal regulatory domain.

Ste20-like kinases function as MAP4Ks, triggering activation of MAPK cascade[27-29] and transmitting signals from extra-cellular stimuli that activate transcription factors (Figure 2). The resulting changes in gene expression, in turn, regulate cellular functions[27-31] that are important in the maintenance of epithelial barrier function, apoptosis, growth, morphogenesis, cell permeability, and rearrangements of the cytoskeleton that lead to changes in cell shape and motility. For example, members of the PAK subfamily of Ste20 kinases have been shown to increase endothelial permeability[32,33]. The pro-inflammatory cytokine, TNF-α, stimulates expression of the yeast Ste20 homolog, Map4k4, through TNF-α-receptor-1-mediated signaling to c-Jun[34], the chemokine CXCL12 and the complement factor C5a. The resulting increase in Map4k4 activity triggers cell migration *via* a PAK1/2-p38α MAPK-MAPKAP-K2-HSP27 pathway[35]. Other relevant examples include: (1) Ste20-like kinase (SLK)[36], Ste20-like oxidant stress-activated kinase (SOK)[37] and prostate-derived Ste20-like kinase 1-α (PSK1-α)[38], which induce apoptosis by activating the JNK pathway; (2) lymphocyte-oriented kinase (LOK)[39] and SLK[40], which regulate Rac1-mediated actin reorganization during cell adhesion and spreading; (3) mixed lineage kinase-3 (MLK-3)[41], which activates the SAPK/JNK and p38/RK pathways *via* SEK1 and MKK3/6; and (4) hematopoietic progenitor kinase 1 (HPK1), which is activated by prostaglandin E2 (PGE2) through a G-protein coupled receptor (GPCR) pathway, and negatively regulates transcription of the *fos* gene[42].

Ste20-like kinases has been reported to be activated by at least three pathogen-associated molecular patterns (PAMPs)-lipopolysaccharide, peptidoglycan, and flagellin-produced by invading microbial pathogens, and has been shown to initiate innate immune responses by binding to pattern recognition receptors (PRRs)[43]. PAMPs activate GCKs (Ste-20 family of kinases), which signal through MLK-2 and -3 to recruit JNK, p38 and their effectors[43]. These findings indicate an important role for GCKs and MLKs in PAMP-stimulated MAPK pathway activation, and therefore in stimulating the expression of pro-inflammatory genes involved in intestinal inflammation.

STE20-RELATED PROLINE/ALANINE-RICH KINASE (SPAK) IS A STE20-LIKE KINASES INVOLVED IN INTESTINAL INFLAMMATION

The GCKs may be divided into eight subfamilies based on homologies in their C-terminal domains (GCKI-VII). The Ste20-like kinase SPAK[44], PASK (the rat SPAK homolog)[45,46] and OSR1[47] share GCK Ⅵ homologies. Among these, SPAK and OSR1 are ubiquitously expressed. PASK is also expressed in most rat tissues, but its expression is particularly notable in cells with high ion-transport activity[45,48]. Both SPAK and PASK are highly expressed in epithelia and neurons[49]. On the other hand, PASK is found only in negligible levels in the liver and skeletal muscle[50]. SPAK, OSR1 and PASK contain a series of proline and alanine repeats (PAPA box) at the extreme N-terminus, followed by a serine/threonine kinase domain, a nuclear localization signal, a consensus caspase cleavage recognition motif, and a C-terminal regulatory region. However, the colonic SPAK isoform is unique in that it lacks the PAPA box and N-terminal F-alpha helix loop, due to the presence of a 5' splice junction-like sequence within exon-1[51]. Given its ubiquitous expression and diverse functional domains, the SPAK protein may be associated with diverse biological roles. It has been shown that under hyper-osmotic (but not hypo-osmotic) stress conditions, SPAK translocates from the cytosolic pool to a Triton X-100-insoluble fraction; although present in both fractions, SPAK associated with the Triton X-100-insoluble pool is dephosphorylated[52]. Our laboratory has observed that upon SPAK over-expression[51] or under TNF-α stress conditions (unpublished data), SPAK is cleaved and the N-terminal fragment is translocated to the nucleus.

The Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1), a member of the Slc12 family of solute carriers and target of SPAK, plays a crucial role in cell volume regulation, cell proliferation and survival, and epithelial transport[53]. The activity and expression of NKCC1 can be regulated by cell volume[53] and intracellular chloride concentration[54], which act through NKCC1's N-terminal (R/K) FX (V/I) binding motif. The pro-inflammatory cytokines IL-1β, TNF-α[55] and IL-6[56] also regulate NKCC1 activity. In addition, NKCC1 can be activated by α- and β-adrenergic stimulation *via* the cAMP/PKA-dependent pathway[57-59] and can be stimulated by PKC in a cell-specific manner[60,61]. Notably, NKCC1 can be phosphorylated by hyperosmolarity and, *in vitro*, by JNK, which can also be activated by hyperosmolarity[62,63]. As an upstream kinase to NKCC1, SPAK can associate through its conserved C-terminal domain with the (R/K) FX (V/I) motif of NKCC1 and phosphorylate Thr203, Thr207, and Thr212 residues on NKCC1, thereby playing an important role in inflammation[45,64,65]. However, SPAK alone is unable to activate NKCC1. SPAK is a substrate of WNK1/4, which are serine threonine kinases lacking a lysine in subdomain I of the catalytic domain[66]. SPAK physically associates through its conserved C-terminal domain with the C-terminus of WNK, resulting in phosphorylation and activation of SPAK by WNK. WNK is also unable to activate NKCC1 in the absence of SPAK, indicating that this association of SPAK with WNK is required for SPAK-dependent phosphorylation and activation of NKCC1. A mutation of WNK1 is involved in the pathogenesis of pseudohypoaldosteronism type Ⅱ (PHA Ⅱ), characterized by hypertension and hyperkalemia[67].

SPAK can also activate p38 pathways in different cell types[51,68,69] to play a role in cell differentiation; an observation that may be relevant in the context of the known relationship between the p38 pathway and inflammation[17,70-74]. Interestingly, p38 activation has been noted in damaged corneal epithelial tissue and in an *in vitro* intestinal epithelial restitution model[75-78], suggesting that under some circumstances p38 may be involved in regulating cell motility and wound healing. Protein kinase C θ (PKCθ) is known to be an intestinal inflammation-related kinase[79]. By associating with Rho GTPases, PKCθ migrates from the cytosol to the membrane and the actin cytoskeleton[80], where SPAK may act as both a substrate and target of PKCθ in a TCR/CD28-induced signaling pathway that leads selectively to AP-1 activation, T-cell transformation and proliferation, and IL-2 production[81]. SPAK is also known to associate with F-actin under conditions of stress, which, along with the activation and phosphorylation of myosin light chain kinase (MLCK), leads to cytoskeleton rearrangement[47,52]. Fray, the Drosophila orthologue of mammalian SPAK, has been shown to participate in the activation of the JNK pathway by sorbitol[47]. Fray probably functions by activating MAP3K, leading to activation of MAP2K/MEK4 and MEK7, and ultimately, JNK activation.

Accumulating evidence points to the important role that SPAK plays in the physiology and pathogenesis of intestinal inflammation (Figure 3). First, by activating and phosphorylating p38, Ap-1, NKCC1, as well as p21-activated protein kinase 1 (PAK1, another Ste20 line kinase), SPAK induces the transcription of inflammation-related genes or modulates the function of inflammation-related proteins. Second, SPAK is activated and phosphorylated by WNK1/4, PKCθ and MLCK. In addition, SPAK has been reported to associate with the heat shock protein HSP105, the cytoskeleton protein gelsolin, and the apoptosis-associated tyrosine kinase AATYK. We have observed that SPAK can increase the permeability of Caco2-BBE cells (unpublished observations). Additional unpublished data indicate that colonic epithelial SPAK expression is increased in IBD patients and in mice with experimentally induced colitis. Importantly, we have also found that the pro-inflammatory cytokine, TNF-α, increases colonic SPAK expression, an observation that underscores the importance of SPAK in the pathogenesis of intestinal inflammation.

PERSPECTIVE

Increased permeability across the mucosal epithelial

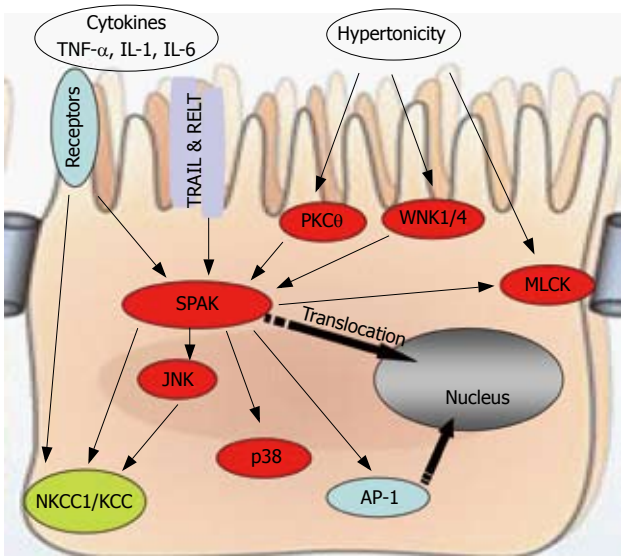


Figure 3 SPAK interacts with other molecules to maintain cellular homeostasis. SPAK can be a substrate, indirectly or directly, for pro-inflammatory cytokines, environmental stress including hypertonicity, some other kinases such as PKCθ, WNK1/4, or other receptors, for example TRAIL & RELT. Also SPAK can function as upstream kinase to JNK, p38, or ion transport NKCC1/KCC, transcription factor AP-1, as well as MLCK. WNK: With no lysine kinase 1/4; TRIL: TNF-related apoptosis-inducing ligand; RELT: Receptor expressed in lymphoid tissues; MLCK: Myosin Ⅱ regulatory light chain kinase; NKCC1: Sodium potassium chloride chloride transporter 1; KCC: Potassium chloride chloride transporter; AP-1: Activating protein 1.

barrier resulting from loss of structural integrity and/or abnormal transepithelial transport is thought to be one of the main functional changes that lead to IBD. Numerous studies have focused on epithelial barrier function, measuring transepithelial electrical resistance (TER), which is known to be decreased in intestinal epithelium by over-expression of SPAK[51]. Other studies have assessed cell adhesion and migration, providing a measure of wound healing. The pro-inflammatory cytokine TNF-α is both necessary and sufficient to trigger the onset of IBD. In fact, nearly half of the drugs used for the treatment of IBD target TNF-α. In *in vitro* studies, we have found that TNF-α increases SPAK expression in intestinal epithelial cells in a dose- and time- dependent manner (unpublished data). It is therefore reasonable to speculate that the regulation of SPAK by TNF-α could account for TNF-α-mediated alterations of barrier function and inflammation in intestinal epithelial cells. Additional studies on the role of SPAK in intestinal barrier function would likely substantially advance the field of IBD.

Intestinal inflammation is usually associated with hyper-osmotic status in the lumen. The WNK1/4-SPAK-NKCC1 pathway has been highlighted in this context as a molecular mechanism that may contribute to ion transport and cell volume changes. This pathway, together with its interactions with other related molecules, such as MLCK, claudin and zo-1, may play an important role in maintaining cell shape, since the epithelial cell tight junctions that play a dominant role in TER would collapse in IBD. In short, more attention should be paid to tight junction and cell volume regulation as important contributing factors in IBD.

It should be evident from this review that SPAK occupies an important intracellular position, integrating extra-cellular pro-inflammatory signals and converting them into pro-inflammatory cellular responses. Given its unique position at the crossroads of multiple pathways, SPAK appears to represent an attractive target for developing effective and efficient strategies to treat IBD. Continuing work along the lines suggested above could make important contributions to the effort to realize the potential of this therapeutic approach.

ACKNOWLEDGMENTS

Dr. Tracy S Obertone proof-read this manuscript, we appreciate her genuine contributions.

REFERENCES

1 **Gaudier E**, Michel C, Segain JP, Cherbut C, Hoebler C. The VSL# 3 probiotic mixture modifies microflora but does not heal chronic dextran-sodium sulfate-induced colitis or reinforce the mucus barrier in mice. *J Nutr* 2005; **135**: 2753-2761
2 **Goyette P**, Labbe C, Trinh TT, Xavier RJ, Rioux JD. Molecular pathogenesis of inflammatory bowel disease: genotypes, phenotypes and personalized medicine. *Ann Med* 2007; **39**: 177-199
3 **Lievin-Le Moal V**, Servin AL. The front line of enteric host defense against unwelcome intrusion of harmful microorganisms: mucins, antimicrobial peptides, and microbiota. *Clin Microbiol Rev* 2006; **19**: 315-337
4 **Hugot JP**, Laurent-Puig P, Gower-Rousseau C, Olson JM, Lee JC, Beaugerie L, Naom I, Dupas JL, Van Gossum A, Orholm M, Bonaiti-Pellie C, Weissenbach J, Mathew CG, Lennard-Jones JE, Cortot A, Colombel JF, Thomas G. Mapping of a susceptibility locus for Crohn's disease on chromosome 16. *Nature* 1996; **379**: 821-823
5 **Satsangi J**, Parkes M, Louis E, Hashimoto L, Kato N, Welsh K, Terwilliger JD, Lathrop GM, Bell JI, Jewell DP. Two stage genome-wide search in inflammatory bowel disease provides evidence for susceptibility loci on chromosomes 3, 7 and 12. *Nat Genet* 1996; **14**: 199-202
6 **van Heel DA**, Fisher SA, Kirby A, Daly MJ, Rioux JD, Lewis CM. Inflammatory bowel disease susceptibility loci defined by genome scan meta-analysis of 1952 affected relative pairs. *Hum Mol Genet* 2004; **13**: 763-770
7 **Peltekova VD**, Wintle RF, Rubin LA, Amos CI, Huang Q, Gu X, Newman B, Van Oene M, Cescon D, Greenberg G, Griffiths AM, St George-Hyslop PH, Siminovitch KA. Functional variants of OCTN cation transporter genes are associated with Crohn disease. *Nat Genet* 2004; **36**: 471-475
8 **Hampe J**, Franke A, Rosenstiel P, Till A, Teuber M, Huse K, Albrecht M, Mayr G, De La Vega FM, Briggs J, Gunther S, Prescott NJ, Onnie CM, Hasler R, Sipos B, Folsch UR, Lengauer T, Platzer M, Mathew CG, Krawczak M, Schreiber S. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat Genet* 2007; **39**: 207-211
9 **Hampe J**, Shaw SH, Saiz R, Leysens N, Lantermann A, Mascheretti S, Lynch NJ, MacPherson AJ, Bridger S, van Deventer S, Stokkers P, Morin P, Mirza MM, Forbes A, Lennard-Jones JE, Mathew CG, Curran ME, Schreiber S. Linkage of inflammatory bowel disease to human chromosome 6p. *Am J Hum Genet* 1999; **65**: 1647-1655
10 **Stoll M**, Corneliussen B, Costello CM, Waetzig GH, Mellgard B, Koch WA, Rosenstiel P, Albrecht M, Croucher PJ, Seegert D, Nikolaus S, Hampe J, Lengauer T, Pierrou

S, Foelsch UR, Mathew CG, Lagerstrom-Fermer M, Schreiber S. Genetic variation in DLG5 is associated with inflammatory bowel disease. *Nat Genet* 2004; **36**: 476-480

11 **Cho JH**, Nicolae DL, Gold LH, Fields CT, LaBuda MC, Rohal PM, Pickles MR, Qin L, Fu Y, Mann JS, Kirschner BS, Jabs EW, Weber J, Hanauer SB, Bayless TM, Brant SR. Identification of novel susceptibility loci for inflammatory bowel disease on chromosomes 1p, 3q, and 4q: evidence for epistasis between 1p and IBD1. *Proc Natl Acad Sci USA* 1998; **95**: 7502-7507

12 **Hampe J**, Frenzel H, Mirza MM, Croucher PJ, Cuthbert A, Mascheretti S, Huse K, Platzer M, Bridger S, Meyer B, Nurnberg P, Stokkers P, Krawczak M, Mathew CG, Curran M, Schreiber S. Evidence for a NOD2-independent susceptibility locus for inflammatory bowel disease on chromosome 16p. *Proc Natl Acad Sci USA* 2002; **99**: 321-326

13 **Duerr RH**, Barmada MM, Zhang L, Achkar JP, Cho JH, Hanauer SB, Brant SR, Bayless TM, Baldassano RN, Weeks DE. Evidence for an inflammatory bowel disease locus on chromosome 3p26: linkage, transmission/disequilibrium and partitioning of linkage. *Hum Mol Genet* 2002; **11**: 2599-2606

14 **Paavola-Sakki P**, Ollikainen V, Helio T, Halme L, Turunen U, Lahermo P, Lappalainen M, Farkkila M, Kontula K. Genome-wide search in Finnish families with inflammatory bowel disease provides evidence for novel susceptibility loci. *Eur J Hum Genet* 2003; **11**: 112-120

15 **Dubuquoy L**, Dharancy S, Nutten S, Pettersson S, Auwerx J, Desreumaux P. Role of peroxisome proliferator-activated receptor gamma and retinoid X receptor heterodimer in hep atogastroenterological diseases. *Lancet* 2002; **360**: 1410-1418

16 **Arulampalam V**, Pettersson S. Uncoupling the p38 MAPK kinase in IBD: a double edged sword? *Gut* 2002; **50**: 446-447

17 **Hollenbach E**, Neumann M, Vieth M, Roessner A, Malfertheiner P, Naumann M. Inhibition of p38 MAP kinase- and RICK/NF-kappaB-signaling suppresses inflammatory bowel disease. *FASEB J* 2004; **18**: 1550-1552

18 **Waetzig GH**, Seegert D, Rosenstiel P, Nikolaus S, Schreiber S. p38 mitogen-activated protein kinase is activated and linked to TNF-alpha signaling in inflammatory bowel disease. *J Immunol* 2002; **168**: 5342-5351

19 **Brinkman BM**, Telliez JB, Schievella AR, Lin LL, Goldfeld AE. Engagement of tumor necrosis factor (TNF) receptor 1 leads to ATF-2- and p38 mitogen-activated protein kinase-dependent TNF-alpha gene expression. *J Biol Chem* 1999; **274**: 30882-30886

20 **Hoffmeyer A**, Grosse-Wilde A, Flory E, Neufeld B, Kunz M, Rapp UR, Ludwig S. Different mitogen-activated protein kinase signaling pathways cooperate to regulate tumor necrosis factor alpha gene expression in T lymphocytes. *J Biol Chem* 1999; **274**: 4319-4327

21 **Yoshinari D**, Takeyoshi I, Koibuchi Y, Matsumoto K, Kawashima Y, Koyama T, Ohwada S, Morishita Y. Effects of a dual inhibitor of tumor necrosis factor-alpha and interleukin-1 on lipopolysaccharide-induced lung injury in rats: involvement of the p38 mitogen-activated protein kinase pathway. *Crit Care Med* 2001; **29**: 628-634

22 **Hommes D**, van den Blink B, Plasse T, Bartelsman J, Xu C, Macpherson B, Tytgat G, Peppelenbosch M, Van Deventer S. Inhibition of stress-activated MAP kinases induces clinical improvement in moderate to severe Crohn's disease. *Gastroenterology* 2002; **122**: 7-14

23 **Carter AB**, Monick MM, Hunninghake GW. Both Erk and p38 kinases are necessary for cytokine gene transcription. *Am J Respir Cell Mol Biol* 1999; **20**: 751-758

24 **Mitsuyama K**, Suzuki A, Tomiyasu N, Tsuruta O, Kitazaki S, Takeda T, Satoh Y, Bennett BL, Toyonaga A, Sata M. Pro-inflammatory signaling by Jun-N-terminal kinase in inflammatory bowel disease. *Int J Mol Med* 2006; **17**: 449-455

25 **Moon DO**, Jin CY, Lee JD, Choi YH, Ahn SC, Lee CM, Jeong SC, Park YM, Kim GY. Curcumin decreases binding of Shiga-like toxin-1B on human intestinal epithelial cell line HT29 stimulated with TNF-alpha and IL-1beta: suppression of p38, JNK and NF-kappaB p65 as potential targets. *Biol Pharm Bull* 2006; **29**: 1470-1475

26 **Raitt DC**, Posas F, Saito H. Yeast Cdc42 GTPase and Ste20 PAK-like kinase regulate Sho1-dependent activation of the Hog1 MAPK pathway. *EMBO J* 2000; **19**: 4623-4631

27 **Bagrodia S**, Cerione RA. Pak to the future. *Trends Cell Biol* 1999; **9**: 350-355

28 **Sells MA**, Chernoff J. Emerging from the Pak: the p21-activated protein kinase family. *Trends Cell Biol* 1997; **7**: 162-167

29 **Kyriakis JM**. Signaling by the germinal center kinase family of protein kinases. *J Biol Chem* 1999; **274**: 5259-5262

30 **Widmann C**, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev* 1999; **79**: 143-180

31 **Ip YT**, Davis RJ. Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development. *Curr Opin Cell Biol* 1998; **10**: 205-219

32 **Stockton RA**, Schaefer E, Schwartz MA. p21-activated kinase regulates endothelial permeability through modulation of contractility. *J Biol Chem* 2004; **279**: 46621-46630

33 **Stockton R**, Reutershan J, Scott D, Sanders J, Ley K, Schwartz MA. Induction of vascular permeability: beta PIX and GIT1 scaffold the activation of extracellular signal-regulated kinase by PAK. *Mol Biol Cell* 2007; **18**: 2346-2355

34 **Tesz GJ**, Guilherme A, Guntur KV, Hubbard AC, Tang X, Chawla A, Czech MP. Tumor necrosis factor alpha (TNFalpha) stimulates Map4k4 expression through TNFalpha receptor 1 signaling to c-Jun and activating transcription factor 2. *J Biol Chem* 2007; **282**: 19302-19312

35 **Rousseau S**, Dolado I, Beardmore V, Shpiro N, Marquez R, Nebreda AR, Arthur JS, Case LM, Tessier-Lavigne M, Gaestel M, Cuenda A, Cohen P. CXCL12 and C5a trigger cell migration via a PAK1/2-p38alpha MAPK-MAPKAP-K2-HSP27 pathway. *Cell Signal* 2006; **18**: 1897-1905

36 **Sabourin LA**, Rudnicki MA. Induction of apoptosis by SLK, a Ste20-related kinase. *Oncogene* 1999; **18**: 7566-7575

37 **Pombo CM**, Bonventre JV, Molnar A, Kyriakis J, Force T. Activation of a human Ste20-like kinase by oxidant stress defines a novel stress response pathway. *EMBO J* 1996; **15**: 4537-4546

38 **Zihni C**, Mitsopoulos C, Tavares IA, Baum B, Ridley AJ, Morris JD. Prostate-derived sterile 20-like kinase 1-alpha induces apoptosis. JNK- and caspase-dependent nuclear localization is a requirement for membrane blebbing. *J Biol Chem* 2007; **282**: 6484-6493

39 **Endo J**, Toyama-Sorimachi N, Taya C, Kuramochi-Miyagawa S, Nagata K, Kuida K, Takashi T, Yonekawa H, Yoshizawa Y, Miyasaka N, Karasuyama H. Deficiency of a STE20/PAK family kinase LOK leads to the acceleration of LFA-1 clustering and cell adhesion of activated lymphocytes. *FEBS Lett* 2000; **468**: 234-238

40 **Wagner S**, Flood TA, O'Reilly P, Hume K, Sabourin LA. Association of the Ste20-like kinase (SLK) with the microtubule. Role in Rac1-mediated regulation of actin dynamics during cell adhesion and spreading. *J Biol Chem* 2002; **277**: 37685-37692

41 **Tibbles LA**, Ing YL, Kiefer F, Chan J, Iscove N, Woodgett JR, Lassam NJ. MLK-3 activates the SAPK/JNK and p38/RK pathways via SEK1 and MKK3/6. *EMBO J* 1996; **15**: 7026-7035

42 **Sawasdikosol S**, Russo KM, Burakoff SJ. Hematopoietic progenitor kinase 1 (HPK1) negatively regulates prostaglandin E2-induced fos gene transcription. *Blood* 2003; **101**: 3687-3689

43 **Zhong J**, Kyriakis JM. Dissection of a signaling pathway by which pathogen-associated molecular patterns recruit the JNK and p38 MAPKs and trigger cytokine release. *J Biol Chem* 2007; **282**: 24246-24254

44 **Johnston AM**, Naselli G, Gonez LJ, Martin RM, Harrison LC, DeAizpurua HJ. SPAK, a STE20/SPS1-related kinase that activates the p38 pathway. *Oncogene* 2000; **19**: 4290-4297

45 **Dowd BF**, Forbush B. PASK (proline-alanine-rich STE20-related kinase), a regulatory kinase of the Na-K-Cl cotransporter (NKCC1). *J Biol Chem* 2003; **278**: 27347-27353

46 **Ushiro H**, Tsutsumi T, Suzuki K, Kayahara T, Nakano K. Molecular cloning and characterization of a novel Ste20-related protein kinase enriched in neurons and transporting epithelia. *Arch Biochem Biophys* 1998; **355**: 233-240

47 **Chen W**, Yazicioglu M, Cobb MH. Characterization of OSR1, a member of the mammalian Ste20p/germinal center kinase subfamily. *J Biol Chem* 2004; **279**: 11129-11136

48 **Moriguchi T**, Urushiyama S, Hisamoto N, Iemura S, Uchida S, Natsume T, Matsumoto K, Shibuya H. WNK1 regulates phosphorylation of cation-chloride-coupled cotransporters via the STE20-related kinases, SPAK and OSR1. *J Biol Chem* 2005; **280**: 42685-42693

49 **Piechotta K**, Garbarini N, England R, Delpire E. Characterization of the interaction of the stress kinase SPAK with the Na+-K+-2Cl- cotransporter in the nervous system: evidence for a scaffolding role of the kinase. *J Biol Chem* 2003; **278**: 52848-52856

50 **Miao N**, Fung B, Sanchez R, Lydon J, Barker D, Pang K. Isolation and expression of PASK, a serine/threonine kinase, during rat embryonic development, with special emphasis on the pancreas. *J Histochem Cytochem* 2000; **48**: 1391-1400

51 **Yan Y**, Nguyen H, Dalmasso G, Sitaraman SV, Merlin D. Cloning and characterization of a new intestinal inflammation-associated colonic epithelial Ste20-related protein kinase isoform. *Biochim Biophys Acta* 2007; **1769**: 106-116

52 **Tsutsumi T**, Ushiro H, Kosaka T, Kayahara T, Nakano K. Proline- and alanine-rich Ste20-related kinase associates with F-actin and translocates from the cytosol to cytoskeleton upon cellular stresses. *J Biol Chem* 2000; **275**: 9157-9162

53 **Russell JM**. Sodium-potassium-chloride cotransport. *Physiol Rev* 2000; **80**: 211-276

54 **Breitwieser GE**, Altamirano AA, Russell JM. Osmotic stimulation of Na(+)-K(+)-Cl- cotransport in squid giant axon is [Cl-]i dependent. *Am J Physiol* 1990; **258**: C749-C753

55 **Topper JN**, Wasserman SM, Anderson KR, Cai J, Falb D, Gimbrone MA Jr. Expression of the bumetanide-sensitive Na-K-Cl cotransporter BSC2 is differentially regulated by fluid mechanical and inflammatory cytokine stimuli in vascular endothelium. *J Clin Invest* 1997; **99**: 2941-2949

56 **Sun D**, Lytle C, O'Donnell ME. IL-6 secreted by astroglial cells regulates Na-K-Cl cotransport in brain microvessel endothelial cells. *Am J Physiol* 1997; **272**: C1829-C1835

57 **Liedtke CM**. Bumetanide-sensitive NaCl uptake in rabbit tracheal epithelial cells is stimulated by neurohormones and hypertonicity. *Am J Physiol* 1992; **262**: L621-L627

58 **Paulais M**, Turner RJ. Beta-adrenergic upregulation of the Na(+)-K(+)-2Cl- cotransporter in rat parotid acinar cells. *J Clin Invest* 1992; **89**: 1142-1147

59 **Andersen GO**, Enger M, Thoresen GH, Skomedal T, Osnes JB. Alpha1-adrenergic activation of myocardial Na-K-2Cl cotransport involving mitogen-activated protein kinase. *Am J Physiol* 1998; **275**: H641-H652

60 **Homma T**, Burns KD, Harris RC. Agonist stimulation of Na+/K+/Cl- cotransport in rat glomerular mesangial cells. Evidence for protein kinase C-dependent and Ca2+/calmodulin-dependent pathways. *J Biol Chem* 1990; **265**: 17613-17620

61 **Torchia J**, Yi Q, Sen AK. Carbachol-stimulated phosphorylation of the Na-K-Cl cotransporter of avian salt gland. Requirement for Ca2+ and PKC Activation. *J Biol Chem* 1994; **269**: 29778-29784

62 **O'Neill WC**, Klein JD. Regulation of vascular endothelial cell volume by Na-K-2Cl cotransport. *Am J Physiol* 1992; **262**: C436-C444

63 **Klein JD**, Lamitina ST, O'Neill WC. JNK is a volume-sensitive kinase that phosphorylates the Na-K-2Cl cotransporter in vitro. *Am J Physiol* 1999; **277**: C425-C431

64 **Piechotta K**, Lu J, Delpire E. Cation chloride cotransporters interact with the stress-related kinases Ste20-related proline-alanine-rich kinase (SPAK) and oxidative stress response 1 (OSR1). *J Biol Chem* 2002; **277**: 50812-50819

65 **Gagnon KB**, England R, Delpire E. Characterization of SPAK and OSR1, regulatory kinases of the Na-K-2Cl cotransporter. *Mol Cell Biol* 2006; **26**: 689-698

66 **Xu B**, English JM, Wilsbacher JL, Stippec S, Goldsmith EJ, Cobb MH. WNK1, a novel mammalian serine/threonine protein kinase lacking the catalytic lysine in subdomain II. *J Biol Chem* 2000; **275**: 16795-16801

67 **Wilson FH**, Disse-Nicodeme S, Choate KA, Ishikawa K, Nelson-Williams C, Desitter I, Gunel M, Milford DV, Lipkin GW, Achard JM, Feely MP, Dussol B, Berland Y, Unwin RJ, Mayan H, Simon DB, Farfel Z, Jeunemaitre X, Lifton RP. Human hypertension caused by mutations in WNK kinases. *Science* 2001; **293**: 1107-1112

68 **Johnston AM**, Naselli G, Gonez LJ, Martin RM, Harrison LC, DeAizpurua HJ. SPAK, a STE20/SPS1-related kinase that activates the p38 pathway. *Oncogene* 2000; **19**: 4290-4297

69 **Dan I**, Watanabe NM, Kusumi A. The Ste20 group kinases as regulators of MAP kinase cascades. *Trends Cell Biol* 2001; **11**: 220-230

70 **Chen S**, Supakar PC, Vellanoweth RL, Song CS, Chatterjee B, Roy AK. Functional role of a conformationally flexible homopurine/homopyrimidine domain of the androgen receptor gene promoter interacting with Sp1 and a pyrimidine single strand DNA-binding protein. *Mol Endocrinol* 1997; **11**: 3-15

71 **Guan Z**, Buckman SY, Pentland AP, Templeton DJ, Morrison AR. Induction of cyclooxygenase-2 by the activated MEKK1 --> SEK1/MKK4 --> p38 mitogen-activated protein kinase pathway. *J Biol Chem* 1998; **273**: 12901-12908

72 **Badger AM**, Cook MN, Lark MW, Newman-Tarr TM, Swift BA, Nelson AH, Barone FC, Kumar S. SB 203580 inhibits p38 mitogen-activated protein kinase, nitric oxide production, and inducible nitric oxide synthase in bovine cartilage-derived chondrocytes. *J Immunol* 1998; **161**: 467-473

73 **Craxton A**, Shu G, Graves JD, Saklatvala J, Krebs EG, Clark EA. p38 MAPK is required for CD40-induced gene expression and proliferation in B lymphocytes. *J Immunol* 1998; **161**: 3225-3236

74 **Pietersma A**, Tilly BC, Gaestel M, de Jong N, Lee JC, Koster JF, Sluiter W. p38 mitogen activated protein kinase regulates endothelial VCAM-1 expression at the post-transcriptional level. *Biochem Biophys Res Commun* 1997; **230**: 44-48

75 **Sharma GD**, He J, Bazan HE. p38 and ERK1/2 coordinate cellular migration and proliferation in epithelial wound healing: evidence of cross-talk activation between MAP kinase cascades. *J Biol Chem* 2003; **278**: 21989-21997

76 **Lu L**, Reinach PS, Kao WW. Corneal epithelial wound healing. *Exp Biol Med* (Maywood) 2001; **226**: 653-664

77 **Yu CF**, Sanders MA, Basson MD. Human caco-2 motility redistributes FAK and paxillin and activates p38 MAPK in a matrix-dependent manner. *Am J Physiol Gastrointest Liver Physiol* 2000; **278**: G952-G966

78 **Frey MR**, Golovin A, Polk DB. Epidermal growth factor-stimulated intestinal epithelial cell migration requires Src family kinase-dependent p38 MAPK signaling. *J Biol Chem* 2004; **279**: 44513-44521

79 **Hokama A**, Mizoguchi E, Sugimoto K, Shimomura Y, Tanaka Y, Yoshida M, Rietdijk ST, de Jong YP, Snapper SB, Terhorst C, Blumberg RS, Mizoguchi A. Induced reactivity of intestinal CD4(+) T cells with an epithelial cell lectin, galectin-4, contributes to exacerbation of intestinal inflammation. *Immunity* 2004; **20**: 681-693

80 **Sedwick CE**, Altman A. Perspectives on PKCtheta in T cell activation. *Mol Immunol* 2004; **41**: 675-686

81 **Li Y**, Hu J, Vita R, Sun B, Tabata H, Altman A. SPAK kinase is a substrate and target of PKCtheta in T-cell receptor-induced AP-1 activation pathway. *EMBO J* 2004; **23**: 1112-1122

S- Editor Tian L **L- Editor** Anand BS **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6122

World J Gastroenterol 2008 October 28; 14(40): 6122-6132
World Journal of Gastroenterology ISSN 1007-9327

REVIEW

Natural heme oxygenase-1 inducers in hepatobiliary function

Giovanni Li Volti, David Sacerdoti, Claudia Di Giacomo, Maria Luisa Barcellona, Antonio Scacco, Paolo Murabito, Antonio Biondi, Francesco Basile, Diego Gazzolo, Raul Abella, Alessandro Frigiola, Fabio Galvano

Giovanni Li Volti, Claudia Di Giacomo, Maria Luisa Barcellona, Antonio Scacco, Fabio Galvano, Department of Biological Chemistry, Medical Chemistry and Molecular Biology, University of Catania, Catania 95125, Italy
David Sacerdoti, Department of Clinical and Experimental Medicine, University of Padova, Padova 35100, Italy
Paolo Murabito, Department of Surgery, section of Anesthesiology, University of Catania, Catania 95125, Italy
Antonio Biondi, Francesco Basile, Department of Surgery, Section of General and Oncological Surgery, University of Catania, Catania 95125, Italy
Diego Gazzolo, Neonatal Intensive Care Unit "Cesare Arrigo" Children Hospital, Alessandria 15100, Italy
Diego Gazzolo, Department of Pediatrics, G. Gaslini Children's University Hospital, Genoa 16100, Italy
Raul Abella, Alessandro Frigiola, Perinatal Research Laboratory IRCSS San Donato Milanese, Milan 20100, Italy
Author contributions: All authors contributed equally to this work.
Supported by Grants (ex 60%) from MURST (Ministero dell' Università e della Ricerca Scientifica e Tecnologica), Rome, Italy
Correspondence to: Giovanni Li Volti, MD, PhD, Professor of Biochemistry, Department of Biological Chemistry, Medical Chemistry and Molecular Biology, University of Catania, Viale Andrea Doria, 6, Catania 95125, Italy. livolti@unict.it
Telephone: +39-95-7384081 Fax: +39-95-7384220
Received: March 23, 2008 Revised: July 23, 2008
Accepted: July 30, 2008
Published online: October 28, 2008

Abstract

Many physiological effects of natural antioxidants, their extracts or their major active components, have been reported in recent decades. Most of these compounds are characterized by a phenolic structure, similar to that of α-tocopherol, and present antioxidant properties that have been demonstrated both *in vitro* and *in vivo*. Polyphenols may increase the capacity of endogenous antioxidant defences and modulate the cellular redox state. Changes in the cellular redox state may have wide-ranging consequences for cellular growth and differentiation. The majority of *in vitro* and *in vivo* studies conducted so far have attributed the protective effect of bioactive polyphenols to their chemical reactivity toward free radicals and their capacity to prevent the oxidation of important intracellular components. However, in recent years a possible novel aspect in the mode of action of these compounds has been suggested; that is, the ultimate stimulation of the heme oxygenase-1 (HO-1) pathway is likely to account for the established and powerful antioxidant/anti-inflammatory properties of these polyphenols. The products of the HO-catalyzed reaction, particularly carbon monoxide (CO) and biliverdin/bilirubin have been shown to exert protective effects in several organs against oxidative and other noxious stimuli. In this context, it is interesting to note that induction of HO-1 expression by means of natural compounds contributes to protection against liver damage in various experimental models. The focus of this review is on the significance of targeted induction of HO-1 as a potential therapeutic strategy to protect the liver against various stressors in several pathological conditions.

Key words: Heme oxygenase; Hepatobiliary function; Natural inducers; Carbon monoxide; Oxidative stress

Peer reviewer: Maurizio Parola, Professor, Dip. Medicina e Oncologia Sperimentale, Universita degli Studi di torino, Corso Raffaello 30, Torino 10125, Italy

Li Volti G, Sacerdoti D, Di Giacomo C, Barcellona ML, Scacco A, Murabito P, Biondi A, Basile F, Gazzolo D, Abella R, Frigiola A, Galvano F. Natural heme oxygenase-1 inducers in hepatobiliary function. *World J Gastroenterol* 2008; 14(40): 6122-6132 Available from: URL: http://www.wjgnet.com/1007-9327/14/6122.asp DOI: http://dx.doi.org/10.3748/wjg.14.6122

INTRODUCTION

Heme oxygenase (HO) is the first, and the rate limiting enzyme in the catabolism of heme[1], to yield equimolar amounts of biliverdin, carbon monoxide (CO) and free iron (Figure 1). To date, two isoforms of HO designated as HO-1 and HO-2 have been identified in mammals[2]. HO-1 is also known as heat shock protein 32. Its human form is composed of 288 amino acids with a molecular mass of 32800 Da and shares about 80% amino acid sequence identity with rat HO-1[3]. On the other hand, human HO-2 is a 36-kDa protein that consists of 316

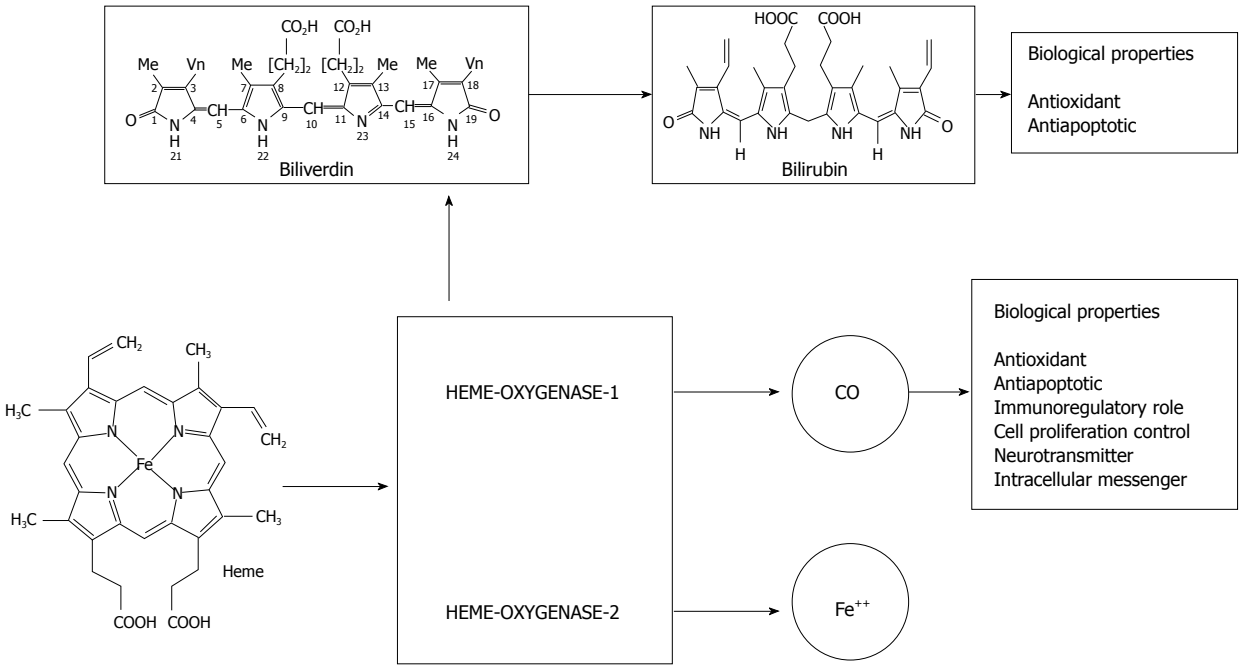


Figure 1 Schematic representation of heme degradation with biological properties of its byproducts.

amino acids with three cysteine residues[4]. HO-1 is highly inducible by hemin and other chemical and physical agents such as ultraviolet, hydrogen peroxide, heavy metals, hypoxia, and nitric oxide[5,6]. Immunohistochemical studies with specific monoclonal antibodies have revealed the distribution of HO-1 and HO-2 in rat liver with distinct topographical patterns[7]: HO-1 has been shown to be expressed principally in Kupffer cells, while HO-2 is expressed in parenchymal cells[8]. Trakshel *et al*[2] have demonstrated that under unstimulated conditions, the activity of HO-2 was two- to three-fold higher than that of HO-1, while the activity of HO-1 increased more than 100-fold in the presence of cadmium or cobalt. Under conditions of oxidative stress, hypoxia or hyperthermia, the induction of HO-1 accounts for the majority of heme breakdown, leading to the formation of bilirubin and CO. Since HO-1 is induced as a protective mechanism in response to various stimuli, targeted induction of this stress-response enzyme may be considered as an important therapeutic strategy for the protection against inflammatory processes and oxidative tissue damage (Figure 1). In this article, recent findings on the implications of HO-1 induction on the cellular adaptive cytoprotective response to various insults and inflammatory conditions are reviewed, with particular emphasis placed on targeted HO-1 induction by natural compounds for hepatoprotection.

NATURAL INDUCERS OF HO-1

A number of natural antioxidant compounds contained in foods and plants have been demonstrated to be effective non-stressful and non-cytotoxic inducers of the response protein HO-1 in hepatic cellular models. Most of these compounds are contained in plants that, besides being widely used as food, spices or flavoring, since ancient times, also represent locally traditional medicinal plants.

Curcumin

Curcumin (diferuloylmethane, Figure 2) is the most investigated natural HO-1 inducer. Curcumin is a yellow pigment obtained by populations living in Asian tropical regions by drying and powdering the rhizome of turmeric (*Curcuma longa Linn*). Widely used as food flavouring, it also plays an important role in traditional medicine because of its anti-inflammatory, anticarcinogenic and antioxidant properties. Curcumin has been demonstrated to be a potent HO-1 inducer in several cellular models (for a Review see Lin[9]). However, the ability of curcumin to induce HO-1 in human hepatocytes has been demonstrated only recently by McNally *et al*[10]. Interestingly, curcumin is able to confer, at non-toxic doses, a significant protective effect in two transplant-related models of cellular injury, such as cold preservation and warm reperfusion. In a successive study[11], these authors confirmed the HO-1 induction ability of curcumin and elucidated the possible biochemical mechanism. Indeed, at both non-toxic and toxic doses, curcumin treatment resulted in ROS generation, activation of Nrf2 and mitogen-activated protein kinases (MAPKs) and in the inhibition of phosphatase activity. They concluded that at non-toxic doses these multiple pathways converged to induce HO-1.

Flavonoids

Flavonoids are naturally occurring antioxidants belonging to the large family of polyphenols. They are widely distributed in plants used as food, as well as traditional medicines, because of their peculiar variety of clinically relevant properties, such as anti-tumor, antiplatelet, anti-ischemic, and anti-inflammatory activities. Antioxidants with strong free-radical scavenging properties contribute

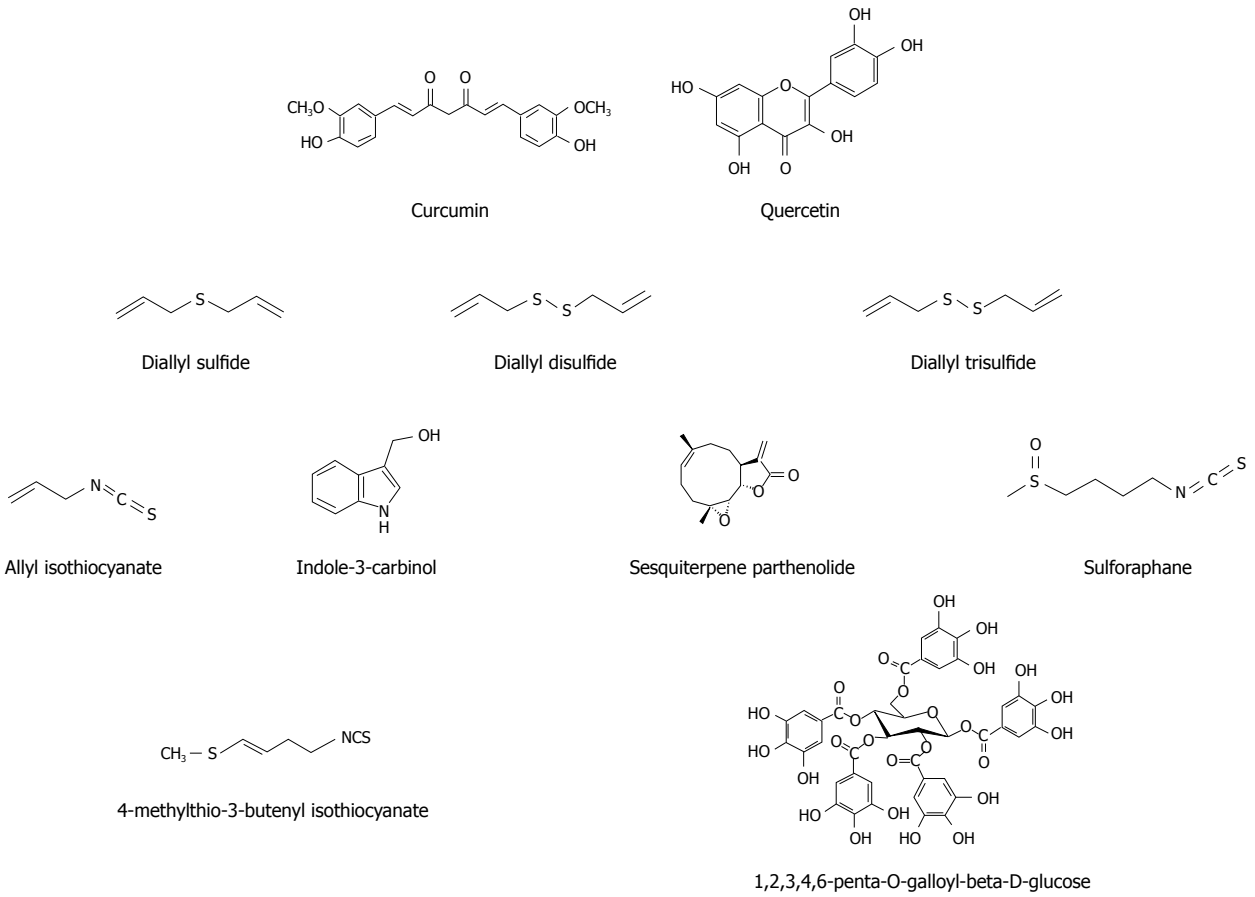


Figure 2 Chemical structures of various natural HO-1 inducers.

to their biological effects mainly by the Michael reaction acceptor function. However, recent studies[12] have demonstrated the ability of flavonoids to exert their protective properties also by influencing signaling pathways, thus indirectly interacting with the endogenous antioxidative defense system.

Quercetin (Figure 2) is one of the most common flavonoids and, probably, overall the most investigated. In human hepatocytes, quercetin is able to attenuate ethanol-induced oxidative damage by HO-1 induction *via* p38 and, especially, *via* ERK/Nrf2 transduction pathway[13,14]. Recently, in the same cellular model, Kluth *et al*[15] have confirmed that quercetin is able to activate the gene expression regulated by the EpRE of HO-1, although its ability was about 10 times less than that of thyme.

Garlic-derived organosulfur compounds

Diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS) (Figure 2), the three major garlic (*Allium sativum*) organosulfur compounds, have been demonstrated to be HO-1 inducers in hepatic cellular models. In human hepatoma HepG2 cells, Chen *et al*[16] observed that garlic organosulfur compounds induced HO-1 as the result of Nrf2 activation. Gong *et al*[17] have confirmed the involvement of Nrf2 activation in the induction of HO-1 by garlic DAS in HepG2 cells. Additionally, in this latter study a different pathway of HO-1 induction has been revealed for DATS, leading the authors to argue that structural differences in terms of the number of sulfur moieties and the length of alkyl side chains can explain the differential effects of garlic-derived organosulfur compounds on the MAPK-mediated activation of Nrf2 and HO-1 induction.

Isothiocyanates

Cruciferous vegetables, particularly Brassica vegetables (i.e. broccoli, Brussels sprouts and cabbage), contain high concentrations of glucosinolates (β-thioglucoside *N*-hydroxysulfates) that are the precursor of isothiocyanates, potent inducers of cytoprotective enzymes and inhibitors of carcinogenesis. Noteworthy, the isothiocyanate sulforaphane, due to its peculiar ability to inhibit phase I enzymes and induce phase Ⅱ enzymes (i.e. HO-1), exerts valuable pleiotropic pharmacologic effects.

Jeong *et al*[18] have investigated the regulatory role of allyl isothiocyanate, indole-3-carbinol, the sesquiterpene parthenolide and sulforaphane (Figure 2) in the expression and degradation of Nrf2 and the induction of the antioxidant enzyme HO-1. Allyl isothiocyanate is an effective inducer of Nrf2 protein expression, ARE-reporter gene and HO-1, but had little effect on delaying the degradation of Nrf2 protein. Parthenolide and indole-3-carbinol also induced ARE-reporter gene expression and Nrf2, although to a lesser extent if compared to sulforaphane and allyl isothiocyanate. Nonetheless, parthenolide considerably induces the HO-1 expression at a level comparable to sulforaphane, while indole-

3-carbinol shows no effect. Of note, sulforaphane strongly induces Nrf2 protein expression and ARE-mediated transcription activation, retards degradation of Nrf2 through inhibiting Keap1, thereby activating the transcriptional expression of HO-1. In the same cellular model, Keum *et al*[19] have confirmed that transcriptional activation of Nrf2/ARE is critical in sulforaphane-mediated induction of HO-1. Further evidence of the ability of isothiocyanate to activate ARE-mediated HO-1 gene transcription through Nrf2/ARE signaling pathway has been provided by a study from Prawan *et al*[20] on HepG2-C8 cells.

Hanlon *et al*[21] have demonstrated that 4-methylthio-3-butenyl isothiocyanate (Figure 2), the isothiocyanate metabolite of glucoraphasatin, a glucosinolate uniquely contained at high concentrations in Spanish black radishes, significantly induces HO-1.

Other compounds and plant extracts

1,2,3,4,6-Penta-*O*-galloyl-beta-d-glucose (PGG), a bioactive tannin contained in many medicinal plants, is able to induce HO-1 in hepatic cells (Hep-G2)[22]. PGG confers hepatoprotection against oxidative injury by inducing HO-1 expression *via* stimulating NF-E2-related factor 2 nuclear translocation in an ERK-dependent manner.

In an *in vivo* study, Yao *et al*[14] have demonstrated that a standardized *Ginko biloba* extract, containing not identified terpenes and flavonol heterosides, is able to induce hepatic microsomal HO-1 on mRNA, protein expression and enzymatic activity, providing a hepato-protective effect in ethanol challenged animals.

Hep-G2 cells have been used as a cellular model to demonstrate that the α-methylene-γ-butyrolactone moiety in dehydrocostus lactone, one of the bioactive constituents of the medicinal plant *Saussurea lappa*, increases cellular resistance to oxidant injury in HepG2 cells, presumably through Nrf2/ARE-dependent HO-1 expression[18]. The same biochemical mechanism is supposed to explain the upregulation of HO-1 synthesis induced by inchinkoto, a Chinese/Japanese herbal medicine[23].

HEME OXYGENASE AS A REGULATOR OF HEPATOBILIARY FUNCTION

In rat liver, HO-1 is prominent in Kupffer cells, whereas HO-2 is most abundant in hepatocytes[7]. Upon stimulation with lipopolysaccharide (LPS), HO-1 not only occurs in tissue macrophages, but is also markedly induced in hepatocytes. On the other hand, HO-2 does not change. Sinusoidal endothelial cells and/or hepatic stellate cells have little if any HO-1, but in culture, appear to express HO-2[8]. HO mRNA levels are high in fetal rat liver during prenatal maturation (9 d before birth) and reach a maximum 24 h after birth, when levels decline but remain above adult levels for at least 1 mo. This correlates with a greater capacity of the liver for bilirubin production in fetuses compared with adults and such circumstances could render the fetus more susceptible to drug injuries because of a depressed heme-cytochrome P450 system[24]. However, Dennery *et al*[25] have found that serum bilirubin protects against oxidative damage in the first few days of life in neonatal Gunn rats exposed to hypoxia.

Another condition in which there is induction of HO-1 is liver regeneration following 2/3 hepatectomy[26]. In HO-1-deficient mice, there is an increased susceptibility of the liver to endotoxin and interruption of a major pathway of iron recycling[27]. Most importantly, the phenotype of the first human case of HO-1 deficiency includes endothelial cell damage, iron accumulation in the liver and kidney, and an increasing cell susceptibility to heme overloading *in vitro*[28].

The major source of CO in animals is the degradation of heme by HO; CO produced by HO may serve as an important cellular signal in the microenvironment. In 1994, Suematsu *et al*[29] have shown that CO is present at submicromolar levels in the liver effluent and that inhibition of HO with ZnPP increases perfusion pressure in the isolated perfused rat liver, an effect that can be reversed by adding CO or a cGMP analogue to the perfusate. CO serves as an endogenous factor that reduces sinusoidal tone involving, at least in part, hepatic stellate cells[30]. The importance of CO as an endogenous modulator of vascular portal perfusion has been confirmed by Pannen *et al*[31] who demonstrated how NO serves as a potent vasodilator in the hepatic arterial circulation, but exerts only a minor vasodilatory effect in the portal venous vascular bed, while CO does not regulate hepatic artery tone, but is able to maintain portal vascular tone in a relaxed state. Wakabayashi *et al*[32] have confirmed these results, by showing that the induction of HO-1 with hemin causes a decrease in baseline resistance and in the response to endothelin-1 through an increase in CO production in the extrasinusoidal compartment. We have also shown that overproduction of CO by induction of HO with $CoCl_2$, reduces the response to the vasoconstrictor endothelin-1, but not to phenylephrine, in the isolated perfused rat liver[33].

Elimination of constitutive CO generation through administration of ZnPP not only increases sinusoidal tone, but also stimulates bile-acid-dependent bile flow[34]. The choleretic action coincides with an increase in microvascular tone and oxygen consumption and may thus reflect a prolonged duration of bile acid uptake by hepatocytes. Exogenous CO at micromolar concentrations completely reverses these changes. The effect of ZnPP is mimicked by administration of methylene blue, a soluble guanylate cyclase inhibitor, but not fully reversed by membrane-permeable 8Br-cGMP, suggesting some involvement of cGMP-independent mechanisms. CO generated in hepatocytes may also affect bile excretion by altering the contractility of the bile canaliculus (BC)[34]. Inhibition of CO with ZnPP shortens intervals of contraction of BCs and increases intracellular Ca^{2+}, an effect that is reversed by CO at a micromolar level without increasing cGMP[35].

As to mechanisms of the CO effect, several lines of experimental evidence suggest that CO modulates BC functions through its action on cytochrome P450-mediated calcium mobilization[36].

Jaundice

Bilirubin production is two- to three-fold greater in newborns than in adults. This increase in plasma bilirubin levels is due in large part to the combination of the rapid degradation of fetal hemoglobin in the first few days of life and the immaturity of the hepatic bilirubin conjugating system, thus leading to an increase in unconjugated bilirubin. If the levels of unconjugated bilirubin become too high, the bilirubin may cross the blood-brain barrier, resulting in bilirubin encephalopathy or kernicterus. Phototherapy is the method of choice to lower serum bilirubin levels, but its safety and efficiency have been called into question and is still a matter of debate. The clinical use of HO inhibitors is an alternative therapy. Sn-PP produces a significant decrease in the levels of serum (mean decrease, 38%) and biliary bilirubin (mean decrease, 47%) in normal subjects. The decrease in these parameters lasts for a minimum of 4 d after administration of the metalloporphyrin[37]. The tin porphyrins have been used on newborns with ABO incompatibility[38], on patients with hereditary porphyria[39], liver disease[37], or Crigler-Najjar type I syndrome[40]. Results indicate that the use of SnMP within 24 h of birth in premature newborns substantially moderates the development of hyperbilirubinemia and reduces the requirement for phototherapy markedly (> 75%) in inhibitor-treated infants compared with control subjects[41]. When administered at the appropriate time to near-term and term newborns with hyperbilirubinemia, it can entirely eliminate the need for phototherapy. In patients with biliary cirrhosis and hemochromatosis, Sn-PP is able to reduce bilirubin levels for about 4 d. Biliary bilirubin concentrations decreased (mean decrease, 49%) in hemochromatosis patients after Sn-PP administration. No decrease in biliary bilirubin concentrations can be detected in primary biliary cirrhosis patients under the same conditions[37].

HEME OXYGENASE CONFERS PROTECTION VERSUS DIFFERENT TYPES OF INSULT

Hypoxia, ischemia/reperfusion (IR) and transplantation

Hemorrhagic shock (HS) causes severe hepatic dysfunction or acute failure related to decreased hepatic microcirculatory flow, and results in enhanced hepatic expression of HO-1[8]. Furthermore, the increase in portal resistance, upon blockade of the HO-CO pathway, is much more pronounced after HS compared with sham controls. After HS is endogenously generated, CO preserves sinusoidal perfusion, mitochondrial redox state, and secretory function in the isolated perfused rat liver[42]. This protective role of CO is mediated *via* a relaxing mechanism, in part, involving Ito cells. Similar results have been obtained by Kyokane *et al*[43] in endotoxemic rats overexpressing both inducible nitric oxide synthase (i-NOS) and HO-1. In this condition, inhibition of CO, but not of NO, causes marked vasoconstriction and cholestasis. Thus, CO may exert a protective function against hepatobiliary dysfunction after HS and endotoxemia.

Reperfusion injury has been defined as the conversion of reversibly injured cells (myocardial, endothelial, *etc.*) to irreversibly injured cells, and is mediated by a burst of free-radical generation as the previously hypoxic cells are flooded with oxygen. HO mRNA increases within 4 h of reperfusion of non-necrogenic ischemic rat liver[44]. Redaelli *et al*[45] have shown that the significant effects of heat preconditioning on liver transplantation after cold storage are prevented by inhibition of HO with tin protoporphyrin, and can be reproduced by administration of cobalt protoporphyrin, an inducer of HO. Thus, overexpression of HO-1 improves post-transplantation survival from 3 d to 3 wk and graft function after prolonged cold ischemia preservation. The mechanism underlying these beneficial effects does not appear to be the prevention of apoptosis. The same beneficial effects of induction of HO-1 with cobalt protoporphyrin (CoPP) or with adenoviral HO-1 (Ad-HO-1) transfection have been shown in steatotic livers[46]. Following cold ischemia/isotransplantation, HO-1 over-expression extended animal survival from 40% in untreated controls to about 80% after CoPP or Ad-HO-1 therapy. This effect is correlated with the preserved hepatic architecture, improves liver function, depresses infiltration by T cells and macrophages, causes suppression of local expression of i-NOS, and modulates the pro- and anti-apoptotic pathways[47]. More recent data have shown that HO-1 modulates pro-inflammatory responses that are triggered *via* TLR4 signaling, a putative HO-1 repressor[48].

The role of CO in protecting liver grafts from cold I/R injury associated with liver transplantation has been studied by Kaizu *et al*[49]. Inhalation of CO reduces hepatic injury and is associated with marked downregulation of early mRNA expression for tumor necrosis factor TNF-α, interleukin IL-6, and NOS. CO significantly inhibits phosphorylation of ERK1/2 MAPK and its upstream MEK1/2 and downstream transcriptional factor c-Myc. CO also significantly inhibits I/R injury-induced STAT1 and STAT3 activation. In contrast, CO does not inhibit p38 or JNK MAPK pathways during hepatic I/R injury. These results demonstrate that exogenous CO suppresses early pro-inflammatory and stress-response gene expression and efficiently improves hepatic I/R injury by downregulation of the MEK/ERK1/2 signaling pathway with CO. CO production, evaluated by CO-Hb, is associated with improved function in liver-transplanted patients[50]. Furthermore, an increase in HO-1 during transplantation is more protective than high HO-1 expression before transplantation[51].

Buis *et al*[52] have shown that donor HO-1 genetic polymorphism may influence the outcome of liver transplantation. Allele genotype is associated with increased graft survival. Graft survival at 1 year is significantly better for A-allele genotype compared to TT-genotype (84% *vs* 63%). Graft loss, due to primary dysfunction (PDF), occurs more frequently in TT-genotype compared to A-receivers ($P = 0.03$). Recipients of a liver with TT-genotype have significantly higher serum transaminases after transplantation and hepatic HO-1 mRNA levels are significantly lower compared to the A-allele livers. No differences are found for any outcome variable between class S and LL-variant of the (GT) (n) polymorphism. Haplotype analysis has confirmed dominance of the A (-413) T single nucleotide polymorphism over the (GT) (n) polymorphism. In conclusion, HO-1 genotype is associated with outcome after liver transplantation, suggesting that HO-1 mediates graft survival after liver transplantation. Excessive shear stress secondary to portal hypertension is probably involved in the augmented HO-1 expression in small-for-size graft liver[53]. In this model, recombinant Ad-HO-1 administered to donors 48 h before transplantation enhances HO-1 expression in both whole and small-for-size allografts, with a predominant augmentation in the small-for-size allografts, suggesting favorable conditions for the induction of HO-1 expression in small-for-size allografts. In close relation to the expression level of HO-1, Ad-HO-1 significantly prolongs both whole and small-for size allograft survivals, with a remarkable effect in the small-for-size allograft group. The prolongation of allograft survival is blocked by the HO-1 inhibitor (Zinc protoporphyrin Ⅸ). The non-treated small-for-size allografts demonstrate impaired liver function during the early period after reperfusion, which can be improved by over-expression of HO-1, but reversed by the HO-1 inhibitor. The markedly increased expression of HO-1 in small-for-size allografts is associated with lower levels of adhesion molecules and pro-inflammatory cytokines in the early phase after reperfusion[54]. Also, in aged liver, HO-1 overexpression can provide potent protection against cold I/R injury. This effect depends, at least in part, on HO-1-mediated inhibition of the anti-apoptotic mechanism, as an active form of pro-apoptotic caspase-3 (p20) protein, and was found to be 2.9-fold lower at 24 h in the hemin-pretreated group, as compared to saline liver transplant controls[55].

The other product of HO activity, biliverdin, also exerts protective effects against liver I/R injury[56]. Adjunctive biliverdin improves portal venous blood flow from the beginning of reperfusion and increases bile production as compared with the control group. I/R-induced hepatocellular damage, as measured by GOT/GPT release, is diminished by biliverdin. Improved liver function by biliverdin is accompanied by preservation of the histologic structure. Additionally[56], biliverdin adjuvant after orthotopic liver transplantation (OLT) decreases endothelial expression of cellular adhesion molecules (P-selectin and intracellular adhesion molecule 1), and decreases the extent of infiltration by neutrophils and inflammatory macrophages. Biliverdin also inhibits expression of i-NOS and pro-inflammatory cytokines (IL-1β, TNF-α, and IL-6) in OLTs. Finally, biliverdin therapy promotes an increased expression of anti-apoptotic molecules independently of HO-1 expression, consistent with biliverdin, being an important mediator through which HO-1 prevents cellular death.

Alcohol and non-alcoholic steatosis

Steato-hepatitis is a liver disease characterized by fat accumulation, inflammation, necrosis, and fibrosis. It can be caused by alcohol, or be independent from alcohol and defined as non-alcoholic steato-hepatitis (NASH). In hepatic NASH, Malaguarnera *et al*[57] have shown that HO-1 expression is significantly increased, and the increase reflects the severity of the disease. They observed a significant correlation between the increased levels of HO-1 and ferritin, and between the increased levels of HO-1 and lipid peroxidation. Moreover, NASH patients with lower levels of GSH exhibit higher expression of HO-1. Thus, the induction of HO-1 seems an adaptive response against oxidative damage elicited by lipid peroxidation, and it may be critical in the progression of the disease.

The only data on alcoholic steato-hepatitis are those of Yao *et al*[14] who have shown that the induction of HO-1 by *Ginkgo biloba* is associated with a decrease in liver damage caused by ethanol feeding for 90 d in rats. This is probably due to the enhanced anti-oxidative capacity against the ethanol-induced oxidative stress and the maintenance of cellular redox balance.

Liu *et al*[13] have shown that ethanol dose-dependently induces HO-1 and increases HO activity in human hepatocytes in culture, and that HO-1 mRNA increases after 30 min of exposure. Induction of HO-1 with CoPP prevents damage from ethanol. These results have been confirmed by Yao *et al*[43], who showed that quercetin prevents ethanol toxicity in human hepatocytes, an effect which is mediated by HO-1 induction. HO activity is also increased by chronic ethanol consumption in rats[58]. While 2.5-mo-old rats respond to acute ethanol intoxication by displaying increased expression of liver HO-1 mRNA, and 6-mo-old rats exhibit a mild response, 18-mo-old rats do not show any response, probably because of a decreased transcriptional ability to respond to stress in older animals[59].

Cirrhotic and pre-hepatic portal hypertension

In cirrhosis induced by bile duct ligation, Wei *et al*[60] have shown that HO-1 mRNA and protein expression is increased in hepatocytes and some Kupffer cells in the early phase of the disease, while HO-2 expression is unchanged. HO-1 induction is also related to iNOS induction.

In cirrhotic livers, mainly biliary cirrhosis, both HO-1 and HO-2 were found to be increased by Goh *et al*[61]. HO-1 was localized mainly in Kupffer cells, while HO-2 was localized in the cytoplasm of the

hepatocytes. Similar results were obtained in patients with post-hepatic cirrhosis by Makino *et al*[62]. They have shown that HO-1 is increased in the liver, being mainly distributed in Kupffer cells and hepatocytes. By contrast, in livers in which portal hypertension is idiopathic and due to increased perisinusoidal resistance, there is a decreased expression of HO-1 in Kupffer cells and an absence in hepatocytes. A study in cirrhotic patients undergoing liver transplantation has shown that HO-1 is up-regulated through heme-independent stimuli according to the development of portal hypertension and that induced HO-1 plays a pathophysiological role in portal hypertension through CO production[63].

In cirrhotic patients CO-Hb is increased, as demonstrated by Tran *et al*[64], but does not correlate with disease severity (MELD score, Child Turcotte Pugh score, or other biochemical or clinical measurements). In cirrhotic patients with spontaneous bacterial peritonitis, CO production, evaluated as CO concentration in the exhaled air and blood CO-Hb level, is further increased and may participate in circulatory alterations[65].

A clear role of the HO-CO system in the pathophysiology of hemodynamic alterations related to experimental cirrhotic portal hypertension in the rat is now established.

Decreased HO-2 expression in the liver is associated with increased portal resistance[33], while mesenteric artery dilatation and hypo-reactivity to vasoconstrictors, phenylephrine, KCl, endothelin-1, is associated with induction of HO-1 and increased HO-2. As a confirmation of these findings, transfection of normal rats with human HO-1 mimics mesenteric arterial alterations of portal hypertension[66]. Hyper-expression of HO-1 is particularly relevant in cirrhotic rats with ascites and its function is mediated by large-conductance calcium-activated potassium channels[67]. The alpha subunits of these channels, in particular, are increased in cirrhotic animals and their increase may be mediated by the increased HO-1[68].

In experimental pre-hepatic portal hypertension, obtained by partial portal vein ligation in rats, Fernandez *et al*[69] have shown that HO activity is increased in the liver. HO-1 expression is present in hepatocytes and Kupffer cells of portal hypertensive rats but not of normal animals, while HO-2 is similarly expressed in all liver cell types of normal and portal-vein ligated rats. They have also evaluated the role of CO in hyporeactivity of the mesenteric vascular beds of prehepatic portal hypertension in rat[69]. In this model, inhibition of HO with ZnMP does not modify the hypo-reactivity to KCl that is partially attenuated by NOS inhibition and completely corrected by simultaneous inhibition of HO and NOS. Also the hypo-reactivity to methoxamine is not affected by ZnMP, but it is completely overcome by L-NAME, without any increase in response after combined inhibition of NOS and HO. In cirrhotic patients with hepatopulmonary syndrome (HPS), characterized by decreased arterial pO_2 levels and increased alveolar-arterial oxygen gradient, CO-Hb levels are increased, compared to those without the syndrome, and are correlated with pO_2 ($P < 0.001$) and Aa pO_2 ($P < 0.001$) levels. Thus, CO may contribute to human HPS[70]. These data confirm what was experimentally found: in cirrhosis experimentally induced in the rat by bile duct ligation, NO-mediated up-regulation of HO-1 expression has been shown to participate in HPS[71]. In the same model, HO-1 mRNA transcription and protein expression are significantly increased in cirrhotic hearts compared with sham-operated controls, whereas there is no difference in HO-2 mRNA or protein levels. Total HO activity and cGMP levels are significantly increased in cirrhotic ventricles *vs* controls, and treatment with ZnPP significantly decreases cGMP production and improves the blunted papillary muscle contractility, whereas it has no effect on control muscles. CO perfusion inhibits papillary muscle contractility, an effect completely blocked by methylene blue and partially blocked by ZnPP. Thus, activation of the HO-CO-cGMP pathway is involved in the pathogenesis of cirrhotic cardiomyopathy[72].

Renal HO-1 expression is decreased in cirrhotic rats (bile ligation) in renal tubules and interlobular arterioles, while it is increased in the liver. The decreased HO-1 is related to renal dysfunction[73].

During HPS caused by liver cirrhosis, pulmonary endothelial NOS expression and NO production are increased. Increased NO contributes to the blunted hypoxic pressure response (HPR) during cirrhosis and may induce HO-1 expression and CO production, exacerbating the blunted HPR. We hypothesized that NO regulates the expression of HO-1 during cirrhosis, contributing to HPS. Cirrhosis was induced in rats by common bile duct ligation (CBDL). Rats were studied 2 wk and 5 wk after CBDL or sham surgery. Lung HO-1 expression was elevated 5 wk after CBDL. Liver HO-1 was increased at 2 wk and remained elevated at 5 wk. In catheterized rats, the blunted HPR was partially restored by HO inhibition. Rats treated with the NOS inhibitor *N*(G)-nitro-l-arginine methyl ester for the entire 2 wk or 5-wk duration had normalized HO-1 expression and HPR. These data provide *in vivo* evidence for the NO-mediated up-regulation of HO-1 expression and support the concept that HPS is multifactorial, involving not only NO, but also HO-1 and CO.

In kidneys from CBDL rats, Miyazono *et al*[73] have shown that HO-1 protein expression is increased slightly at 2 wk but is abolished at 5 wk. In addition, histologically, HO-1 expression was suppressed in renal tubules and interlobular arterioles in 5-wk-old CBDL rats. Conversely, HO-1 expression in liver was strongly increased. Consistent with the development of cirrhosis and renal dysfunction, mean arterial pressure (MAP), glomerular filtration rate (GFR), and renal blood flow (RBF) are decreased in CBDL rats, compared with sham-operated controls. In sham rats, treatment with the selective HO inhibitor ZnPP markedly decreases GFR and RBF to values similar to those measured in CBDL rats without decreasing MAP. In conclusion, decreased renal HO-1 expression contributes to deteriorated renal function and hemodynamics during cirrhosis. This finding provides a novel mechanism for the pathophysiology of renal dysfunction during cirrhosis.

Hepatitis

Interactions between hepatitis viruses B and C and HO have been described, both directly and through the effects on the immune response.

Hepatitis B

Protzer *et al*[74] have investigated the effects of HO-1 induction in models of human hepatitis B virus (HBV) infection. Adenoviral transfer of an HBV 1.3 genome into wild-type mice was used as a model for acute hepatitis B. HBV transgenic animals were used as a model for chronic HBV infection. To investigate HO-1 effects on HBV replication at a molecular level, stably HBV-transfected hepatoma cells were used. In the acute hepatitis B model, liver injury was reduced significantly after HO-1 induction. In addition, HO-1 showed a pronounced antiviral effect, which was confirmed in stably HBV-transfected hepatoma cells and in persistently HBV replicating transgenic mice. HO-1 induction repressed HBV replication directly in hepatocytes at a post-transcriptional step by reducing stability of HBV core protein and thus blocking refill of nuclear HBV covalently closed circular DNA. Small interfering RNA directed against HO-1 proved that this effect was dependent on the expression level of HO-1. The authors[74] concluded that, besides its hepato-protective effect, HO-1 showed a pronounced antiviral activity in HBV infection.

Hepatitis C

Conflicting data are available on HO-1 in hepatitis C. Ghaziani *et al*[75] have shown that human hepatoma cells expressing HCV have increased HO-1 and decreased Bach1 expression. Abdalla *et al*[76], on the contrary, have found a clear decrease in HO-1 and HO-1 mRNA in liver biopsies from HCV-infected patients. The expression of HO-1 was also reduced in cell lines that stably express HCV core protein, which suggests that core gene products are capable of regulating the expression of HO-1. These results are confirmed by Wen *et al*[77] have who shown that HCV core protein attenuates the induction of HO-1 by heme, heavy metals, and peroxides and contributes to hepatocellular damage by increasing both steady-state levels of pro-oxidants and the susceptibility of hepatocytes to damage by impairing their response to other sources of oxidative stress. Concerning the effects of HO-1 induction on hepatitis C, Shan *et al*[78] have shown a decrease in HCV replication, an effect similar to that described by Protzer *et al*[74] in HBV hepatitis.

CRITICAL CONSIDERATIONS AND FUTURE STUDIES

The amount of experimental data that demonstrate important properties of many ingredients and/or bioactive substances from plants and food plants is vast and continues to increase rapidly. The use of terms such as nutraceuticals, functional foods, herbal extracts, bioactive dietary constituents, phytochemicals and similar is becoming copious. In many cases marketing strategies abuse these terms and health properties are claimed although far from being scientifically demonstrated. Thus, researchers are requested to have scientific objectivity in evaluating health properties of food ingredients. It is possible to sustain those diverse bioactive substances from plants and food plants are promising candidates as natural HO-1 hepatic inducers. However, some critical evaluations on literature data are necessary. It is important to note that the majority of studies were conducted in cellular models, whereas only two studies were conducted on rats. Thus, the reproduction of natural HO-1 hepatic inducers in more relevant *in vivo* models is certainly necessary. With regards to the inductive mechanism of natural HO-1 hepatic inducers, although other pathways cannot be excluded, it seems quite clear that the prevalent mechanism is an ARE-mediated HO-1 gene transcription through the Nrf2/ARE signaling pathway.

Other uncertainties derive from the fact that the referred studies have reported data on natural HO-1 inducers considered both as single chemicals and food extracts. In some cases, scarce or no information has been provided about (1) the quantitative measurements of the proposed active compound; (2) methods of analysis and, (3) extraction procedures. Obviously, the above information is essential to enable other researchers to reproduce the experiments and to obtain comparable data.

When considering a possible therapeutic use of future natural HO-1-inducer-based drugs, the amount of work to perform is even more significant. Indeed, exhaustive information on absorption, distribution, metabolism and excretion by the main possible routes (oral, intraperitoneal, intravenous, intrathecal) are largely insufficient. A potential point of strength of natural HO-1 hepatic inducers is that, generally, they have no toxic effects, and it is presumed that they should not have side effects or teratogenic properties.

REFERENCES

1 **Maines MD**. The heme oxygenase system: a regulator of second messenger gases. *Annu Rev Pharmacol Toxicol* 1997; **37**: 517-554

2 **Trakshel GM**, Kutty RK, Maines MD. Purification and characterization of the major constitutive form of testicular heme oxygenase. The noninducible isoform. *J Biol Chem* 1986; **261**: 11131-11137

3 **Yoshida T**, Biro P, Cohen T, Muller RM, Shibahara S. Human heme oxygenase cDNA and induction of its mRNA by hemin. *Eur J Biochem* 1988; **171**: 457-461

4 **McCoubrey WK Jr**, Ewing JF, Maines MD. Human heme oxygenase-2: characterization and expression of a full-length cDNA and evidence suggesting that the two HO-2 transcripts may differ by choice of polyadenylation signal. *Arch Biochem Biophys* 1992; **295**: 13-20

5 **Keyse SM**, Tyrrell RM. Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. *Proc Natl Acad Sci USA* 1989; **86**: 99-103

6 **Motterlini R**, Foresti R, Bassi R, Calabrese V, Clark JE, Green CJ. Endothelial heme oxygenase-1 induction by

hypoxia. Modulation by inducible nitric-oxide synthase and S-nitrosothiols. *J Biol Chem* 2000; **275**: 13613-13620

7 **Goda N**, Suzuki K, Naito M, Takeoka S, Tsuchida E, Ishimura Y, Tamatani T, Suematsu M. Distribution of heme oxygenase isoforms in rat liver. Topographic basis for carbon monoxide-mediated microvascular relaxation. *J Clin Invest* 1998; **101**: 604-612

8 **Bauer I**, Wanner GA, Rensing H, Alte C, Miescher EA, Wolf B, Pannen BH, Clemens MG, Bauer M. Expression pattern of heme oxygenase isoenzymes 1 and 2 in normal and stress-exposed rat liver. *Hepatology* 1998; **27**: 829-838

9 **Lin JK**. Molecular targets of curcumin. *Adv Exp Med Biol* 2007; **595**: 227-243

10 **McNally SJ**, Harrison EM, Ross JA, Garden OJ, Wigmore SJ. Curcumin induces heme oxygenase-1 in hepatocytes and is protective in simulated cold preservation and warm reperfusion injury. *Transplantation* 2006; **81**: 623-626

11 **McNally SJ**, Harrison EM, Ross JA, Garden OJ, Wigmore SJ. Curcumin induces heme oxygenase 1 through generation of reactive oxygen species, p38 activation and phosphatase inhibition. *Int J Mol Med* 2007; **19**: 165-172

12 **Rahman I**, Biswas SK, Kirkham PA. Regulation of inflammation and redox signaling by dietary polyphenols. *Biochem Pharmacol* 2006; **72**: 1439-1452

13 **Liu LG**, Yan H, Zhang W, Yao P, Zhang XP, Sun XF, Nussler AK. Induction of heme oxygenase-1 in human hepatocytes to protect them from ethanol-induced cytotoxicity. *Biomed Environ Sci* 2004; **17**: 315-326

14 **Yao P**, Li K, Song F, Zhou S, Sun X, Zhang X, Nussler AK, Liu L. Heme oxygenase-1 upregulated by Ginkgo biloba extract: potential protection against ethanol-induced oxidative liver damage. *Food Chem Toxicol* 2007; **45**: 1333-1342

15 **Kluth D**, Banning A, Paur I, Blomhoff R, Brigelius-Flohe R. Modulation of pregnane X receptor- and electrophile responsive element-mediated gene expression by dietary polyphenolic compounds. *Free Radic Biol Med* 2007; **42**: 315-325

16 **Chen C**, Pung D, Leong V, Hebbar V, Shen G, Nair S, Li W, Kong AN. Induction of detoxifying enzymes by garlic organosulfur compounds through transcription factor Nrf2: effect of chemical structure and stress signals. *Free Radic Biol Med* 2004; **37**: 1578-1590

17 **Yang LQ**, Yu WF, Cao YF, Gong B, Chang Q, Yang GS. Potential inhibition of cytochrome P450 3A4 by propofol in human primary hepatocytes. *World J Gastroenterol* 2003; **9**: 1959-1962

18 **Jeong WS**, Keum YS, Chen C, Jain MR, Shen G, Kim JH, Li W, Kong AN. Differential expression and stability of endogenous nuclear factor E2-related factor 2 (Nrf2) by natural chemopreventive compounds in HepG2 human hepatoma cells. *J Biochem Mol Biol* 2005; **38**: 167-176

19 **Keum YS**, Yu S, Chang PP, Yuan X, Kim JH, Xu C, Han J, Agarwal A, Kong AN. Mechanism of action of sulforaphane: inhibition of p38 mitogen-activated protein kinase isoforms contributing to the induction of antioxidant response element-mediated heme oxygenase-1 in human hepatoma HepG2 cells. *Cancer Res* 2006; **66**: 8804-8813

20 **Prawan A**, Keum YS, Khor TO, Yu S, Nair S, Li W, Hu L, Kong AN. Structural influence of isothiocyanates on the antioxidant response element (ARE)-mediated heme oxygenase-1 (HO-1) expression. *Pharm Res* 2008; **25**: 836-844

21 **Hanlon PR**, Webber DM, Barnes DM. Aqueous extract from Spanish black radish (Raphanus sativus L. Var. niger) induces detoxification enzymes in the HepG2 human hepatoma cell line. *J Agric Food Chem* 2007; **55**: 6439-6446

22 **Pae HO**, Oh GS, Jeong SO, Jeong GS, Lee BS, Choi BM, Lee HS, Chung HT. 1,2,3,4,6-penta-O-galloyl-beta-D-glucose up-regulates heme oxygenase-1 expression by stimulating Nrf2 nuclear translocation in an extracellular signal-regulated kinase-dependent manner in HepG2 cells. *World J Gastroenterol* 2006; **12**: 214-221

23 **Chan SC**, Chang YS, Wang JP, Chen SC, Kuo SC. Three new flavonoids and antiallergic, anti-inflammatory constituents from the heartwood of Dalbergia odorifera. *Planta Med* 1998; **64**: 153-158

24 **Rahman I**, Biswas SK, Kirkham PA. Regulation of inflammation and redox signaling by dietary polyphenols. *Biochem Pharmacol* 2006; **72**: 1439-1452

25 **Dennery PA**, McDonagh AF, Spitz DR, Rodgers PA, Stevenson DK. Hyperbilirubinemia results in reduced oxidative injury in neonatal Gunn rats exposed to hyperoxia. *Free Radic Biol Med* 1995; **19**: 395-404

26 **Solangi K**, Sacerdoti D, Goodman AI, Schwartzman ML, Abraham NG, Levere RD. Differential effects of partial hepatectomy on hepatic and renal heme and cytochrome P450 metabolism. *Am J Med Sci* 1988; **296**: 387-391

27 **Poss KD**, Tonegawa S. Reduced stress defense in heme oxygenase 1-deficient cells. *Proc Natl Acad Sci USA* 1997; **94**: 10925-10930

28 **Yachie A**, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, Ohta K, Kasahara Y, Koizumi S. Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. *J Clin Invest* 1999; **103**: 129-135

29 **Suematsu M**, Kashiwagi S, Sano T, Goda N, Shinoda Y, Ishimura Y. Carbon monoxide as an endogenous modulator of hepatic vascular perfusion. *Biochem Biophys Res Commun* 1994; **205**: 1333-1337

30 **Suematsu M**, Goda N, Sano T, Kashiwagi S, Egawa T, Shinoda Y, Ishimura Y. Carbon monoxide: an endogenous modulator of sinusoidal tone in the perfused rat liver. *J Clin Invest* 1995; **96**: 2431-2437

31 **Pannen BH**, Bauer M. Differential regulation of hepatic arterial and portal venous vascular resistance by nitric oxide and carbon monoxide in rats. *Life Sci* 1998; **62**: 2025-2033

32 **Wakabayashi Y**, Takamiya R, Mizuki A, Kyokane T, Goda N, Yamaguchi T, Takeoka S, Tsuchida E, Suematsu M, Ishimura Y. Carbon monoxide overproduced by heme oxygenase-1 causes a reduction of vascular resistance in perfused rat liver. *Am J Physiol* 1999; **277**: G1088-G1096

33 **Sacerdoti D**, Abraham NG, Oyekan AO, Yang L, Gatta A, McGiff JC. Role of the heme oxygenases in abnormalities of the mesenteric circulation in cirrhotic rats. *J Pharmacol Exp Ther* 2004; **308**: 636-643

34 **Suematsu M**, Ishimura Y. The heme oxygenase-carbon monoxide system: a regulator of hepatobiliary function. *Hepatology* 2000; **31**: 3-6

35 **Dufour JF**, Turner TJ, Arias IM. Nitric oxide blocks bile canalicular contraction by inhibiting inositol trisphosphate-dependent calcium mobilization. *Gastroenterology* 1995; **108**: 841-849

36 **Shinoda Y**, Suematsu M, Wakabayashi Y, Suzuki T, Goda N, Saito S, Yamaguchi T, Ishimura Y. Carbon monoxide as a regulator of bile canalicular contractility in cultured rat hepatocytes. *Hepatology* 1998; **28**: 286-295

37 **Berglund L**, Angelin B, Hultcrantz R, Einarsson K, Emtestam L, Drummond G, Kappas A. Studies with the haeme oxygenase inhibitor Sn-protoporphyrin in patients with primary biliary cirrhosis and idiopathic haemochromatosis. *Gut* 1990; **31**: 899-904

38 **Kappas A**, Drummond GS, Munson DP, Marshall JR. Sn-Mesoporphyrin interdiction of severe hyperbilirubinemia in Jehovah's Witness newborns as an alternative to exchange transfusion. *Pediatrics* 2001; **108**: 1374-1377

39 **Galbraith RA**, Kappas A. Pharmacokinetics of tin-mesoporphyrin in man and the effects of tin-chelated porphyrins on hyperexcretion of heme pathway precursors in patients with acute inducible porphyria. *Hepatology* 1989; **9**: 882-888

40 **Galbraith RA**, Drummond GS, Kappas A. Suppression of bilirubin production in the Crigler-Najjar type I syndrome: studies with the heme oxygenase inhibitor tin-mesoporphyrin. *Pediatrics* 1992; **89**: 175-182

41 **Valaes T**, Petmezaki S, Henschke C, Drummond GS,

Kappas A. Control of jaundice in preterm newborns by an inhibitor of bilirubin production: studies with tin-mesoporphyrin. *Pediatrics* 1994; **93**: 1-11
42 **Pannen BH**, Kohler N, Hole B, Bauer M, Clemens MG, Geiger KK. Protective role of endogenous carbon monoxide in hepatic microcirculatory dysfunction after hemorrhagic shock in rats. *J Clin Invest* 1998; **102**: 1220-1228
43 **Kyokane T**, Norimizu S, Taniai H, Yamaguchi T, Takeoka S, Tsuchida E, Naito M, Nimura Y, Ishimura Y, Suematsu M. Carbon monoxide from heme catabolism protects against hepatobiliary dysfunction in endotoxin-treated rat liver. *Gastroenterology* 2001; **120**: 1227-1240
44 **Tacchini L**, Schiaffonati L, Pappalardo C, Gatti S, Bernelli-Zazzera A. Expression of HSP 70, immediate-early response and heme oxygenase genes in ischemic-reperfused rat liver. *Lab Invest* 1993; **68**: 465-471
45 **Redaelli CA**, Tian YH, Schaffner T, Ledermann M, Baer HU, Dufour JF. Extended preservation of rat liver graft by induction of heme oxygenase-1. *Hepatology* 2002; **35**: 1082-1092
46 **Amersi F**, Buelow R, Kato H, Ke B, Coito AJ, Shen XD, Zhao D, Zaky J, Melinek J, Lassman CR, Kolls JK, Alam J, Ritter T, Volk HD, Farmer DG, Ghobrial RM, Busuttil RW, Kupiec-Weglinski JW. Upregulation of heme oxygenase-1 protects genetically fat Zucker rat livers from ischemia/reperfusion injury. *J Clin Invest* 1999; **104**: 1631-1639
47 **Coito AJ**, Buelow R, Shen XD, Amersi F, Moore C, Volk HD, Busuttil RW, Kupiec-Weglinski JW. Heme oxygenase-1 gene transfer inhibits inducible nitric oxide synthase expression and protects genetically fat Zucker rat livers from ischemia-reperfusion injury. *Transplantation* 2002; **74**: 96-102
48 **Shen XD**, Ke B, Zhai Y, Gao F, Busuttil RW, Cheng G, Kupiec-Weglinski JW. Toll-like receptor and heme oxygenase-1 signaling in hepatic ischemia/reperfusion injury. *Am J Transplant* 2005; **5**: 1793-1800
49 **Kaizu T**, Ikeda A, Nakao A, Tsung A, Toyokawa H, Ueki S, Geller DA, Murase N. Protection of transplant-induced hepatic ischemia/reperfusion injury with carbon monoxide via MEK/ERK1/2 pathway downregulation. *Am J Physiol Gastrointest Liver Physiol* 2008; **294**: G236-G244
50 **Ali Y**, Negmi H, Elmasry N, Sadek M, Riaz A, Al Ouffi H, Khalaf H. Early graft function and carboxyhemoglobin level in liver transplanted patients. *Middle East J Anesthesiol* 2007; **19**: 513-525
51 **Geuken E**, Buis CI, Visser DS, Blokzijl H, Moshage H, Nemes B, Leuvenink HG, de Jong KP, Peeters PM, Slooff MJ, Porte RJ. Expression of heme oxygenase-1 in human livers before transplantation correlates with graft injury and function after transplantation. *Am J Transplant* 2005; **5**: 1875-1885
52 **Buis CI**, van der Steege G, Visser DS, Nolte IM, Hepkema BG, Nijsten M, Slooff MJ, Porte RJ. Heme oxygenase-1 genotype of the donor is associated with graft survival after liver transplantation. *Am J Transplant* 2008; **8**: 377-385
53 **Kobayashi T**, Sato Y, Yamamoto S, Takeishi T, Hirano K, Watanabe T, Takano K, Naito M, Hatakeyama K. Augmentation of heme oxygenase-1 expression in the graft immediately after implantation in adult living-donor liver transplantation. *Transplantation* 2005; **79**: 977-980
54 **Yang ZF**, Tsui TY, Ho DW, Tang TC, Fan ST. Heme oxygenase-1 potentiates the survival of small-for-size liver graft. *Liver Transpl* 2004; **10**: 784-793
55 **Wang XH**, Wang K, Zhang F, Li XC, Li J, De W, Guo J, Qian XF, Fan Y. Heme oxygenase-1 alleviates ischemia/reperfusion injury in aged liver. *World J Gastroenterol* 2005; **11**: 690-694
56 **Fondevila C**, Katori M, Lassman C, Carmody I, Busuttil RW, Bach FH, Kupiec-Weglinski JW. Biliverdin protects rat livers from ischemia/reperfusion injury. *Transplant Proc* 2003; **35**: 1798-1799
57 **Malaguarnera L**, Madeddu R, Palio E, Arena N, Malaguarnera M. Heme oxygenase-1 levels and oxidative stress-related parameters in non-alcoholic fatty liver disease patients. *J Hepatol* 2005; **42**: 585-591
58 **Okuno F**, Arai M, Sujita K, Eto S, Ishii H. Alterations in hepatic delta-aminolevulinic acid synthetase and heme oxygenase activities after chronic ethanol consumption in rats. *Alcohol* 1991; **8**: 449-451
59 **Patriarca S**, Furfaro AL, Cosso L, Pesce Maineri E, Balbis E, Domenicotti C, Nitti M, Cottalasso D, Marinari UM, Pronzato MA, Traverso N. Heme oxygenase 1 expression in rat liver during ageing and ethanol intoxication. *Biogerontology* 2007; **8**: 365-372
60 **Wei CL**, Lee KH, Khoo HE, Hon WM. Expression of haem oxygenase in cirrhotic rat liver. *J Pathol* 2003; **199**: 324-334
61 **Goh BJ**, Tan BT, Hon WM, Lee KH, Khoo HE. Nitric oxide synthase and heme oxygenase expressions in human liver cirrhosis. *World J Gastroenterol* 2006; **12**: 588-594
62 **Makino N**, Suematsu M, Sugiura Y, Morikawa H, Shiomi S, Goda N, Sano T, Nimura Y, Sugimachi K, Ishimura Y. Altered expression of heme oxygenase-1 in the livers of patients with portal hypertensive diseases. *Hepatology* 2001; **33**: 32-42
63 **Matsumi M**, Takahashi T, Fujii H, Ohashi I, Kaku R, Nakatsuka H, Shimizu H, Morita K, Hirakawa M, Inagaki M, Sadamori H, Yagi T, Tanaka N, Akagi R. Increased heme oxygenase-1 gene expression in the livers of patients with portal hypertension due to severe hepatic cirrhosis. *J Int Med Res* 2002; **30**: 282-288
64 **Tran TT**, Martin P, Ly H, Balfe D, Mosenifar Z. Carboxyhemoglobin and its correlation to disease severity in cirrhotics. *J Clin Gastroenterol* 2007; **41**: 211-215
65 **De las Heras D**, Fernandez J, Gines P, Cardenas A, Ortega R, Navasa M, Barbera JA, Calahorra B, Guevara M, Bataller R, Jimenez W, Arroyo V, Rodes J. Increased carbon monoxide production in patients with cirrhosis with and without spontaneous bacterial peritonitis. *Hepatology* 2003; **38**: 452-459
66 **Sacerdoti D**, Abraham NG, Oyekan AO, Yang L, Gatta A, McGiff JC. Role of the heme oxygenases in abnormalities of the mesenteric circulation in cirrhotic rats. *J Pharmacol Exp Ther* 2004; **308**: 636-643
67 **Bolognesi M**, Sacerdoti D, Di Pascoli M, Angeli P, Quarta S, Sticca A, Pontisso P, Merkel C, Gatta A. Haeme oxygenase mediates hyporeactivity to phenylephrine in the mesenteric vessels of cirrhotic rats with ascites. *Gut* 2005; **54**: 1630-1636
68 **Bolognesi M**, Sacerdoti D, Piva A, Di Pascoli M, Zampieri F, Quarta S, Motterlini R, Angeli P, Merkel C, Gatta A. Carbon monoxide-mediated activation of large-conductance calcium-activated potassium channels contributes to mesenteric vasodilatation in cirrhotic rats. *J Pharmacol Exp Ther* 2007; **321**: 187-194
69 **Fernandez M**, Lambrecht RW, Bonkovsky HL. Increased heme oxygenase activity in splanchnic organs from portal hypertensive rats: role in modulating mesenteric vascular reactivity. *J Hepatol* 2001; **34**: 812-817
70 **Arguedas MR**, Drake BB, Kapoor A, Fallon MB. Carboxyhemoglobin levels in cirrhotic patients with and without hepatopulmonary syndrome. *Gastroenterology* 2005; **128**: 328-333
71 **Carter EP**, Hartsfield CL, Miyazono M, Jakkula M, Morris KG Jr, McMurtry IF. Regulation of heme oxygenase-1 by nitric oxide during hepatopulmonary syndrome. *Am J Physiol Lung Cell Mol Physiol* 2002; **283**: L346-L353
72 **Liu H**, Song D, Lee SS. Role of heme oxygenase-carbon monoxide pathway in pathogenesis of cirrhotic cardiomyopathy in the rat. *Am J Physiol Gastrointest Liver Physiol* 2001; **280**: G68-G74
73 **Miyazono M**, Garat C, Morris KG Jr, Carter EP. Decreased renal heme oxygenase-1 expression contributes to decreased renal function during cirrhosis. *Am J Physiol Renal Physiol* 2002; **283**: F1123-F1131
74 **Protzer U**, Seyfried S, Quasdorff M, Sass G, Svorcova M, Webb D, Bohne F, Hosel M, Schirmacher P, Tiegs G. Antiviral activity and hepatoprotection by heme

oxygenase-1 in hepatitis B virus infection. *Gastroenterology* 2007; **133**: 1156-1165

75 **Ghaziani T**, Shan Y, Lambrecht RW, Donohue SE, Pietschmann T, Bartenschlager R, Bonkovsky HL. HCV proteins increase expression of heme oxygenase-1 (HO-1) and decrease expression of Bach1 in human hepatoma cells. *J Hepatol* 2006; **45**: 5-12

76 **Abdalla MY**, Britigan BE, Wen F, Icardi M, McCormick ML, LaBrecque DR, Voigt M, Brown KE, Schmidt WN. Down-regulation of heme oxygenase-1 by hepatitis C virus infection in vivo and by the in vitro expression of hepatitis C core protein. *J Infect Dis* 2004; **190**: 1109-1118

77 **Wen F**, Brown KE, Britigan BE, Schmidt WN. Hepatitis C core protein inhibits induction of heme oxygenase-1 and sensitizes hepatocytes to cytotoxicity. *Cell Biol Toxicol* 2008; **24**: 175-188

78 **Shan Y**, Zheng J, Lambrecht RW, Bonkovsky HL. Reciprocal effects of micro-RNA-122 on expression of heme oxygenase-1 and hepatitis C virus genes in human hepatocytes. *Gastroenterology* 2007; **133**: 1166-1174

S- Editor Zhong XY **L- Editor** Alpini GD **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6133

World J Gastroenterol 2008 October 28; 14(40): 6133-6139
World Journal of Gastroenterology ISSN 1007-9327

REVIEW

Nutritional management of newborn infants: Practical guidelines

Xiao-Ming Ben

Xiao-Ming Ben, Department of Neonatology, Nanjing Children's Hospital of Nanjing Medical University, Nanjing 210008, Jiangsu Province, China
Author contributions: Ben XM contributed all to this paper.
Correspondence to: Xiao-Ming Ben, MD, PhD, Department of Neonatology, Nanjing Children's Hospital of Nanjing Medical University, Nanjing 210008, Jiangsu Province, China. benxm@163.com
Telephone: +86-25-51554501 Fax: +86-25-83304239
Received: February 15, 2008 Revised: September 25, 2008
Accepted: October 1, 2008
Published online: October 28, 2008

Abstract

The requirements of growth and organ development create a challenge in nutritional management of newborn infants, especially premature newborn and intestinal-failure infants. Since their feeding may increase the risk of necrotizing enterocolitis, some high-risk infants receive a small volume of feeding or parenteral nutrition (PN) without enteral feeding. This review summarizes the current research progress in the nutritional management of newborn infants. Searches of MEDLINE (1998-2007), Cochrane Central Register of Controlled Trials (The Cochrane Library, Issue 3, 2007), abstracts and conference proceedings, references from relevant publications in the English language were performed, showing that breast milk is the preferred source of nutrients for enteral feeding of newborn infants. The number of nutrients found in human milk was recommended as a guideline in establishing the minimum and maximum levels in infant formulas. The fear of necrotizing enterocolitis and feeding intolerance are the major factors limiting the use of the enteral route as the primary means of nourishing premature infants. PN may help to meet many of the nutritional needs of these infants, but has significant detrimental side effects. Trophic feedings (small volume of feeding given at the same rate for at least 5 d) during PN are a strategy to enhance the feeding tolerance and decrease the side effects of PN and the time to achieve full feeding. Human milk is a key component of any strategy for enteral nutrition of all infants. However, the amounts of calcium, phosphorus, zinc and other nutrients are inadequate to meet the needs of the very low birth weight (VLBW) infants during growth. Therefore, safe and effective means to fortify human milk are essential to the care of VLBW infants.

Key words: Breast milk; Infant formula; Trophic feeding; Parenteral nutrition

Peer reviewers: Shingo Tsuji, Professor, Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine (A8), 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan; Samuel B Ho, MD, Chief, Gastroenterology Section (111D), VA San Diego Healthcare System, 3350 La Jolla Village Drive, San Diego CA 92161, United States

Ben XM. Nutritional management of newborn infants: Practical guidelines. *World J Gastroenterol* 2008; 14(40): 6133-6139 Available from: URL: http://www.wjgnet.com/1007-9327/14/6133.asp DOI: http://dx.doi.org/10.3748/wjg.14.6133

INTRODUCTION

The requirements of growth and organ development create a challenge in nutritional management of newborn infants. The stress of critical illness further complicates the delivery of adequate nutrients. Enteral feeding has several advantages over parenteral nutrition (PN), such as preservation of the gastrointestinal mucosa and decreasing the occurrence of sepsis related to bacterial translocation. Although feeding through the gastrointestinal tract is the preferred route for nutritional management, there are specific instances when PN as an adjunctive or sole therapy is necessary to meet nutritional needs. When meticulous attention is paid to the requirements of fluid, calory, protein, and fat along with monitoring the metabolic status of patients, it is possible to provide full nutritional support for critically ill newborn infants.

MACRO-NUTRIENTS OF BREAST MILK AND INFANT FORMULA

Breast milk is the preferred source of nutrients for newborn infants, and the number of nutrients found in human milk is recommended as a guideline in

establishing the minimum and maximum levels in infant formulas[1]. Following macronutrients (e.g. proteins, fatty acids and carbohydrates) for infant formulas based on scientific investigations of breast milk during the last decades were recommended by the American Academy of Pediatrics, Committee on Nutrition (AAP-CON) in 2003, and approved by Food Safety and Applied Nutrition, Food and Drug Administration (FDA)[2].

Proteins and amino acids

A minimum protein content of 1.7 g/100 kcal (i.e. total nitrogen × 6.25) and a maximum total protein content of 3.4 g/100 kcal in infant formulas have been recommended[2,3]. The current maximum protein content of 4.5 g/100 kcal is too high because there is no physiological reason to provide protein at this level. Milk contains two primary sources of protein: caseins and whey[2,3]. It has been reported that human breast milk contains whey/caseins at a ratio of 9/1 to 6/4 in different lactating periods. However, most of the marketing formulas for infants contain whey/caseins at a ratio of 6/4 to 4/6[2,3].

Whey, a protein complex derived from milk, is touted as a functional food with a number of health benefits. The biological components of whey, including lactoferrin, β-lactoglobulin, α-lactalbumin, glycomacropeptide, and immunoglobulins, demonstrate a variety of immune-enhancing properties[2,3]. In addition, whey, an antioxidant, can act as an antihypertensive, antitumor, hypolipidemic, antiviral, antibacterial, and chelating agent. The primary mechanism by which whey exerts its effects is by intracellular conversion of amino acid cysteine to glutathione, a potent intracellular antioxidant. A number of clinical trials have successfully been performed using whey in the treatment of cancer, human immunodeficiency virus (HIV) infection, hepatitis B, cardiovascular disease, osteoporosis, and as an antimicrobial agent[3].

Lactoferrin is an important protein in human milk (range 0.02-0.2 g/dL) at different lactating stages[2,3]. Although it is technically feasible to add bovine lactoferrin or transgenic human transferrin to infant formulas, bovine lactoferrin does not bind consistently to human lactoferrin receptors and whether it increases iron absorption remains unknown. The efficacy and safety of adding human lactoferrin to infant formulas have not been adequately evaluated. Given the emerging knowledge about the biological importance of human lactoferrin in infant nutrition, lactoferrin supplementation is worthy of consideration. However, clinical studies will be essential to demonstrate the efficacy and safety of such addition.

A minimum carnitine content of 1.2 mg/100 kcal (a level similar to that in human milk) and a maximum content of 2.0 mg/100 kcal in infant formulas (a value similar to the upper limit in human milk), have been recommended[2,3]. Although evidence that dietary carnitine is essential for infants, biochemical changes are noted when infants are fed with a carnitine-free diet. A few anecdotal reports are available on the abnormal clinical manifestations associated with diets low in carnitine[2]. Infants nourished with soy-protein-based formulas with a low carnitine content have lower plasma and urine carnitine levels and altered lipid metabolism, but no significant difference in rates of growth compared with those not nourished with soy-protein-based formulas with a low carnitine content.

Glutamine and taurine are free amino acids commonly detected in human breast milk[2-4]. The addition of glutamine to infant formulas is not recommended because no convincing evidence is available on glutamine requirement in diet. Also, no compelling evidence mandates the addition of taurine to formulas for infants. However, taurine has been used in some commercially available formulas. Currently, a minimum taurine content of zero and a maximum taurine content of 12 mg/100 kcal in infant formulas (a value similar to the limit in human milk) are recommended.

There are few compelling reasons for the addition of nucleotides to infant formulas[2,3,5]. The beneficial effects of nucleotide supplementation to infant formulas are intriguing, and further research in this area is strongly urged. When data from long-term, large-scale clinical trials are available, the question of adding nucleotides to infant formulas should be reconsidered. A maximum content with 16 mg/100 kcal of nucleotides and their precursors in infant formulas, a value similar to the upper limit in human milk, is recommended.

Fat and fatty acids

A minimum fat content of 4.4 g/100 kcal (40% of total energy) and a maximum fat content of 6.4 g/100 kcal (57.2% of total energy) in infant formulas have been recommended[2,3,5,6]. With the proposed minimum protein of 1.7 g/100 kcal (6.8 kcal/100 kcal) and minimum carbohydrate of 9 g/100 kcal (36 kcal/100 kcal), a maximum value for fat may not, therefore, exceed 57.2 kcal/100 kcal, which is equivalent to 6.4 g/100 kcal.

Medium-chain triglyceride (MCT) is not recommended to be supplemented in infant formulas, with the exception of certain exempt formulas for infants with impaired fat digestion or absorption.

Linoleic acid (LA) is recommended with a minimum content of 8% of total fatty acids in infant formulas. With a minimum fat content of 4.4 g/100 kcal, the minimum LA content is, therefore, 350 mg/100 kcal. Concentrations of LA in human milk vary widely as a reflection of maternal dietary intake, but values less than 8% of fatty acids are rarely reported. Currently, marketed infant formulas provide more than 8% of fatty acids as LA. A maximum LA content of 35% of total fatty acids in infant formulas is recommended. With a maximum fat content of 6.4 g/100 kcal, the maximum LA content is, therefore, 2 240 mg/100 kcal. The polyunsaturated vegetable oils (corn, safflower, and soybean oils) used in manufacture of infant formulas contain an abundant amount of LA (usually 45%-70% of total fatty acids). Historically, infant formulas, particularly corn-oil-based

formulas, contained LA exceeding 35% of fatty acids, with no adverse effects. Moreover, this value (35% of fatty acids) is within the limit reported for individual human milk samples[2,3].

α-Linolenic acid (ALA) is recommended with a minimum content of 1.75% of fatty acids in infant formulas, with the further stipulation that the ratio of LA:ALA should not exceed 16 to 1. With the minimum total fat content of 4.4 g/100 kcal, the minimum content of ALA is 77 mg/100 kcal, approximately 0.7% of energy. This recommendation is based on the essentiality of ALA as a precursor of the n-3 series of long-chain polyunsaturated fatty acids (LCPUFAs). Studies showed that formulas providing ALA at levels below this may be associated with delayed development of visual function and lower levels of docosahexaenoic acid (DHA) in the brain[6]. The recommended upper limit for the ratio of LA:ALA (16:1) is intended to prevent an inappropriate combination of high LA content with low ALA content, which might interfere with the formation of longer-chain fatty acids of the n-3 series. A maximum ALA content of 4% total fatty acids in formulas can be additionally stipulated that the ratio of LA:ALA is not less than 6 to 1. With a maximum fat content of 6.4 g/100 kcal, 4% of fatty acids from ALA amounts to 256 mg/100 kcal. The maximum is based on the long history of use of formulas containing soy oil (soybean oils typically contain 6%-9% ALA) as the source of unsaturated fatty acids. The recommended minimum ratio of 6 to 1 is intended to ensure that combination of the minimum LA content with the maximum ALA level does not interfere with the production of longer-chain fatty acids of the n-6 series[2,3].

LCPUFAs, including arachidonic acid (AA) and DHA, are not recommended in infant formulas. Breast milk contains adequate AA and DHA (range 5-20 mg/dL). However, whether AA + DHA should be added to infant-formula milk is uncertain. The results of studies on the growth and neurodevelopment in infants fed with milk formula supplemented with AA + DHA are inconsistent[6,7], suggesting that LCPUFAs are not essential in the diet of infants. Because of the uncertain efficacy and safety, LCPUFAs should not be added to infant formulas. The FDA expert panel plans to revisit this field in 5 years when more data from larger studies are available[2,3,6,7].

Carbohydrate and oligosaccharides

An energy density of 63-71 kcal/dL in infant formulas is recommended. Carbohydrate is the most important nutrient for energy. A minimum total carbohydrate content of 9 g/100 kcal in infant formula is recommended. This minimum is based on a theoretical calculation taking into account the amount of glucose needed for obligatory central nervous system oxidation. A maximum of 13 g/100 kcal is recommended for total carbohydrate in infant formulas. This value is obtained by subtracting from 100% of the total energy (63 to 71 kcal/dL), the minimum energy provided by protein (1.7 g protein/100 kcal = 6.8 kcal) and the minimum energy from fat (4.4 g fat/100 kcal = 39.6 kcal), resulting in a maximum of 53.6 kcal from carbohydrate, which is equivalent to 13.4 g/100 kcal[2,3].

The addition of glucose to infant formulas is not recommended, because inclusion of glucose in infant formulas offers no biological advantage over other carbohydrate sources and would unnecessarily increase the osmolality of formulas. Lactose is safe and appropriate for use in formulas by most healthy infants, and may be used as a sole carbohydrate source. However, it should not be used at a level higher than the recommended maximum value for total carbohydrate (i.e. 13 g/100 kcal). Also, addition of sucrose to infant formulas is safe and may be used for the palatability of some formulas (e.g. protein-hydrolysate-based formulas).

The concentration and composition of oligosaccharides in breast milk are increased in a dynamic process. The highest amount of oligosaccharides, 2 g/dL milk, is reached on the fourth day of life. On days 30 and 120 of lactation, it decreases to 20% and 40%, respectively, in comparison to that on day 4. Most studies have reported that oligosaccharide in human milk consists of approximately 70%-90% galacto-oligosaccharides (GOSs) and 10%-30% fructo-oligosaccharides (FOSs) in the first few months. The available data are insufficient at present to establish a minimum or a maximum level of these substances in infant formulas. However, some infant formulas supplemented with GOS 0.2-0.4 g/dL and FOS 0.05-0.1 g/dL are available on the market. Although glucose polymers are safe and appropriate for use in formulas by most healthy infants, either a minimum or a maximum level of such substances is not recommended. The amount of carbohydrate from glucose polymers in a formula should be within the lower and upper limits of total carbohydrate. Inclusion of modified food starches in infant formulas involves toxicologic concerns rather than nutritional concerns. Therefore, such food starches in infant formulas are not recommended[2,3,8].

TROPHIC AND ENTERAL FEEDING

The provision of adequate enteral nutrition for preterm infants is one of the major clinical challenges facing neonatologists throughout the world. Many preterm infants are too ill to receive substantial enteral feeds and require prolonged PN. It was reported that normal gastrointestinal structure and function are lost, villi become shorter, mucosal DNA is lost, protein content and enzymatic activity are reduced both in animal models and in children, although an anabolic state is maintained by PN[9]. In a rat model, atrophy occurred after only 3 d of no enteric intake, while gastrointestinal atrophy and dysfunction were reversed following enteral feeding[9].

Trophic feeding (synonyms include minimal enteral feeding or nutrition, gastrointestinal priming, gut priming, and early hypocaloric feeding) is a relatively recent concept that has been introduced into clinical practice in an attempt to counter the effects of enteral starvation[9,10]. It may be defined as the practice of feeding nutritionally insignificant volumes of enteral

Table 1 Evidence-based enteral nutrition in preterm newborns

	Evidence-based enteral nutrition
Human milk	Human milk from the preterm infant's own mother is the first choice. Human milk can be stored at room temperature for up to 24 h after expression in colostrum and up to 6 h for mature milk. Beyond that, it should be stored at 3-4℃ before use. If not used for more than 5 d, it should be frozen
Human milk fortifier	Human milk fortifier is indicated in preterm infants < 31 wk and/or < 1500 g. Human milk (100 mL/kg) is given per day and discontinued when the infant has established full breast-feeding
Formula milk	If human milk from the preterm infant's own mother is not available, the only acceptable alternative is a preterm formula. A concentration of about 60 kcal/100 mL or 20 kcal/oz is recommended, but should be increased to 80 kcal/100 mL or 24 kcal/oz when the infant has achieved full enteral feeds
Feeding methods	Gavage feeding is given via an indwelling nasogastric tube during mechanical ventilation. An indwelling orogastric tube is used after endotracheal extubation. Intermittent intragastric feeding is the first choice method, but continuous transpyloric feeding can be tried in selected preterm infants with extremely poor gastric emptying and symptomatic gastro-esophageal reflux
Commencement of feeds	Hourly feeds of 1 mL are generally used in infants weighing less than 1000 g, 2-h 2 mL for infants weighing 1000-1500 g, 3-h 3 mL for infants weighing 1500-2000 g, and 4-h 4 mL for infants weighing more than 2000 g, unless there is significant respiratory distress, when the infant remains on 1-2-h feeds. If this might not be tolerated, milk may be commenced at 1 mL every 2 h, even less than 1 mL every 4-6 h. Such trophic feeding should begin as soon as possible after birth, and definitely within the first 3-4 d
Progression of feeds	Daily increment in the range of 10-30 mL/kg of milk feeds is safe. Demand feeding is started after infants have established full milk feeds on a 4 h regimen. Non-nutritive sucking is beneficial without side effects
Supplements	Multivitamin supplement is started when the infant has established full enteral feeds, and iron is started when the infant has doubled their birth weight (usually at 2 mo). Medium-chain triglycerides can be used as an energy supplement for preterm infants who fail to thrive

substrate to sick neonates, to supply nutrients to, and directly stimulate, the developing gastrointestinal system without increasing disease severity. Typically, a milk volume of 10-20 mL/kg per day is given at the same rate for at least 5 d. Several studies have examined the clinical outcome after trophic feeding[9-11], showing that milk tolerance, liver function, metabolic bone disease, days to hospital discharge, and weight gain are improved after trophic feeding. Nosocomial infections due to PN (because of its interference with the immune system and translocation of enteric pathogenic microorganisms into the circulation) may be reduced either because of improved gastrointestinal mucosal barrier function or because of beneficial alteration of the enteric flora.

Since premature infants are unable to coordinate sucking, swallowing, and breathing, orogastric tube-feeding is necessary. The most common methods used are continuous milk infusion and intermittent (bolus) milk delivery (usually every 3 h). Recent studies have suggested that bolus feeding promotes more "normal" feed-fasting hormonal concentrations that potentially benefit intestinal development and nutrient partitioning, and marked differences are observed in feeding tolerance and growth between continuous *vs* bolus tube-feeding methods[9-13]. Since continuous feeding is associated with more significant feeding intolerance, more infants are switched to bolus feeding. Importantly, throughout hospitalization, the continuous feeding method is associated with slower growth compared with the bolus group. Thus, bolus feeding is more advantageous than continuous infusion for premature infants with relatively healthy gastrointestinal tracts.

Current data support the practice of starting GI priming early, which does not add complications of neonatal intensive care[9,10,12]. Further studies are needed to determine if early feeding can be advanced in volume so that the use of PN can be reduced. Bolus feeding results in better feeding tolerance and growth than continuous tube-feeding and also obviates the need for costly infusion pumps and support care. The use of human milk, however, may have the most profound effects because of its association with a decrease in morbidity. The evidence-based guidelines for enteral nutrition in preterm infants are listed in Table 1[14].

PN

PN can meet neonates' requirement for growth and development when their size or condition precludes enteral feeding. Although feeding through the gastrointestinal tract is the preferred route for nutritional management, there are specific conditions for which PN as an adjunctive or a sole therapy is necessary. In very low birth weight (VLBW) premature infants, enteral feeding cannot be established in the first few days of life, due to the immaturity of the gastrointestinal system. PN can successfully meet the nutritional demands in critically ill neonates, neonates with protracted diarrhea and neonates undergone a major gastrointestinal surgery. The evidence-based guidelines for PN in preterm infants are summarized in Table 2[14].

Fluids and energy requirements

PN is a fundamental part of neonatal intensive care[15,16]. Fluid intake volume varies from 60 to 150 mL/kg per day, depending on maturity of the infant and environmental conditions influencing insensible water loss from the skin. An energy intake of 50 kcal/kg per day is adequate to match ongoing expenditure but an additional energy intake of 70 kcal/kg per day is required to achieve optimal growth. The ideal distribution of calories should be 60% carbohydrate, 10%-15%

Table 2 Evidence-based PN in preterm newborns

	Evidence-based PN
Fluids	D 1: 60-80 mL/kg per day. Infants < 28 wk gestation are nursed in a maximally humidified environment (90% humidity) for at least 7 d. Postnatal weight loss of 5% per day to a maximum of 15% is acceptable, which is achieved by progressively increasing the fluid intake to 120-150 mL/kg per day at 1 wk of age
Energy	An intake of 50 kcal/kg per day is sufficient to match ongoing expenditure, but it does not meet additional requirements of growth. The goal energy intake is 120 kcal/kg per day, which is higher in infants with chronic lung diseases
Protein	Optimal parenteral amino acid intake is 3.5 g/kg per day. Parenteral amino acids can begin from day 1 at a dose of 1.75 g/kg per day
Carbohydrate	From day 1, 6-10 g/kg per day can be infused and adjusted to maintain blood glucose level of 2.6-10 mmol/L. Insulin is only used in infants whose blood glucose level is higher than 15 mmol/L and associated with glycosuria and osmotic diuresis, even after glucose intake has been decreased to 6 g/kg per day. Carbohydrate is given as a continuous infusion commencing at a rate of 0.05 U/kg per hour, and increased as required for persistent hyperglycemia
Fat	Intravenous fat, 1 g/kg per day, can be started from day 1, or when intravenous amino acids are started. The dose of intravenous fat is increased to 2 g/kg and 3 g/kg per day over the next 2 d. Twenty percent intravenous fat is delivered as a continuous infusion via a syringe pump, separated from the infusate containing amino acids and glucose. The syringe and infusion line should be shielded from ambient light
Minerals	Minerals should include sodium (3-5 mmol/kg per day), chloride (3-5 mmol/kg per day), potassium (1-2 mmol/kg per day), calcium (1.5-2.2 mmol/kg per day), phosphorus (1.5-2.2 mmol/kg per day), and magnesium (0.3-0.4 mmol/kg per day)
Trace elements	Trace elements should include zinc (6-8 μmol/kg per day), copper (0.3-0.6 μmol/kg per day), selenium (13-25 nmol/kg per day), manganese (18-180 nmol/kg per day), iodine (8 nmol/kg per day), chromium (4-8 nmol/kg per day), and molybdenum (2-10 nmol/kg per day)
Vitamins	Vitamins must be added to the fat emulsion to minimize loss of vitamins due to adherence to tubes and photodegradation

protein, and 30% fat. A 10% dextrose solution provides 0.34 kcal/mL, a 10% lipid solution provides 0.9 kcal/mL. Although protein is a potential energy substrate, it should be utilized only for tissue growth. Glucose and lipids can provide sufficient calories to avoid protein catabolism. A preterm neonate needs 100-150 kcal/kg per day, whereas a term neonate needs 100-120 kcal/kg per day.

Carbohydrate requirements

Glucose is the most widely used intravenous carbohydrate for neonates because it is readily available to the brain. A preterm infant has a higher glucose demand and hence early administration of glucose is vital. It is important to balance non-protein calories between carbohydrates and fats, and a 2:1 ratio is recommended. Excess use of glucose would result in lipogenesis, excess production of CO_2 and hyperglycemia leading to osmotic diuresis. Hyperglycaemia during PN can be minimized by starting glucose infusion at a rate of 4-6 mg/kg permin (6-8 g/kg per day) with progressive increase to 12-15 mg/kg per min (16-20 g/kg per day) for 2-3 wk after birth[15,16].

Treatment of hyperglycemia is initiated with insulin if blood glucose is > 200 mg/dL, although the dextrose infusion is below 5 mg/kg per min. Insulin can be started at a dose of 0.05-0.1 U/kg per hour. Insulin infusion rate should be adjusted to 0.05 U/kg per hour to keep the glucose level at 150-200 mg/dL. When the glucose level decreases to < 100 mg/dL, the glucose is monitored every 4 h once the target level is achieved[15,16]. Other causes of hyperglycemia like sepsis, intraventricular hemorrhage, and steroids should be ruled out before insulin is used.

Protein requirements

The goal of giving proteins is to limit catabolism, maintain endogenous protein stores, and provide sufficient energy and protein to support growth. It has been reported that early administration of PN is safe and efficacious with no metabolic derangements[17]. The concept put forth by the American Academy of Pediatrics that nutrition should support postnatal growth that approximates the in utero growth of a normal fetus should be accepted. Parenteral nitrogen requirement is 30-35 mmol/kg per day, which is equivalent to 3.0-3.5 mg/kg amino acids per day. These solutions contain nine essential amino acids and cysteine, tyrosine, taurine and arginine as the semi-essential amino acids. In the absence of an exogenous protein source, a preterm infant catabolizes 1 g/kg of its own body protein per day to meet its metabolic needs. Excess protein administration causes a rise in blood urea, ammonia and high levels of potentially toxic amino acids such as phenylalanine[18]. In our unit, we usually start amino acids (1 g/kg per day) on the second day of life for extremely low birth weight (ELBW) infants and increase to 3 g/kg per day with 1 g/kg daily increments per day. Protein with a maximum of 15% calories should be given.

Glutamine, one of the most abundant amino acids in both plasma and breast milk, is not included in amino acid preparations for PN. Glutamine, which is unstable in solution, is usually regarded as a non-essential amino acid. However, glutamine provides an important metabolic fuel for rapidly dividing cells of the gastrointestinal tract and immune system, and is an intermediate in a large number of metabolic pathways, and a precursor that donates nitrogen for the synthesis of purines, pyramidines, nucleotides and amino sugars. In addition, glutamine plays a key part in acid-base balance by acting as the most important substrate for renal ammonia production. It was reported that glutamine supplementation may decrease sepsis and mortality in critically ill adult patients[19]. In view of the

important metabolic roles of glutamine, further clinical evaluation is required in neonates.

Lipid requirements

Lipid is a major source of non-protein energy and has a nitrogen sparing effect. Serving as a source of essential fatty acids and LCPUFA, lipid is a major source of non-protein energy and has a nitrogen-sparing effect. The commercial intravenous lipid emulsions are aqueous suspensions containing neutral triglycerides derived from soybean, safflower oil and egg yolk to emulsify and adjust glycerin tonicity. Hydrolysis of triglycerides by hepatic and lipoprotein lipase results in formation of free fatty acids. Circulating free fatty acids can be used as an energy source or they enter adipose tissue where they are re-esterified to form triglycerides[15,16].

Parenteral fat is introduced at 1 g/kg per day, and gradually increased to 3 g/kg per day, given as a continuous infusion. In our unit, we usually start lipids on the third day of life in ELBW infants when the most acute phase of respiratory distress or other life-threatening events are controlled. We start 1 g/kg of lipids per day and increase it to 3 g/kg per day. At present, a 20% lipid emulsion is preferred over 10% emulsion, because the higher phospholipid content in 10% solution impedes plasma triglyceride clearance, resulting in higher concentrations of triglyceride and plasma cholesterol. Also, combined MCT/long-chain triglyceride (LCT) and lipid emulsion is preferred over LCT emulsion in preterm and critical neonates, because MCT/LCT is more easily metabolized. Advantages of lipid emulsions over concentrated glucose solutions include their isotonicity and greater energy density, the latter means that a low volume is required per calorie[15,16].

Minerals, trace elements and vitamins

Minerals and trace elements delivered with PN are calculated to meet in-utero accretion rates. Sodium, potassium, chloride, calcium, magnesium and phosphorus levels need to be closely monitored and the infusion needs to be prescribed accordingly. Neonates on long-term TPN may develop trace element deficiencies which should be checked regularly. TPN can provide the daily requirements for water and fat-soluble vitamins. The dose of water-soluble vitamins is 1 mL/kg per day, which should be added to the dextrose-electrolyte solution. The dose of fat-soluble vitamins is 1 mL/kg per day, which should be added to the lipid emulsions[14-16].

PN-associated cholestasis

In neonatal intensive care units where appropriate medical, nursing, pharmacy and laboratory experts are available, the potential benefits of PN outweigh its hazards. However, PN-associated cholestasis, onset of hyperbilirubinemia with direct bilirubin > 2 mg/dL within 2 wk after starting PN, are the common complications of PN, along with hepatomegaly, and mild elevation of conjugated bilirubin, alkaline phosphatase and transaminases[20,21]. Liver function generally becomes normal within 1-4 mo after stopping PN, but prolonged liver dysfunction and even fibrosis have been reported in certain cases[20,21]. The following factors may contribute to PN-associated liver diseases, including prolonged duration of PN therapy, sepsis, low serum albumin, excessive caloric load, enteral fasting, deficiency in nutritional taurine, carnitine, manganese, oxidative stress and hormonal factors such as elevated insulin/glucagon ratio, gut hormones and biliary stasis. Simple interventions such as minimizing the duration of therapy, early detection and treatment of sepsis, and choosing enteral nutrition rather than PN whenever possible, can minimize liver injury[20,21]. Ursodeoxycholic acid (UDCA) is used in the treatment of cholestasis[20], because it increases the hydrophilic non-hepatotoxic bile acid pool, decreases hepatocyte display of histocompatibility antigens and gives direct cytoprotection. The dose of UDCA is 20-30 mg/kg per day.

REFERENCES

1 **Mattar CN**, Chan YS, Chong YS. Breastfeeding: it's an important gift. *Obstet Gynecol* 2003; **102**: 1414
2 **Carver JD**. Advances in nutritional modifications of infant formulas. *Am J Clin Nutr* 2003; **77**: 1550S-1554S
3 **Lonnerdal B**. Nutritional and physiologic significance of human milk proteins. *Am J Clin Nutr* 2003; **77**: 1537S-1543S
4 **van den Berg A**, van Elburg RM, Westerbeek EA, Twisk JW, Fetter WP. Glutamine-enriched enteral nutrition in very-low-birth-weight infants and effects on feeding tolerance and infectious morbidity: a randomized controlled trial. *Am J Clin Nutr* 2005; **81**: 1397-1404
5 **Aggett PJ**, Agostini C, Goulet O, Hernell O, Koletzko B, Lafeber HL, Michaelsen KF, Rigo J, Weaver LT. The nutritional and safety assessment of breast milk substitutes and other dietary products for infants: a commentary by the ESPGHAN Committee on Nutrition. *J Pediatr Gastroenterol Nutr* 2001; **32**: 256-258
6 **Clandinin MT**, Van Aerde JE, Merkel KL, Harris CL, Springer MA, Hansen JW, Diersen-Schade DA. Growth and development of preterm infants fed infant formulas containing docosahexaenoic acid and arachidonic acid. *J Pediatr* 2005; **146**: 461-468
7 **Ben XM**, Zhou XY, Zhao WH, Yu WL, Pan W, Zhang WL, Wu SM, Van Beusekom CM, Schaafsma A. Growth and development of term infants fed with milk with long-chain polyunsaturated fatty acid supplementation. *Chin Med J* (Engl) 2004; **117**: 1268-1270
8 **Ben XM**, Zhou XY, Zhao WH, Yu WL, Pan W, Zhang WL, Wu SM, Van Beusekom CM, Schaafsma A. Supplementation of milk formula with galacto-oligosaccharides improves intestinal micro-flora and fermentation in term infants. *Chin Med J* (Engl) 2004; **117**: 927-931
9 **Dunn L**, Hulman S, Weiner J, Kliegman R. Beneficial effects of early hypocaloric enteral feeding on neonatal gastrointestinal function: preliminary report of a randomized trial. *J Pediatr* 1988; **112**: 622-629
10 **Meetze WH**, Valentine C, McGuigan JE, Conlon M, Sacks N, Neu J. Gastrointestinal priming prior to full enteral nutrition in very low birth weight infants. *J Pediatr Gastroenterol Nutr* 1992; **15**: 163-170
11 **Berseth CL**. Effect of early feeding on maturation of the preterm infant's small intestine. *J Pediatr* 1992; **120**: 947-953
12 **Troche B**, Harvey-Wilkes K, Engle WD, Nielsen HC, Frantz ID 3rd, Mitchell ML, Hermos RJ. Early minimal feedings promote growth in critically ill premature infants. *Biol Neonate* 1995; **67**: 172-181

13 **Silvestre MA**, Morbach CA, Brans YW, Shankaran S. A prospective randomized trial comparing continuous versus intermittent feeding methods in very low birth weight neonates. *J Pediatr* 1996; **128**: 748-752
14 **Yu VY**. Extrauterine growth restriction in preterm infants: importance of optimizing nutrition in neonatal intensive care units. *Croat Med J* 2005; **46**: 737-743
15 **Koletzko B**, Goulet O, Hunt J, Krohn K, Shamir R. 1. Guidelines on Paediatric Parenteral Nutrition of the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) and the European Society for Clinical Nutrition and Metabolism (ESPEN), Supported by the European Society of Paediatric Research (ESPR). *J Pediatr Gastroenterol Nutr* 2005; **41** Suppl 2: S1-S87
16 **Acra SA**, Rollins C. Principles and guidelines for parenteral nutrition in children. *Pediatr Ann* 1999; **28**: 113-120
17 **Ziegler EE**, Thureen PJ, Carlson SJ. Aggressive nutrition of the very low birthweight infant. *Clin Perinatol* 2002; **29**: 225-244
18 **Thureen PJ**, Melara D, Fennessey PV, Hay WW Jr. Effect of low versus high intravenous amino acid intake on very low birth weight infants in the early neonatal period. *Pediatr Res* 2003; **53**: 24-32
19 **Poindexter BB**, Ehrenkranz RA, Stoll BJ, Wright LL, Poole WK, Oh W, Bauer CR, Papile LA, Tyson JE, Carlo WA, Laptook AR, Narendran V, Stevenson DK, Fanaroff AA, Korones SB, Shankaran S, Finer NN, Lemons JA. Parenteral glutamine supplementation does not reduce the risk of mortality or late-onset sepsis in extremely low birth weight infants. *Pediatrics* 2004; **113**: 1209-1215
20 **Chaudhari S**, Kadam S. Total parenteral nutrition in neonates. *Indian Pediatr* 2006; **43**: 953-964
21 **Puntis JW**. Nutritional support in the premature newborn. *Postgrad Med J* 2006; **82**: 192-198

S- Editor Li DL **L- Editor** Wang XL **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6140

World J Gastroenterol 2008 October 28; 14(40): 6140-6144
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Toru Ishikawa, MD, *Series Editor*

Secondary prevention of recurrence by interferon therapy after ablation therapy for hepatocellular carcinoma in chronic hepatitis C patients

Toru Ishikawa

Toru Ishikawa, Department of Gastroenterology and Hepatology, Saiseikai Niigata Second Hospital, Niigata 950-1104, Japan
Author contributions: Ishikawa T contributed all to this paper.
Correspondence to: Toru Ishikawa, MD, Department of Gastroenterology and Hepatology, Saiseikai Niigata Second Hospital, Teraji 280-7, Niigata 950-1104, Japan. toruishi@ngt.saiseikai.or.jp
Telephone: +81-25-2336161 Fax: +81-25-2338880
Received: March 31, 2008 Revised: May 13, 2008
Accepted: May 20, 2008
Published online: October 28, 2008

Peer reviewer: Hisato Nakajima, MD, Department of Gastroenterology and Hepatology, The Jikei University School of Medicine, 3-25-8, Nishi-Shinbashi, Minato-ku, Tokyo 105-8461, Japan

Ishikawa T. Secondary prevention of recurrence by interferon therapy after ablation therapy for hepatocellular carcinoma in chronic hepatitis C patients. *World J Gastroenterol* 2008; 14(40): 6140-6144 Available from: URL: http://www.wjgnet.com/1007-9327/14/6140.asp DOI: http://dx.doi.org/10.3748/wjg.14.6140

Abstract

Chronic hepatitis C is a leading cause of hepatocellular carcinoma (HCC) worldwide. Interferon (IFN) therapy decreases the incidence of HCC in patients with chronic hepatitis C. Prevention of chronic-hepatitis-C-related HCC is one of the most important issues in current hepatology. We have previously reported that male gender and high titer of hepatitis C virus (HCV) RNA are predictive factors for the development of HCC in HCV-related cirrhosis. Clinical efforts at eradicating or reducing the viral load may reduce the risk for HCC. Furthermore, because HCC often recurs after ablation therapy, we performed a trial of IFN in patients with chronic liver disease caused by HCV to see whether IFN therapy decreases recurrence after ablation therapy of HCV-related HCC. By using IFN therapy as a secondary prevention, patients with HCC who had received complete tumor ablation showed better survival, primarily as a result of the preservation of liver function and also probably prevention of recurrence. Postoperative IFN therapy appears to decrease recurrence after ablation therapy such as radiofrequency ablation (RFA) therapy of HCV-related HCC. We believe that there is a survival benefit in secondary prevention using IFN therapy. However, a controlled study is essential to obtain conclusive evidence of the efficacy of this strategy.

Key words: Hepatocellular carcinoma; Radiofrequency ablation; Interferon; Secondary prevention

INTRODUCTION

In Japan, the overwhelming majority of hepatocellular carcinoma (HCC) is caused by chronic hepatitis and liver cirrhosis due to persistent hepatitis C or B, mostly hepatitis C virus (HCV) infection. The onset of HCC can be roughly divided into intrahepatic metastasis and multicentric carcinogenesis. The latter can be further divided into synchronous multicentric carcinogenesis, in which HCC occurs in multiple locations simultaneously, and asynchronous multicentric carcinogenesis, in which HCC occurs some time after localized therapy such as partial hepatectomy, percutaneous ethanol injection therapy (PEIT), or percutaneous radiofrequency ablation (RFA). With asynchronous multicentric carcinogenesis, the prognosis of patients can be improved by preventing carcinogenesis in the remaining liver. HCC is treated by analyzing multiple factors, including: (1) decreased hepatic function due to chronic hepatitis and cirrhosis; (2) multicentric carcinogenesis (synchronous/asynchronous) due to persistent infection; and (3) early intrahepatic metastasis due to portal invasion, which is one of the characteristics of HCC. In other words, unlike other cancers, it is necessary to assess not only cancer progression, but also the hepatic reserve. The main objective of interferon (IFN) therapy for chronic hepatitis C infection is to end persistent infection and prevent the progression of liver disease. The present article discusses the significance of IFN therapy as secondary prevention after localized therapy for HCC, particularly IFN therapy combining pegylated IFN (PEG-IFN) and ribavirin.

Table 1 Univariate and multivariate analyses on the carcinogenic factors for HCC in patients with HCV cirrhosis

Variables	Number of patients	Univariate analysis			Multivariate analysis		
		P-value[1]	RR[2]	95% CI	*P*-value[3]	RR[2]	95% CI
Sex							
Male	85	$P = 0.001$	1.971	1.298-2.991	$P = 0.005$	2.107	1.198-2.987
Female	80		1			1	
Alcohol							
Yes	49	$P = 0.012$	1.681	1.108-2.550	NS ($P = 0.496$)	1.234	0.673-2.262
No	116		1			1	
ALT							
⩾ 100	77	$P = 0.013$	1.657	1.103-2.489	NS ($P = 0.876$)	1.050	0.572-1.927
< 100	88		1			1	
LDH							
⩾ 480	74	NS ($P = 0.064$)	-	-	NS ($P = 0.112$)	0.673	0.413-1.096
< 480	91					1	
HCV-RNA							
⩾ 1.0 Meq/mL	110	$P = 0.018$	1.709	1.086-2.695	$P = 0.028$	1.658	1.125-2.315
< 1.0 Meq/mL	55		1			1	
Ant i-HBc							
Positive	78	NS ($P = 0.834$)	-	-	NS ($P = 0.577$)	1.136	0.724-1.782
Negative	87					1	

[1]*P*-values were obtained by using the log-rank test; [2]RR were calculated by comparing classes with Cox regression analysis; [3]*P*-values were obtained by using Cox regression analysis. RR: Relative risks; CI: Confidence interval; NS: Not significant.

ABLATION THERAPY FOR HCC

Unlike other cancers, the treatment of HCC involves not only the stage of the carcinoma itself, but also the stage of underlying chronic hepatitis. In patients with advanced HCC or extrahepatic metastasis, chemotherapy is mostly performed and its usefulness has been shown[1-5]. However, in patients with stage I or Ⅱ HCC, including early-stage HCC, percutaneous therapy is useful because its impact on normal hepatocytes is relatively small. Percutaneous therapy began with PEIT[6] and advanced to percutaneous microwave coagulation therapy (PMCT)[7], and today, RFA that combines the advantages associated with the previous two techniques is often performed[8-10]. In Japan, RFA was first performed in 1999, and it is still premature to discuss its long-term results, but regarding overseas results, Rossi *et al*[8-10] have reported that the survival rate for RFA was 94% at 1 year, 68% at 3 years, and 40% at 5 years, and that the rate of local recurrence was 5% with an average follow-up of 22.6 mo. At present, three RFA needles are available: Radionics Cooltip (single needle), RTC LeVeen probe (expandable needle), and RITA Model 90/70 (expandable needle). In our department, different RFA needles are used depending on tumor site and size, and according to our data, the extent of thermocoagulation per single ablation for RITA Model 90 is 43.2 mL, which is significantly greater when compared to the others. The rate of local recurrence within a range of 20 mm or 30 mm is significantly lower for RITA Model 90 (data not shown). Ablation therapy appears useful for the local control of HCC, but even if local control is sufficient, it is necessary to take into account background liver factors when suppressing recurrence. In other words, as in chronic hepatitis B [11], it is important to treat chronic hepatitis C using IFN.

CARCINOGENIC FACTORS IN HCV-RELATED CHRONIC HEPATITIS

While the onset mechanism of HCV HCC has not been elucidated, it has been suggested that persistent HCV-induced inflammation causes abnormally high levels of transaminase and results in excessive cellular turnover consisting of hepatocyte necrosis and regeneration, thus increasing the risk for genetic abnormalities leading to carcinogenesis. We examined carcinogenic factors in patients with HCV cirrhosis and advanced liver fibrosis; long-term follow-up examinations revealed that high viral titer, sex (male), and age (elderly) were significant onset factors (Tables 1 and 2)[12]. Hence, it is necessary to prevent HCC in patients with these risk factors.

PRIMARY PREVENTION OF HCV-RELATED CHRONIC HEPATITIS BY IFN THERAPY

Many studies have documented that IFN significantly suppresses the onset of HCC from chronic hepatitis and liver cirrhosis. Studies have found that IFN therapy for HCV infection is useful in suppressing carcinogenesis and improving liver function[13,14] and that IFN therapy eliminates HCV RNA and clearly suppresses the onset of HCC in patients with normalized transaminase levels[15]. Additionally, even if a complete response is not achieved, IFN therapy suppresses HCC when compared to untreated cases[16].

Furthermore, even in the presence of advanced chronic hepatitis, cirrhosis improves in about half of patients with a sustained response to IFN therapy[17], and IFN therapy lowers transaminase, maintains platelet counts, and reduces carcinogenesis[13]. This suggests

Table 2 Univariate and multivariate analyses of the carcinogenic factors for HCC in male patients with HCV cirrhosis

Variables	Number of patients	Univariate analysis			Multivariate analysis		
		P-value[1]	RR[2]	95% CI	*P*-value[3]	RR[2]	95% CI
Age (yr)							
⩾ 60	43	*P* = 0.032	1.726	1.032-2.881	*P* = 0.035	4.469	1.271-5.723
< 60	42		1			1	
Alcohol							
Yes	45	*P* = 0.826	1.058	0.632-1.771	NS (*P* = 0.676)	0.877	0.473-1.025
No	40		1			1	
Smoking							
Yes	36	*P* = 0.566	0.863	0.517-1.440	NS (*P* = 0.696)	0.893	0.504-1.580
No	49		1			1	
AST							
⩾ 100	47	*P* = 0.213	1.376	0.824-2.298	NS (*P* = 0.151)	1.863	0.797-4.350
< 100	38		1			1	
ALT							
⩾ 100	46	*P* = 0.805	1.064	0.643-1.763	NS (*P* = 0.485)	0.752	0.337-1.667
< 100	39		1			1	
γ-GTP							
⩾ 80	41	*P* = 0.509	1.182	0.714-1.954	NS (*P* = 0.561)	1.178	0.679-2.041
< 80	44		1			1	
Anti-HBc							
Positive	43	*P* = 0.111	1.522	0.898-2.577	NS (*P* = 0.099)	1.609	0.914-2.835
Negative	42		1			1	

[1]*P*-values were obtained by using the log-rank test; [2]RR were calculated by comparing classes with Cox regression analysis; [3]*P*-values were obtained by using Cox regression analysis. RR: Relative risks; CI: Confidence interval; NS: Not significant.

Table 3 Studies in which IFN was administered after treatments for HCV-related HCC in Japan

Authors	Treated *vs* untreated	Treatment	Follow-up (mo)	Recurrence (%)	Survival (%)
Ikeda[21]	20 *vs* 10	IFN-β	25	10 *vs* 70 ($P = 0.0004$)	
Kubo[22]	15 *vs* 15	IFN-α	36	33 *vs* 80 ($P = 0.037$)	
Suou[23]	18 *vs* 28	IFN-α	60	28 *vs* 82 ($P < 0.01$)	0 *vs* 27 ($P < 0.05$)
Shiratori[24]	49 *vs* 25	IFN-α	84	80 *vs* 92[1]	53 *vs* 23

[1]IFN therapy did not markedly lower the rate of recurrence the first time, it significantly lowered the rate of recurrence the second and third times.

that IFN suppresses persistent hepatitis in liver cirrhosis and carcinogenesis. Regarding the onset of HCC, it is not clear if it is important to maintain low transaminase levels or suppress liver fibrosis, but it is highly likely that blocking fibrosis is important in suppressing carcinogenesis. Therefore, IFN therapy appears to prevent liver fibrosis in liver cirrhosis.

Ever since the national health insurance system began covering IFN therapy in 1992, antiviral therapy for hepatitis C has steadily advanced and at present, therapy combining PEG-IFN and ribavirin is considered the most potent. The combination therapy was markedly effective in about 90% of patients with genotype-2 HCV when administered for 24 wk[18], and it was markedly effective in about 50% of patients with intractable hepatitis (genotype-1 HCV or high viral load) when administered for 48 wk[19]. In Japan, PEG-IFN and ribavirin combination therapy has improved the therapeutic results for intractable chronic hepatitis C.

IFN THERAPY AS SECONDARY PREVENTION FOR RECURRENT HCC

IFN therapy has been performed to prevent recurrent HCC (Table 3). One study retrospectively investigated recurrence after curative resection of HCV HCC, and found that alanine aminotransferase levels remained high[20]. In other words, hepatocyte necrosis and inflammation appear to be closely involved with recurrence. If IFN is successful in lowering HCV to an undetectable level, necrotic inflammation is naturally improved. At the same time, carcinogenesis is believed to be suppressed even in biochemical responders. Ikeda *et al*[21] have investigated the suppression of recurrent HCC by IFN-β following surgical resection or PEIT for HCC in patients with HCV cirrhosis. They have reported that intermittent IFN-β administration following surgical resection or PEIT for HCV HCC suppresses recurrence.

Kubo *et al*[22] have conducted a randomized controlled trial of postoperative IFN therapy in patients with HCV HCC and have reported that the rate of recurrence is significantly lower for patients with IFN therapy.

Suou *et al*[23] administered 6 MU of IFN-α for 24 wk and reported that the 3-year survival rate for patients without IFN-α was 18% and that of patients with IFN-α was 63%. Additionally, Shiratori *et al*[24] have reported that while IFN therapy does not markedly lower the rate of recurrence the first time, it significantly lowers the rate for the second and third times. Hence, IFN may initially act on tumors to suppress intrahepatic micrometastases following therapy for HCC, and then it may act on the virus to suppress recurrence 3-5 years later. Furthermore, it is reported to the contrary that although the cumulative recurrence rate in the IFN group was found to be lower than in the control group during the first 3 years after commencement of IFN administration, the recurrence rate in the IFN group increased with the lapse of time over 3 years. However, long-term, low-dose, intermittent IFN therapy successfully delayed clinical recurrence of HCC after radical RFA therapy[25]. In these studies, IFN therapy consisted of non-PEG-IFN monotherapy and the rate of sustained viral response was low, at 13%-33%. Therefore, if PEG-IFN and ribavirin combination therapy further improves antiviral effects[26], then recurrence may be suppressed even more. However, many patients with HCV HCC are elderly or have cirrhosis, and the dose and duration of PEG-IFN and ribavirin combination therapy have not been established in these patients. Further investigations are warranted.

IFN therapy following therapy for HCC is safe in selected patients. However, IFN therapy for the prevention of recurrent HCC is different from that for the treatment of primary HCC. Because prevention involves not only inflammation, fibrosis, and HCV, but also HCC-related factors, further investigations, including randomized controlled trials, are needed. Furthermore, antiviral therapy itself may improve liver reserve and expand the therapeutic options at the time of recurrence, thus improving the prognosis of HCC, and this issue also needs to be addressed by further studies including randomized controlled trials.

CONCLUSION

Secondary prevention of HCC is an important clinical issue because the recurrence rates of HCC are extremely high even after effective local treatment with hepatic resection or percutaneous ablation. This involves multicentric carcinogenesis in which new lesions are formed as a result of underlying hepatitis. Therefore, IFN therapy following the treatment for HCC is safe in selected patients and IFN therapy is an effective secondary prevention. In the future, PEG-IFN and ribavirin combination therapy may prove to be effective in preventing recurrence, and further investigations involving more cases are needed.

REFERENCES

1 **Ishikawa T**, Ichida T, Ishimoto Y, Yokoyama J, Nomoto M, Ebe Y, Usuda H, Naito M, Asakura H. Complete remission of multiple hepatocellular carcinomas associated with hepatitis C virus-related, decompensated liver cirrhosis by oral administration of enteric-coated tegafur/uracil. *Am J Gastroenterol* 1999; **94**: 1682-1685

2 **Ishikawa T**, Ichida T, Sugitani S, Tsuboi Y, Genda T, Sugahara S, Uehara K, Inayoshi J, Yokoyama J, Ishimoto Y, Asakura H. Improved survival with oral administration of enteric-coated tegafur/uracil for advanced stage IV-A hepatocellular carcinoma. *J Gastroenterol Hepatol* 2001; **16**: 452-459

3 **Ishikawa T**, Ichida T, Yokoyama J, Matsuda Y, Watanabe T, Asakura H. Complete disappearance of pulmonary metastases in a case of hepatocellular carcinoma treated with docetaxel-based systemic chemotherapy. *J Gastroenterol Hepatol* 2004; **19**: 1423-1426

4 **Ishikawa T**, Imai M, Kamimura H, Tsuchiya A, Togashi T, Watanabe K, Seki K, Ohta H, Yoshida T, Kamimura T. Improved survival for hepatocellular carcinoma with portal vein tumor thrombosis treated by intra-arterial chemotherapy combining etoposide, carboplatin, epirubicin and pharmacokinetic modulating chemotherapy by 5-FU and enteric-coated tegafur/uracil: a pilot study. *World J Gastroenterol* 2007; **13**: 5465-5470

5 **Ishikawa T**. Chemotherapy with enteric-coated tegafur/uracil for advanced hepatocellular carcinoma. *World J Gastroenterol* 2008; **14**: 2797-2801

6 **Notsumata K**, Shimizu M, Unoura M, Mizuno Y, Rhorin H, Motoo Y, Kobayashi K, Hattori N, Matsui O, Izumi R. [Study of percutaneous ethanol injection therapy (PEIT) under ultrasonography in hepatocellular carcinoma] *Nippon Shokakibyo Gakkai Zasshi* 1988; **85**: 2666-2672

7 **Seki T**, Wakabayashi M, Nakagawa T, Itho T, Shiro T, Kunieda K, Sato M, Uchiyama S, Inoue K. Ultrasonically guided percutaneous microwave coagulation therapy for small hepatocellular carcinoma. *Cancer* 1994; **74**: 817-825

8 **Rossi S**, Garbagnati F, Lencioni R, Allgaier HP, Marchiano A, Fornari F, Quaretti P, Tolla GD, Ambrosi C, Mazzaferro V, Blum HE, Bartolozzi C. Percutaneous radio-frequency thermal ablation of nonresectable hepatocellular carcinoma after occlusion of tumor blood supply. *Radiology* 2000; **217**: 119-126

9 **Rossi S**, Buscarini E, Garbagnati F, Di Stasi M, Quaretti P, Rago M, Zangrandi A, Andreola S, Silverman D, Buscarini L. Percutaneous treatment of small hepatic tumors by an expandable RF needle electrode. *AJR Am J Roentgenol* 1998; **170**: 1015-1022

10 **Rossi S**, Di Stasi M, Buscarini E, Quaretti P, Garbagnati F, Squassante L, Paties CT, Silverman DE, Buscarini L. Percutaneous RF interstitial thermal ablation in the treatment of hepatic cancer. *AJR Am J Roentgenol* 1996; **167**: 759-768

11 **Ishikawa T**, Ichida T. [Features of hepatitis B virus-related hepatocellular carcinoma] *Nippon Rinsho* 2001; **59** Suppl 6: 435-439

12 **Ishikawa T**, Ichida T, Yamagiwa S, Sugahara S, Uehara K, Okoshi S, Asakura H. High viral loads, serum alanine aminotransferase and gender are predictive factors for the development of hepatocellular carcinoma from viral compensated liver cirrhosis. *J Gastroenterol Hepatol* 2001; **16**: 1274-1281

13 **Nishiguchi S**, Kuroki T, Nakatani S, Morimoto H, Takeda T, Nakajima S, Shiomi S, Seki S, Kobayashi K, Otani S. Randomised trial of effects of interferon-alpha on incidence of hepatocellular carcinoma in chronic active hepatitis C with cirrhosis. *Lancet* 1995; **346**: 1051-1055

14 **Nishiguchi S**, Shiomi S, Nakatani S, Takeda T, Fukuda K, Tamori A, Habu D, Tanaka T. Prevention of hepatocellular

carcinoma in patients with chronic active hepatitis C and cirrhosis. *Lancet* 2001; **357**: 196-197

15 **Yoshida H**, Shiratori Y, Moriyama M, Arakawa Y, Ide T, Sata M, Inoue O, Yano M, Tanaka M, Fujiyama S, Nishiguchi S, Kuroki T, Imazeki F, Yokosuka O, Kinoyama S, Yamada G, Omata M. Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of Hepatocarcinogenesis by Interferon Therapy. *Ann Intern Med* 1999; **131**: 174-181

16 **Kasahara A**, Hayashi N, Mochizuki K, Takayanagi M, Yoshioka K, Kakumu S, Iijima A, Urushihara A, Kiyosawa K, Okuda M, Hino K, Okita K. Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with chronic hepatitis C. Osaka Liver Disease Study Group. *Hepatology* 1998; **27**: 1394-1402

17 **Shiratori Y**, Imazeki F, Moriyama M, Yano M, Arakawa Y, Yokosuka O, Kuroki T, Nishiguchi S, Sata M, Yamada G, Fujiyama S, Yoshida H, Omata M. Histologic improvement of fibrosis in patients with hepatitis C who have sustained response to interferon therapy. *Ann Intern Med* 2000; **132**: 517-524

18 **Zeuzem S**, Hultcrantz R, Bourliere M, Goeser T, Marcellin P, Sanchez-Tapias J, Sarrazin C, Harvey J, Brass C, Albrecht J. Peginterferon alfa-2b plus ribavirin for treatment of chronic hepatitis C in previously untreated patients infected with HCV genotypes 2 or 3. *J Hepatol* 2004; **40**: 993-999

19 **Manns MP**, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965

20 **Tarao K**, Takemiya S, Tamai S, Sugimasa Y, Ohkawa S, Akaike M, Tanabe H, Shimizu A, Yoshida M, Kakita A. Relationship between the recurrence of hepatocellular carcinoma (HCC) and serum alanine aminotransferase levels in hepatectomized patients with hepatitis C virus-associated cirrhosis and HCC. *Cancer* 1997; **79**: 688-694

21 **Ikeda K**, Arase Y, Saitoh S, Kobayashi M, Suzuki Y, Suzuki F, Tsubota A, Chayama K, Murashima N, Kumada H. Interferon beta prevents recurrence of hepatocellular carcinoma after complete resection or ablation of the primary tumor-A prospective randomized study of hepatitis C virus-related liver cancer. *Hepatology* 2000; **32**: 228-232

22 **Kubo S**, Nishiguchi S, Hirohashi K, Tanaka H, Shuto T, Yamazaki O, Shiomi S, Tamori A, Oka H, Igawa S, Kuroki T, Kinoshita H. Effects of long-term postoperative interferon-alpha therapy on intrahepatic recurrence after resection of hepatitis C virus-related hepatocellular carcinoma. A randomized, controlled trial. *Ann Intern Med* 2001; **134**: 963-967

23 **Suou T**, Mitsuda A, Koda M, Matsuda H, Maruyama S, Tanaka H, Kishimoto Y, Kohno M, Hirooka Y, Kawasaki H. Interferon alpha inhibits intrahepatic recurrence in hepatocellular carcinoma with chronic hepatitis C: a pilot study. *Hepatol Res* 2001; **20**: 301-311

24 **Shiratori Y**, Shiina S, Teratani T, Imamura M, Obi S, Sato S, Koike Y, Yoshida H, Omata M. Interferon therapy after tumor ablation improves prognosis in patients with hepatocellular carcinoma associated with hepatitis C virus. *Ann Intern Med* 2003; **138**: 299-306

25 **Sakaguchi Y**, Kudo M, Fukunaga T, Minami Y, Chung H, Kawasaki T. Low-dose, long-term, intermittent interferon-alpha-2b therapy after radical treatment by radiofrequency ablation delays clinical recurrence in patients with hepatitis C virus-related hepatocellular carcinoma. *Intervirology* 2005; **48**: 64-70

26 **Pol S**, Nalpas B, Bourliere M, Couzigou P, Tran A, Abergel A, Zarski JP, Berthelot P, Brechot C. Combination of ribavirin and interferon-alfa surpasses high doses of interferon-alfa alone in patients with genotype-1b-related chronic hepatitis C. *Hepatology* 2000; **31**: 1338-1344

S- Editor Li DL **L- Editor** Alpini GD **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6145

World J Gastroenterol 2008 October 28; 14(40): 6145-6153
World Journal of Gastroenterology ISSN 1007-9327

Diallyl sulfide protects against *N*-nitrosodiethylamine-induced liver tumorigenesis: Role of aldose reductase

Safinaz S Ibrahim, Noha N Nassar

Safinaz S Ibrahim, Department of Biochemistry, Faculty of Pharmacy, Cairo University, Cairo 11562, Egypt
Noha N Nassar, Department of Pharmacology and Toxicology, Faculty of Pharmacy, Cairo University, Cairo 11562, Egypt
Author contributions: Both authors contributed equally to carrying out the design, execution of the experiments as well as writing up and revision of the manuscript.
Correspondence to: Noha N Nassar, PhD, Department of Pharmacology and Toxicology, Faculty of Pharmacy, Cairo University, Kasr El-Aini, Cairo 11562, Egypt. nnagah@gmail.com
Telephone: +20-22-3639307 Fax: +20-22-25320005
Received: June 21, 2008 Revised: September 20, 2008
Accepted: September 27, 2008
Published online: October 28, 2008

Abstract

AIM: To evaluate the protective effect of diallyl sulfide (DAS) against *N*-nitrosodiethylamine (NDEA)-induced liver carcinogenesis.

METHODS: Male Wistar rats received either NDEA or NDEA together with DAS as protection. Liver energy metabolism was assessed in terms of lactate, pyruvate, lactate/pyruvate, ATP levels, lactate dehydrogenase (LDH) and glucose-6-phosphate dehydrogenase (G6PD) activities. In addition, membrane disintegration of the liver cells was evaluated by measuring lipid-peroxidation products, measured as malondialdehyde (MDA); nitric oxide (NO) levels; glucose-6-phosphatase (G6Pase), catalase (CAT) and superoxide dismutase (SOD) activities. Liver DNA level, glutathione-S-transferase (GST) and cytochrome c oxidase activities were used as DNA fragmentation indices. Aldose reductase (AR) activity was measured as an index for cancer cells resistant to chemotherapy and histopathological examination was performed on liver sections from different groups.

RESULTS: NDEA significantly disturbed liver functions and most of the aforementioned indices. Treatment with DAS significantly restored liver functions and hepatocellular integrity; improved parameters of energy metabolism and suppressed free-radical generation.

CONCLUSION: We provide evidence that DAS exerts a protective role on liver functions and tissue integrity in face of enhanced tumorigenesis caused by NDEA, as well as improving cancer-cell sensitivity to chemotherapy. This is mediated through combating oxidative stress of free radicals, improving the energy metabolic state of the cell, and enhancing the activity of G6Pase, GST and AR enzymes.

Key words: *N*-nitrosodiethylamine; Diallyl sulfide; Liver cancer; Energy metabolism; Aldose reductase

Peer reviewer: Dr. Cintia Siqueira, Center of Gastroenterology, Institute of Molecular Medicine, Av. Prof. Egas Moniz, Lisboa 1649-028, Portugal

Ibrahim SS, Nassar NN. Diallyl sulfide protects against *N*-nitrosodiethylamine-induced liver tumorigenesis: Role of aldose reductase. *World J Gastroenterol* 2008; 14(40): 6145-6153 Available from: URL: http://www.wjgnet.com/1007-9327/14/6145.asp DOI: http://dx.doi.org/10.3748/wjg.14.6145

INTRODUCTION

Primary liver cancer has been classified as the fifth most common cause of cancer and the fourth most common cause of cancer mortality in the world. One of the main pathological subtypes of liver cancer is hepatocellular carcinoma, which constitutes a major contributor to cancer incidence and mortality[1]. The population of Egypt has a heavy burden of liver disease, mostly due to chronic infection with hepatitis C virus. Since the liver offers a very important site for detoxification of xenobiotics, the use of synthetic chemoprotective agents offers potential risk factors[2,3]. Several reports have stressed the importance of many dietary habits in modifying the initiation, promotion and progression stages in carcinogenesis[4]. Garlic (*Allium sativum*), an important flavoring agent, exhibits medicinal properties that include immunomodulatory, hepatoprotective, antioxidant, antimutagenic, and anticarcinogenic effects[5,6]. The anticarcinogenic property of garlic has been documented from both epidemiological and experimental studies which suggests that the consumption of garlic can decrease the incidence of several cancers[2,7,8]. The ability of garlic to reduce the incidence of cancer has been attributed to its content of organosulfur compounds which reportedly suppress carcinogen-induced tumors in various organs of animals including the colorectum, breast and liver[9-11].

A major constituent of garlic, diallyl sulfide (DAS), has been shown to inhibit chemical toxicity and tumorigenesis in several animal models[12]. Nonetheless, the possibility that DAS may exert a protective role against *N*-nitrosodiethylamine (NDEA)-induced liver tumorigenesis cannot be ruled out. In this study, we investigated the cellular and molecular mechanisms of the protective effects of DAS against liver damage induced by NDEA, a potent inducer of liver cancer. We determined the histopathological effect of DAS on liver tissue, as well as on enzymatic and non-enzymatic liver functions. In addition, we investigated the possibility that DAS might act on maintaining liver tissue functions, which were assessed by investigating energy metabolism, membrane disintegration and DNA integrity indices.

MATERIALS AND METHODS

Animals

We used a total of 36 male albino rats of the Wistar strain, weighing 170-200 g, that were obtained from the central animal facility at the Faculty of Pharmacy, Cairo University, Cairo, Egypt. All rats were housed in a room with a controlled environment, at a constant temperature of 23 ± 1℃, humidity of 60% ± 10%, and a 12 h light/dark cycle. The animals were housed in groups and kept at constant nutritional conditions throughout the experimental period. The experimental protocols were approved by the Ethical Committee of Cairo University.

Drugs and chemicals

NDEA, DAS, enzymes and coenzymes were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals were of Analar grade. NDEA was prepared as 8 mg/mL saline, whereas DAS was prepared as 80 mg/mL corn oil.

Induction of liver cancer

Each rat received an oral dose of 20 mg/kg per day NDEA for 5 d per week for 9 wk, followed by 10 mg/kg per day for 5 d per week for another 6 wk.

Protocols and experimental groups

Animals were divided into three groups: Group Ⅰ was the NDEA-induced cancer group, and Group Ⅱ was the DAS-treated group. Cancer was induced in this group by the same protocol. In addition, DAS was co-administered at a daily oral dose of 200 mg/kg per day for 5 d per week for the total period of the experiment (i.e. 15 wk). Group Ⅲ consisted of normal rats that received an oral dose of vehicles (saline, corn oil) for the total period of the experiment.

Biochemical estimations

Blood analysis: At the end of the experimental period, all animals were killed by cervical dislocation. The separated plasma was analyzed for total protein[13] and albumin[14]. The separated serum was analyzed for aspartate aminotransferase (AST), using a kit provided by Bicon, Germany[15]; alkaline phosphatase (ALP) using a kit provided by Biolabo, France[16,17], and gamma glutamyltransferase (GGT) using a kinetic photometric method[18].

Tissue analysis: The liver was removed, rinsed with ice-cold saline and blotted dry. Accurately weighed pieces of liver tissue were treated differently for the separation and estimation of the studied parameters.

Measurement of liver malondialdehyde (MDA) content: A 10% homogenate was prepared in 1.15% KCl, centrifuged at 1000 × *g* at 4℃ for 20 min, and the resultant supernatant was used for the assay of liver MDA content[19].

Measurement of liver cytochrome c oxidase activity and nitric oxide (NO) content: Liver tissue was homogenized in Tris-sucrose buffer, pH 7.4 (5% homogenate), using Potter-Elvejhem glass homogenizer, and centrifuged at 2000 × *g* at 4℃ for 10 min. The resultant supernatant was used for the estimation of cytochrome c oxidase activity[20] and NO content[21].

Measurement of liver glutathione-S-transferase (GST), lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G6PD), superoxide dismutase (SOD) and catalase (CAT) activities: A 10% homogenate was obtained in Tris-sucrose buffer, pH 7.4, and centrifuged at 105 000 × *g* at 4℃ for 30 min, using a Dupont Sorvall Ultracentrifuge (USA), to isolate the cytosolic fraction which was used for the assay of GST[22], SOD[23], LDH[24], G6PD[25] and CAT[26] activities.

Estimation of liver pyruvate and lactate concentrations: Liver tissue was homogenized in 5% meta-phosphoric acid, and centrifuged at 3000 × *g* at 4℃ for 15 min. The resultant supernatant was used for the estimation of pyruvate and lactate concentrations according to the method of Mohun and Cook[27] and David[28], respectively.

Estimation of liver ATP content: Liver tissue was homogenized with 3 mL ice-cold 3 mol/L perchloric acid, using Potter-Elvejhem glass homogenizer. Following that, 12.5 mL of 1 mmol/L EDTA was added and the mixture was centrifuged at 1000 × *g* at 4℃ for 1 h. The supernatant was further treated for the estimation of ATP[29].

Determination of liver glucose-6-phosphatase (G6Pase) activity: Liver tissue was homogenized in ice-cold solution containing 0.15mol/L KCl; 4 mmol/L $MgSO_4$; 4 mmol/L EDTA and 4 mmol/L N-acetylcysteine, pH 7, and centrifuged at 12000 × *g* at 4℃ for 10 min. The resultant supernatant was analyzed for G6Pase activity[30].

Estimation of liver DNA content: Liver tissue was homogenized in 0.25 mol/L sucrose/in TKM buffer (0.05 mol/L Tris-HCl, 0.025 mol/L KCl, 0.005 mol/L

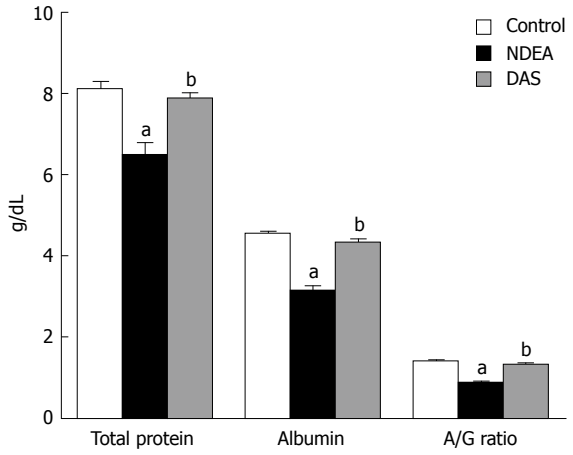


Figure 1 Non-enzymatic liver functions in rats treated with NDEA in absence or presence of DAS (mean ± SE) differed significantly compared to the control and NDEA-treated group ($^aP = 0.045$, $^bP = 0.042$, respectively).

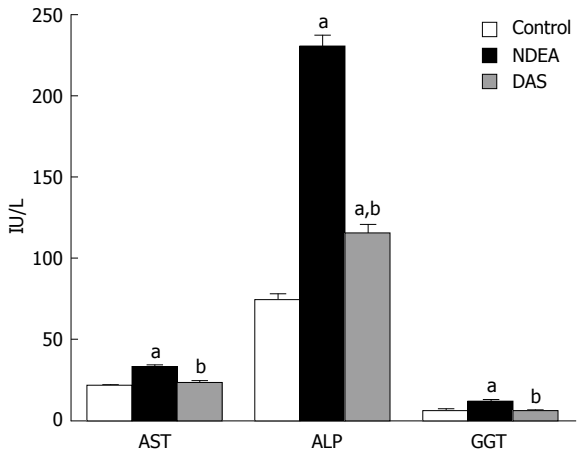


Figure 2 Enzymatic liver functions in rats treated with NDEA in absence or presence of DAS (mean ± SE) differed significantly compared to the control and NDEA-treated group ($^aP = 0.048$, $^bP = 0.044$, respectively).

$MgCl_2$), pH 7.5, to prepare a 15% homogenate. Then 0.1 mL of 0.3 mol/L perchloric acid was added, left to stand at 0℃ for 15 min, centrifuged at 2000 × *g* at 4℃ for 10 min, and the precipitate was used for the estimation of DNA content[31].

Determination of liver aldose reductase (AR) activity: Liver tissue was homogenized in potassium phosphate buffer, pH 7 (20% homogenate), centrifuged at 105000 × *g* for 45 min at 4℃, and the resultant supernatant was used for the estimation of AR activity[32].

Determination of protein concentrations: Protein concentrations of the above supernatants were estimated by the method of Lowry *et al*[33].

Histopathological examination

The portions of liver tissue embedded in paraffin were sectioned at 5 μm. Following sectioning, liver tissue was stained with hematoxylin and eosin. Light microscopy was used to evaluate the pathological changes in liver tissue.

Statistical analysis

The values were expressed as mean ± SE. Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Kruskal-Wallis comparison test. $P \leqslant 0.05$ was considered significant.

RESULTS

The results showed that there were no differences between the various control groups (vehicle-treated groups). Thus, the data from all of the control animals were po-oled and are shown as one normal group.

Effect of NDEA in absence or presence of DAS on non-enzymatic liver functions

NDEA significantly decreased total protein, albumin, and A/G ratio. Co-administration of DAS restored total protein, albumin and A/G ratio (Figure 1). Moreover, NDEA significantly increased total bilirubin (2.43 ± 0.151 mg/dL), compared to their control counterparts (1.19 ± 0.090 mg/dL). DAS significantly decreased total bilirubin (1.68 ± 0.078 mg/dL, $P = 0.045$) when compared to the NDEA-treated group value.

Effect of NDEA in absence or presence of DAS on enzymatic liver functions

NDEA significantly elevated serum AST, ALP and GGT activities when compared to the normal group. DAS co-administration restored serum AST and GGT activities, together with a significant decrease in ALP activity, compared to the NDEA group value ($P = 0.048$) (Figure 2).

Changes in liver energy metabolism indices following NDEA in absence or presence of DAS

Table 1 showed that NDEA significantly decreased ATP level and increased G6PD activity compared to normal group values ($P = 0.046$). Treatment with DAS resulted in a significant increase in ATP level compared to the NDEA-treated group value. In addition, DAS significantly elevated and reduced pyruvate and lactate levels respectively, with a consequent significant reduction in lactate/pyruvate ratio compared to both the NDEA and control counterparts. Also, DAS significantly increased LDH and G6PD activities compared to either NDEA or control groups.

Changes in oxidative stress and membrane disintegration indices following NDEA in absence or presence of DAS

As shown in Table 2, NDEA treatment significantly ($P = 0.048$) elevated MDA and NO contents and reduced G6Pase, CAT and SOD activities when compared to control counterparts. Administration of DAS restored MDA level and G6Pase activity, significantly decreased NO level, and non-significantly changed SOD and CAT activities when compared to NDEA-treated rats.

Table 1 Effect of NDEA in absence or presence of DAS on liver energy metabolism indices (mean ± SE)

Parameters	Normal	NDEA	DAS
Lactate (μmol/g liver)	2.27 ± 0.16	2.08 ± 0.094	1.41 ± 0.08[a,b]
Pyruvate (μmol/g liver)	0.11 ± 0.007	0.103 ± 0.008	0.15 ± 0.007[a,b]
Lac/Pyr	20.6 ± 1.65	17.37 ± 0.9	11.31 ± 0.76[a,b]
LDH (μmol/mg protein per min)	0.9 ± 0.021	0.9 ± 0.043	1.05 ± 0.028[a,b]
ATP (μmol/g liver)	5.69 ± 0.32	4.04 ± 0.12[a]	4.91 ± 0.085[a,b]
G6PD (μmol/mg protein per min)	27.9 ± 2.29	47.4 ± 1.48[a]	54.1 ± 4.04[a,b]

Significantly different from baseline values at [a]P = 0.046; Significantly different from NDEA treatment at [b]P = 0.043.

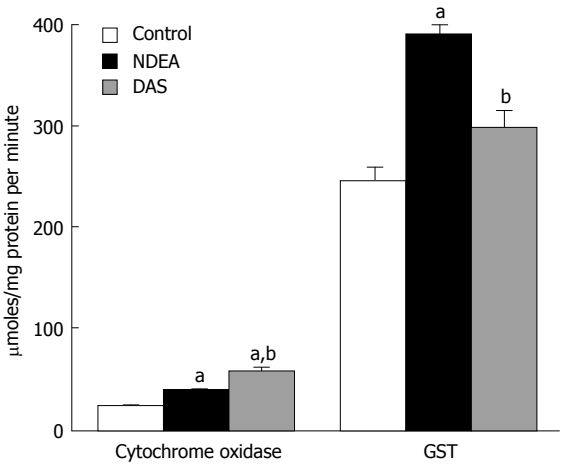


Figure 3 Changes in cytochrome oxidase and GST activities in rats treated with NDEA in absence or presence of DAS (mean ± SE) differed significantly compared to the control and NDEA-treated group values ([a]*P* = 0.043, [b]*P* = 0.045, respectively).

Effect of NDEA in absence or presence of DAS on DNA fragmentation indices

NDEA significantly elevated DNA level (3.97 ± 0.189 mg/gt) compared to control level (2.5 ± 0.199 mg/gt) (P = 0.043). DAS normalized DNA levels (2.38 ± 0.08 mg/gt). NDEA significantly elevated cytochrome c oxidase and GST activities. DAS significantly lowered GST , which approached the normal values. However, DAS significantly enhanced cytochrome c oxidase activity when compared to either control or NDEA-treated group levels (Figure 3).

Effect of NDEA in absence or presence of DAS on AR activity

NDEA significantly elevated AR activity (P = 0.045). Co-administration of DAS restored such enzymatic activity (Figure 4).

Histopathological findings

Examination of liver sections of the different groups illustrated that: Liver tissue of the normal group showed hepatic lobules with normal architecture (Figure 5A). Liver tissue of the NDEA-treated rats showed pleomorphism. Some cells exhibited multiple nucleoli, some of the cells were pyknotic, while others showed

Table 2 Effect of NDEA in absence or presence of DAS on oxidative stress and membrane disintegration indices (mean ± SE)

Parameters	Normal	NDEA	DAS
MDA (nmol/g liver)	55.6 ± 3.4	90.4 ± 8.01[a]	66.8 ± 2.4[b]
NO (nmol/g liver)	139 ± 9.7	344 ± 14.9[a]	187 ± 9.2[a,b]
G6Pase (nmol/mg protein/min)	6.39 ± 0.46	3.38 ± 0.26[a]	7.36 ± 0.3[b]
CAT (IU/mg protein)	173 ± 9.1	141 ± 6.44[a]	138 ± 6.64[a]
SOD (IU/mg protein)	88.3 ± 6.55	55.7 ± 2.7[a]	57.8 ± 5.38[a]

Significantly different from baseline values at [a]P = 0.048; Significantly different from NDEA treatment at [b]P = 0.044.

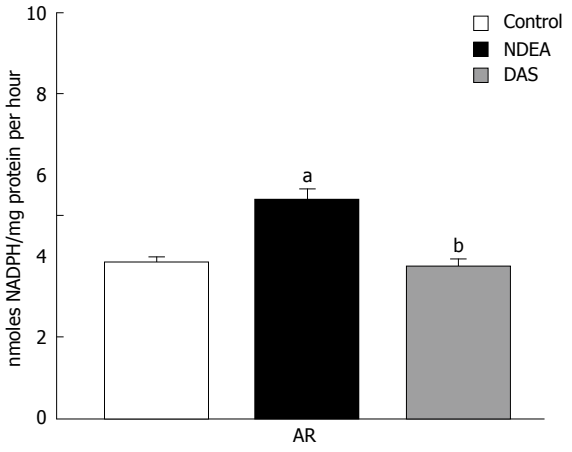


Figure 4 Changes in AR in rats treated with NDEA in absence or presence of DAS (mean ± SE) differed significantly compared to the control and NDEA-treated group values ([a]*P* = 0.045, [b]*P* = 0.046, respectively).

intranuclear vacuoles and cellular infiltration (Figure 5B). Other sections showed massive areas of vacuolated hepatocytes, cellular infiltration, and some cells possessed pyknotic nuclei (Figure 5C). Other section showed hyperchromatic nuclei and numerous Kupffer cells (Figure 5D). In other sections, hyperchromatic malignant nuclei were evident (Figure 5E). Liver tissue from the DAS-treated rats showed fewer degenerative changes, such as vacuolated cytoplasm, few pyknotic nuclei and dilated sinusoids (Figure 5F). Other section showed more or less normal hepatic lobular architecture (Figure 5G).

DISCUSSION

The liver is a multifunctional organ that plays essential roles in metabolism, biosynthesis, excretion, secretion and detoxification. These processes require energy, making the liver a highly aerobic, oxygen-dependent tissue. These processes also cause vulnerability of the liver to anoxia, increased susceptibility to noxious insults, and create a demand for cell replacement after tissue loss. Enhanced liver cell death and impaired regeneration are indeed features of most liver disorders. Proteins play a big role in fighting off infections and building or repairing muscle tissue. Low albumin is a sign of poor health and a predictor of a bad outcome. Thus, a

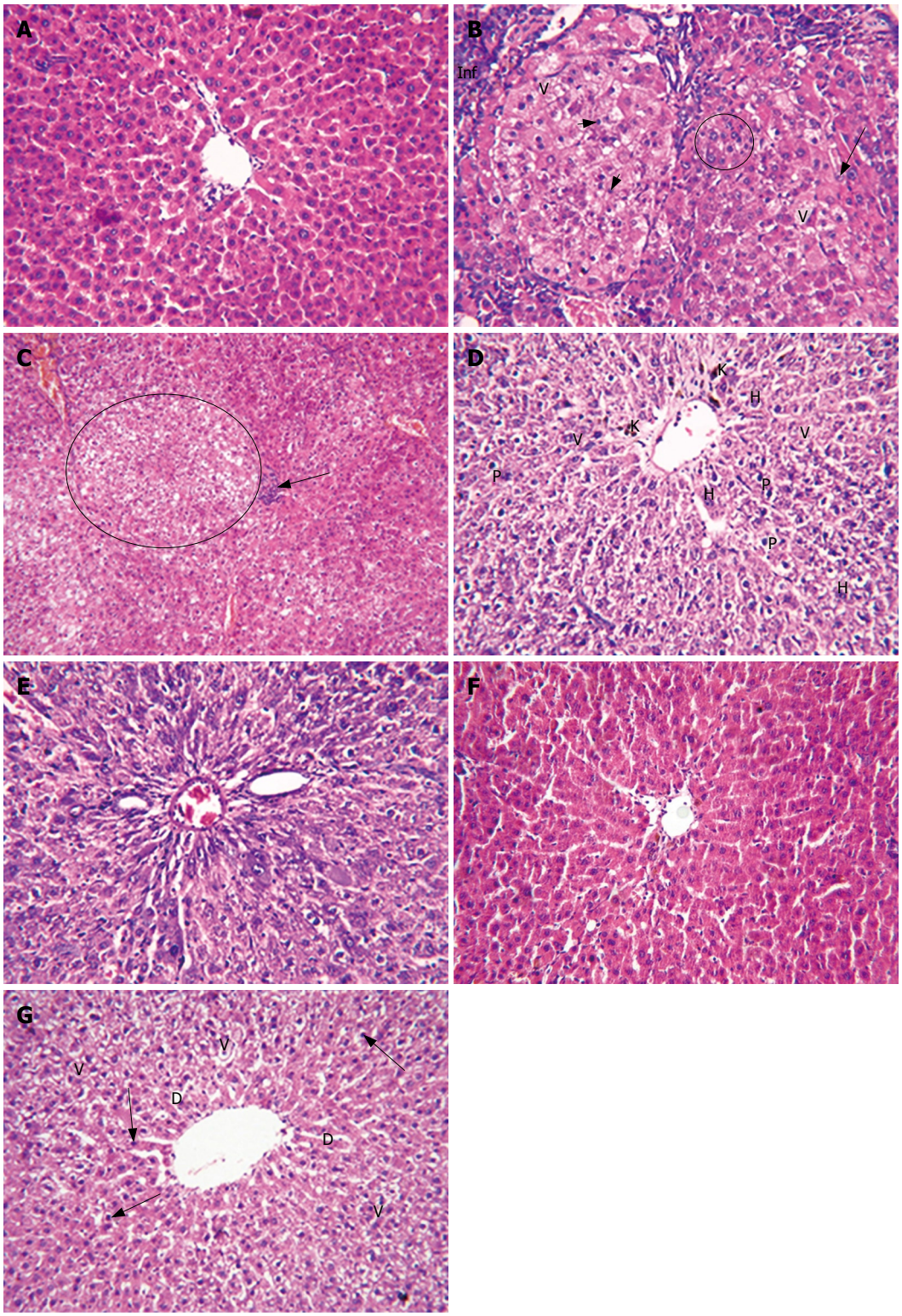


Figure 5 Liver sections of the different groups. A: Liver tissue of the normal group (control) showed hepatic lobule having normal architecture; B: Liver tissue of the NDEA-treated rats showed nuclear pleomorphism; some cells exhibited multiple nucleoli (encircled), pyknotic cells (short arrows), intranuclear vacuoles (arrow), some showed cytoplasmic vacuoles (V) and cellular infiltration (Inf); C: Other sections showed massive areas of vacuolated hepatocytes (encircled); cellular infiltration (arrow) and some cells possessed pyknotic nuclei; D: Other slides showed vacuolated cytoplasm, hyperchromatic nuclei, pyknotic nuclei and numerous Kupffer cells; E: Other section showed hyperchromatic malignant nuclei (H); F: Liver tissue of the DAS-treated rats showed some degenerative changes, vacuolated cytoplasm (V), few pyknotic nuclei (arrows) and dilated sinusoids (D); G: Other slides showed more or less normal hepatic lobular architecture.

decrease in the A/G ratio often indicates the presence of impaired liver function. As shown in our results, NDEA decreased significantly total protein, albumin and A/G levels, which was indicative of poor liver function and inability to fight infections. On the contrary, DAS administration normalized total protein, albumin and A/G levels, indicating the ability of DAS to improve liver function in face of NDEA-induced liver damage.

Serum AST, ALP and GGT are sensitive indicators of hepatic injury. Several reports have shown an increase in the activities of AST and ALT during NDEA-induced hepatocarcinogenesis[34]. Elevated activities of serum AST, ALP and GGT observed in NDEA-treated rats may be due to the NDEA-induced hepatic damage and the subsequent leakage of these enzymes into the circulation. Administration of DAS restored the activities of these enzymes to near normal values, which may be an indication of the hepatoprotective role of DAS.

As shown in our results, NDEA produced a significant decrease in hepatic ATP level. Oncotic necrosis is most often the consequence of metabolic injury, leading to ATP depletion. ATP depletion in hepatocytes is associated with ATP-depletion-dependent cytoskeletal alterations, after which a metastable state develops, characterized by mitochondrial depolarization and lysosomal breakdown. This metastable state culminates in outright rupture of plasma membrane, irreversible breakdown of the plasma membrane permeability barrier, and leakage of cytosolic enzymes and metabolic intermediates[35]. Treatment with DAS resulted in a significant decrease in lactate and lactate/pyruvate ratio, along with significant elevation of pyruvate, ATP levels and liver LDH activity, compared to NDEA-treated rats. The increased activity of LDH could favor pyruvate (aerobic carbohydrate metabolism) against lactate (anaerobic), thus enhancing energy metabolism in the cell and reflecting restoration of normal cellular/metabolic function. The histopathological findings observed in this study support the biochemical ones that liver tissue of the NDEA-treated rats showed drastic changes in the morphology of the liver cells, whereas the DAS-treated rats showed more or less normal hepatic lobular architecture. Accordingly, we presented evidence that DAS substantially improved the liver cell metabolic indices, as well as its synthetic capacity, and further protected against its malignant transformation.

The present data revealed that NDEA produced significant increases in the activity of liver G6PD, which agrees with findings from other studies[36,37]. G6PD is a housekeeping enzyme that produces riboses, which are incorporated into nucleotides and nucleic acids, and NADPH, the major cytoplasmic reducing compound[38]. NADPH is necessary for reduction of oxidized glutathione by glutathione reductase[39], and is a substrate for phase I and Ⅱ biotransformation and detoxification enzymes[36]. G6PD is elevated in response to external stimuli, toxic and oxidative stress[40,41]. G6PD activity is strongly upregulated in proliferating cells such as malignant cells[42]. There is increasing evidence that G6PD activity is of major importance for NADPH production for defense against oxidative stress, rather than for ribose production during proliferation[43]. Interestingly, treatment with DAS resulted in further elevation in the activity of G6PD. This result provides new evidence that DAS might exhibit a compensatory mechanism in enhancing the production of NADPH as a further defense mechanism against proliferating cancer cells, as well as for enhancing cellular antioxidant capacity.

In hepatocellular carcinoma, there is disequilibrium between oxidant and antioxidant balance, which is tilted towards the oxidant side[44]. Reactive oxygen species (ROS) are believed to cause genetic oxidation and damage to DNA and other macromolecules. Unchecked, this oxidative damage may lead to a host of conditions including cancer. Normally, this process is held in check by elaborate endogenous or exogenous antioxidant processes. Various enzymatic and non-enzymatic systems have been developed by the cell to cope with ROS and other free radicals[45]. Since many of the anomalies that are induced by NDEA can arise from oxidative stress, which is also known to accompany cancer development, it was of a prime interest to evaluate oxidative stress levels under those circumstances. As shown in the present study, NDEA produced a significant increase in hepatic MDA and NO levels, along with significant decreases in SOD and CAT activities. MDA was one of the main lipid peroxidation products; its elevated levels can reflect the degree of lipid-peroxidation-induced injury in hepatocytes[46]. On the other hand, it has been reported that SOD and CAT constitute a mutually supportive defense against ROS[47]. The decreased activity of SOD in liver of NDEA-treated rats may have been due to the enhanced lipid peroxidation or inactivation of the antioxidative enzymes. This may have caused an increased accumulation of superoxide radicals, which could have further stimulated lipid peroxidation[48]. Decreased activities of SOD and CAT in NDEA-treated rats, which is in agreement with other reported studies[49], could have been due to over-utilization of these enzymatic antioxidants to scavenge the products of lipid peroxidation. Tumor cells have been reported to sequester essential antioxidants from the circulation in order to meet the demands of the growing tumor cells. On the other hand, the current data demonstrated the ability of DAS to reduce formation of ROS and reactive nitrogen species, measured as MDA and NO, which agrees with findings from other laboratories[7]. These findings conform to previous results on the established, specific antioxidant profile for DAS as an inhibitor of the hepatic ROS generating enzyme CYP2E1. The latter enzyme is known as a prominent trigger of hepatic oxidative stress[50]. Treatment with DAS showed no significant enhancement of the activity of the endogenous antioxidant enzyme SOD or CAT.

G6Pase plays a critical role in blood glucose homoeostasis and its activity can also be considered as an index of the stability of the microsomal membrane[51]. Decreased activity of liver G6Pase was shown in the NDEA-treated rats, which might be attributed to the increased lipid peroxidation caused by NDEA. Consistent with previous studies[52], DAS administration enhanced G6Pase activity significantly, compared to the NDEA-treated group value, suggesting the ability of DAS to preserve membrane integrity.

In this study, an increased activity of liver GST was observed in NDEA-treated rats, with respect to their control counterparts. In addition, we also showed an increased activity of serum GGT in the NDEA-treated

rats, which might have been responsible for the increased level of GST in this group of animals[44]. Initial reports from nitrogen-mustard-resistant cell lines have shown these cells to over-express GST, which also holds true for a number of tumors. The increased level of GST is likely to be the key mediator of drug resistance in cancer chemotherapy[44]. Restoration of GST activity was observed with DAS treatment, suggesting a preservation of the redox system, which reflects a decrease in free-radical production, as well as improving cancer cell sensitivity to chemotherapy.

Cancer is well known to induce uncontrolled cellular proliferation. In this context, our results demonstrated that NDEA increased total DNA level, suggesting enhanced cellular proliferation. Notably, treatment with DAS significantly reduced DNA levels to near normal values, suggesting interference with mitotic pathways and enhancing apoptosis of cancer cells[53]. In addition, the current results showed an enhanced activity of cytochrome c oxidase enzyme in the NDEA-treated rats, which was further enhanced by the co-administration of DAS. However, for this investigation, we measured total cytochrome c oxidase, so it was difficult to delineate whether the increase we observed in the levels of cytochrome c oxidase was attributed to the mitochondrial or cytosolic fraction. According to the observed elevation in hepatic MDA and NO contents in the NDEA-treated group, a state of oxidative stress can exist in such animals, which contributes to mitochondrial membrane leakage and in turn, allows the translocation of cytochrome c oxidase to the cytosolic fractions. Thus, we suggest that the increase in cytochrome c oxidase with NDEA treatment might be of cytosolic origin. Interestingly, we demonstrated, and to the best of our knowledge, for the first time, that DAS markedly enhanced the activity of cytochrome c oxidase. We suggest that such increased cytochrome c oxidase activity might be attributed to a mitochondrial rather than cytosolic origin, which is supported by the observed increase in ATP and decrease in oxidative-stress biomarkers shown in DAS-treated animals. DAS might induce direct perturbation of mitochondria, resulting in apoptotic damage of the cancer cells. This effect has been reported recently with some anticancer agents[54,55].

Our results showed an almost 1.5-fold increase in AR activity in NDEA-treated animals. AR belongs to the aldo-keto reductase (AKR) superfamily. Most of the AKR superfamily proteins are involved in the detoxification of a wide variety of substrates. Several reports have shown that over-expression of AR, in many tumor cells, renders these cells resistant to chemotherapy, and also demonstrate that inhibition of AR enhances cancer cell sensitivity to chemotherapeutic drugs[56,57]. In addition, over-expression of AR enhances production of ROS, which cause membrane damage and cellular leakage[58]. To the best of our knowledge, the present study is the first to show enhanced production of AR in an *in vivo* model of liver tumorigenesis. To our knowledge, this is the first report that identifies the ability of DAS to reduce the expression of AR in NDEA-treated rats. This provides new evidence for its very important potential role in cancer protection. The ability of DAS to reduce the expression of AR suggests that DAS is effective against *in vivo* tumorigenesis by suppressing AR production and subsequently lowering the production of ROS, as well as enhancing cancer cell sensitivity to chemotherapeutic drugs.

Our findings were further supported by the histopathological examination of liver sections, which illustrated that liver tissue of NDEA-treated rats showed damage, manifest as nuclear pleomorphism, intranuclear vacuoles, cellular infiltration, hyperchromatic nuclei, pyknotic nuclei, numerous Kupffer cells and hyperchromatic malignant nuclei. On the contrary, liver tissue of the DAS-treated rats showed more or less normal hepatic lobular architecture.

To conclude, we provide evidence that DAS exerts a protective role on liver tissue in face of enhanced tumorigenesis caused by NDEA, as demonstrated by the following points: (1) DAS could normalize almost all the non-enzymatic and enzymatic liver function tests, indicating its ability to improve liver functions; (2) DAS significantly decreased lactate and lactate/pyruvate ratio, along with elevating pyruvate, ATP levels and liver LDH activity, thus enhancing energy metabolism in the liver tissue and reflecting restoration of normal cellular/metabolic functions; (3) DAS elevated the activity of G6PD, so it might exhibit a compensatory mechanism in enhancing the production of NADPH as a further defense mechanism against proliferating cancer cells, as well as enhancing cellular antioxidant capacity; (4) DAS reduced the formation of free radicals, measured as MDA and NO, providing specific antioxidant profiles for DAS as an inhibitor of the hepatic ROS-generating enzyme; (5) DAS enhanced G6Pase activity significantly, suggesting its ability to preserve liver cell membrane integrity; (6) DAS restored GST activity, suggesting a preservation of the redox system, as well as improving cancer cell sensitivity to chemotherapy; (7) DAS significantly reduced DNA level comparable to that in the NDEA-treated group, and close to the normal value, suggesting interference with mitotic pathways and enhancing apoptosis of cancer cells; (8) DAS markedly enhanced cytochrome c oxidase activity, thus, DAS might induce direct perturbation of mitochondria, resulting in apoptotic damage of the cancer cells; (9) to the best of our knowledge, this is the first report that identifies the ability of DAS to reduce the expression of liver AR in NDEA-treated rats, which suggests a very important potential role in cancer protection and subsequently lowering the production of ROS, as well as enhancing cancer cell sensitivity to chemotherapeutic drugs; (10) our biochemical findings were further supported by the histopathological examination of liver sections, which illustrated that liver tissue of the NDEA-treated rats showed damage, resulting in malignant cell formation. On the contrary, liver tissue of the DAS-treated rats showed more or less normal hepatic lobular architecture.

ACKNOWLEDGMENTS

The authors thank Dr. Sohair Assad, Professor of Histology, Faculty of Medicine, Cairo University, Cairo, Egypt, for her valuable assistance with the staining and the interpretation of the histological slides.

COMMENTS

Background

Diallyl sulfide (DAS), a biologically active garlic constituent, has been demonstrated as a potential cytoprotective agent in many animal models. Garlic (*Allium sativum*), an important flavoring agent, exhibits medicinal properties that include immunomodulatory, hepatoprotective, antioxidant, antimutagenic and anticarcinogenic effects.

Research frontiers

Oncotic necrosis is most often the consequence of metabolic injury, leading to ATP depletion, culminating in leakage of cytosolic enzymes and metabolic intermediates. In hepatocellular carcinoma, disequilibrium exists between oxidant and antioxidant balance, which is tilted towards oxidants. Tumor cells sequester essential antioxidants from the circulation to meet the demands of the growing tumor cells. We showed that DAS reduced formation of ROS, which agrees with reported findings. Aldose reductase (AR) belongs to the aldo-keto reductase (AKR) superfamily involved in the detoxification processes of a wide variety of substrates. Over-expression of AR, in many tumor cells, renders these cells resistant to chemotherapy.

Innovations and breakthroughs

DAS protects against *N*-nitrosodiethylamine (NDEA)-induced liver cancer. DAS markedly enhanced, cytochrome c oxidase activity, thus inducing direct perturbation of mitochondria, culminating in apoptotic damage of the cancer cells. The expression of liver AR in NDEA-treated rats was reduced by DAS, subsequently lowering the production of ROS and enhancing cancer cell sensitivity to chemotherapeutic drugs. Histopathological examination illustrated that liver tissue of the NDEA-treated rats showed malignant cell formation, which was prevented by DAS.

Applications

The population of Egypt has a heavy burden of liver disease and the use of synthetic chemoprotective agents has potential risks in this population. Dietary habits may modify carcinogenesis initiation, promotion and progression. Hence, this study indicates the potential protective effect of DAS against NDEA-induced liver cancer. This could be used as a protective method to prevent exacerbation of cancer in developed countries that cannot afford the burden of expensive chemotherapy.

Terminology

DAS: Diallyl sulfide (a major constituent of garlic); NDEA: *N*-nitrosodiethylamine (inducer of liver cancer); LDH: Lactate dehydrogenase; G6PD: Glucose-6-phosphate dehydrogenase; MDA: Malondialdehyde; NO: Nitric oxide; G6Pase: Glucose-6-phosphatase; CAT: Catalase; SOD: Superoxide dismutase; GST: Glutathione-S-transferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transferase; AR: Aldose reductase.

Peer review

The authors demonstrated in this study that DAS had anti-oxidant properties in NDEA-treated rats. This work is very interesting. The authors bring some novelty and innovation to their research. The references are appropriate and updated.

REFERENCES

1 **McGlynn KA**, Tsao L, Hsing AW, Devesa SS, Fraumeni JF Jr. International trends and patterns of primary liver cancer. *Int J Cancer* 2001; **94**: 290-296
2 **D'Alessandro N**, Poma P, Montalto G. Multifactorial nature of hepatocellular carcinoma drug resistance: could plant polyphenols be helpful? *World J Gastroenterol* 2007; **13**: 2037-2043
3 **Steiner M**, Seule M, Steiner B, Bauer I, Freund M, Kohne CH, Schuff-Werner P. 5-Fluorouracil/irinotecan induced lethal toxicity as a result of a combined pharmacogenetic syndrome: report of a case. *J Clin Pathol* 2005; **58**: 553-555
4 **Schorah CJ**. Micronutrients, vitamins, and cancer risk. *Vitam Horm* 1999; **57**: 1-23
5 **Singh SV**, Mohan RR, Agarwal R, Benson PJ, Hu X, Rudy MA, Xia H, Katoh A, Srivastava SK, Mukhtar H, Gupta V, Zaren HA. Novel anti-carcinogenic activity of an organosulfide from garlic: inhibition of H-RAS oncogene transformed tumor growth in vivo by diallyl disulfide is associated with inhibition of p21H-ras processing. *Biochem Biophys Res Commun* 1996; **225**: 660-665
6 **Agarwal KC**. Therapeutic actions of garlic constituents. *Med Res Rev* 1996; **16**: 111-124
7 **Aquilano K**, Filomeni G, Baldelli S, Piccirillo S, De Martino A, Rotilio G, Ciriolo MR. Neuronal nitric oxide synthase protects neuroblastoma cells from oxidative stress mediated by garlic derivatives. *J Neurochem* 2007; **101**: 1327-1337
8 **Shukla Y**, Kalra N. Cancer chemoprevention with garlic and its constituents. *Cancer Lett* 2007; **247**: 167-181
9 **Jakubikova J**, Sedlak J. Garlic-derived organosulfides induce cytotoxicity, apoptosis, cell cycle arrest and oxidative stress in human colon carcinoma cell lines. *Neoplasma* 2006; **53**: 191-199
10 **Arunkumar A**, Vijayababu MR, Venkataraman P, Senthilkumar K, Arunakaran J. Chemoprevention of rat prostate carcinogenesis by diallyl disulfide, an organosulfur compound of garlic. *Biol Pharm* Bull 2006; **29**: 375-379
11 **El-Bayoumy K**, Sinha R, Pinto JT, Rivlin RS. Cancer chemoprevention by garlic and garlic-containing sulfur and selenium compounds. *J Nutr* 2006; **136**: 864S-869S
12 **Herman-Antosiewicz A**, Singh SV. Signal transduction pathways leading to cell cycle arrest and apoptosis induction in cancer cells by Allium vegetable-derived organosulfur compounds: a review. *Mutat Res* 2004; **555**: 121-131
13 **Weichselbaum TE**, Varney PL. A new method of flame photometry. *Proc Soc Exp Biol Med* 1949; **71**: 570-572
14 **Doumas BT**, Watson WA, Biggs HG. Albumin standards and the measurement of serum albumin with bromcresol green. *Clin Chim Acta* 1971; **31**: 87-96
15 **Reitman S**, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *Am J Clin Pathol* 1957; **28**: 56-63
16 **Kind PR**, King EJ. Estimation of plasma phosphatase by determination of hydrolysed phenol with amino-antipyrine. *J Clin Pathol* 1954; **7**: 322-326
17 **Belfield A**, Goldberg DM. Colorimetric determination of alkaline phosphatase activity. *Enzyme* 1971; **12**: 561-568
18 **Szasz G**. A kinetic photometric method for serum gamma-glutamyl transpeptidase. *Clin Chem* 1969; **15**: 124-136
19 **Yoshioka T**, Kawada K, Shimada T, Mori M. Lipid peroxidation in maternal and cord blood and protective mechanism against activated-oxygen toxicity in the blood. *Am J Obstet Gynecol* 1979; **135**: 372-376
20 **Smith L**. Spectrophotometric assay of cytochrome c oxidase. In Methods of Biochemical Analysis. In: Glick D ed. New York: John Wiley & Sons, 1955: 427-434
21 **Schmidt HH**, Warner TD, Nakane M, Forstermann U, Murad F. Regulation and subcellular location of nitrogen oxide synthases in RAW264.7 macrophages. *Mol Pharmacol* 1992; **41**: 615-624
22 **Habig WH**, Pabst MJ, Jakoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* 1974; **249**: 7130-7139
23 **Marklund S**, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur J Biochem* 1974; **47**: 469-474
24 **Buhl SN**, Jackson KY. Optimal conditions and comparison of lactate dehydrogenase catalysis of the lactate-to-pyruvate and pyruvate-to-lactate reactions in human serum at 25, 30, and 37 degrees C. *Clin Chem* 1978; **24**: 828-831

25 **Sie HG**, Hablanian A, Fishman WH. Divergent effects of actinomycin d on cortisol and on glucose stimulation of glycogenesis in mouse liver. *Biochem J* 1967; **102**: 103-109
26 **Maral J**, Puget K, Michelson AM. Comparative study of superoxide dismutase, catalase and glutathione peroxidase levels in erythrocytes of different animals. *Biochem Biophys Res Commun* 1977; **77**: 1525-1535
27 **Mohun AF**, Cook IJ. Simple methods for measuring serum levels of the glutamic-oxalacetic and glutamic-pyruvic transaminases in routine laboratories. *J Clin Pathol* 1957; **10**: 394-399
28 **David BS**. Carbohydrate. In: Burtis CA, Ashwood ER, eds. Tietz textbook of clinical chemistry, 3rd ed. Philadelphia, Pennsylvania: W.B. Saunders Company, 1999: 788
29 **Lowry OH**, Passommeau JV, Hasselberger FX, Schulz DW. Effect of Ischemia on Known Substrates and Cofactors of the Glycolytic Pathway in Brain. *J Biol Chem* 1964; **239**: 18-30
30 **Harper AE**. Hormonal factors affecting glucose 6-phosphatase activity. 2. Some effects of diet and of alloxan diabetes in the rat. *Biochem J* 1959; **71**: 702-705
31 **Giles KW**, Myers A. An imporoved diphenylamine method for the estimation of deoxyribonucleic acid. *Nature* (Lond) 1965; **206**: 93
32 **Chauncey B**, Leite MV, Goldstein L. Renal sorbitol accumulation and associated enzyme activities in diabetes. *Enzyme* 1988; **39**: 231-234
33 **Lowry OH**, Rosebrough NJ, Farr AL, Ranall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193**: 265-275
34 **Dakshayani KB**, Subramanian P, Manivasagam T, Essa MM, Manoharan S. Melatonin modulates the oxidant-antioxidant imbalance during N-nitrosodiethylamine induced hepatocarcinogenesis in rats. *J Pharm Pharm Sci* 2005; **8**: 316-321
35 **Malhi H**, Gores GJ, Lemasters JJ. Apoptosis and necrosis in the liver: a tale of two deaths? *Hepatology* 2006; **43**: S31-S44
36 **Winzer K**, Van Noorden CJ, Kohler A. Glucose-6-phosphate dehydrogenase: the key to sex-related xenobiotic toxicity in hepatocytes of European flounder (Platichthys flesus L.)? *Aquat Toxicol* 2002; **56**: 275-288
37 **Frederiks WM**, Bosch KS, De Jong JS, Van Noorden CJ. Post-translational regulation of glucose-6-phosphate dehydrogenase activity in (pre)neoplastic lesions in rat liver. *J Histochem Cytochem* 2003; **51**: 105-112
38 **Kletzien RF**, Harris PK, Foellmi LA. Glucose-6-phosphate dehydrogenase: a "housekeeping" enzyme subject to tissue-specific regulation by hormones, nutrients, and oxidant stress. *FASEB J* 1994; **8**: 174-181
39 **Ninfali P**, Biagiotti E, Guidi L, Malatesta M, Gazzanelli G, Del Grande P. Cytochemical and immunocytochemical methods for electron microscopic detection of glucose-6-phosphate dehydrogenase in brain areas. *Brain Res Brain Res Protoc* 2000; **5**: 115-120
40 **Spolarics Z**. Endotoxemia, pentose cycle, and the oxidant/antioxidant balance in the hepatic sinusoid. *J Leukoc Biol* 1998; **63**: 534-541
41 **Salati LM**, Amir-Ahmady B. Dietary regulation of expression of glucose-6-phosphate dehydrogenase. *Annu Rev Nutr* 2001; **21**: 121-140
42 **Kuo W**, Lin J, Tang TK. Human glucose-6-phosphate dehydrogenase (G6PD) gene transforms NIH 3T3 cells and induces tumors in nude mice. *Int J Cancer* 2000; **85**: 857-864
43 **Biagiotti E**, Bosch KS, Ninfali P, Frederiks WM, Van Noorden CJ. Posttranslational regulation of glucose-6-phosphate dehydrogenase activity in tongue epithelium. *J Histochem Cytochem* 2000; **48**: 971-977
44 **Rajkapoor B**, Murugesh N, Chodon D, Sakthisekaran D. Chemoprevention of N-nitrosodiethylamine induced phenobarbitol promoted liver tumors in rat by extract of Indigofera aspalathoides. *Biol Pharm Bull* 2005; **28**: 364-366
45 **Milner JA**. Preclinical perspectives on garlic and cancer. *J Nutr* 2006; **136**: 827S-831S
46 **Yang SS**, Huang CC, Chen JR, Chiu CL, Shieh MJ, Lin SJ, Yang SC. Effects of ethanol on antioxidant capacity in isolated rat hepatocytes. *World J Gastroenterol* 2005; **11**: 7272-7276
47 **Manna PR**, Chandrala SP, King SR, Jo Y, Counis R, Huhtaniemi IT, Stocco DM. Molecular mechanisms of insulin-like growth factor-I mediated regulation of the steroidogenic acute regulatory protein in mouse leydig cells. *Mol Endocrinol* 2006; **20**: 362-378
48 **Park YJ**, Choi EY, Choi JY, Park JG, You HJ, Chung MH. Genetic changes of hOGG1 and the activity of oh8Gua glycosylase in colon cancer. *Eur J Cancer* 2001; **37**: 340-346
49 **Sundaresan S**, Subramanian P. S-allylcysteine inhibits circulatory lipid peroxidation and promotes antioxidants in N-nitrosodiethylamine-induced carcinogenesis. *Pol J Pharmacol* 2003; **55**: 37-42
50 **Park KA**, Kweon S, Choi H. Anticarcinogenic effect and modification of cytochrome P450 2E1 by dietary garlic powder in diethylnitrosamine-initiated rat hepatocarcinogenesis. *J Biochem Mol Biol* 2002; **35**: 615-622
51 **van Schaftingen E**, Gerin I. The glucose-6-phosphatase system. *Biochem J* 2002; **362**: 513-532
52 **Singh A**, Arora A, Shukla Y. Modulation of altered hepatic foci induction by diallyl sulphide in Wistar rats. *Eur J Cancer Prev* 2004; **13**: 263-269
53 **Xiao D**, Pinto JT, Gundersen GG, Weinstein IB. Effects of a series of organosulfur compounds on mitotic arrest and induction of apoptosis in colon cancer cells. *Mol Cancer Ther* 2005; **4**: 1388-1398
54 **Rotem R**, Heyfets A, Fingrut O, Blickstein D, Shaklai M, Flescher E. Jasmonates: novel anticancer agents acting directly and selectively on human cancer cell mitochondria. *Cancer Res* 2005; **65**: 1984-1993
55 **You KR**, Wen J, Lee ST, Kim DG. Cytochrome c oxidase subunit III: a molecular marker for N-(4-hydroxy-phenyl)retinamise-induced oxidative stress in hepatoma cells. *J Biol Chem* 2002; **277**: 3870-3877
56 **Saraswat M**, Mrudula T, Kumar PU, Suneetha A, Rao Rao TS, Srinivasulu M, Reddy B. Overexpression of aldose reductase in human cancer tissues. *Med Sci Monit* 2006; **12**: CR525-CR529
57 **Zeindl-Eberhart E**, Haraida S, Liebmann S, Jungblut PR, Lamer S, Mayer D, Jager G, Chung S, Rabes HM. Detection and identification of tumor-associated protein variants in human hepatocellular carcinomas. *Hepatology* 2004; **39**: 540-549
58 **Thomas T**, Rauscher F, Sanders R, Veltman J, Watkins JB 3rd. Effects of aldose reductase inhibitors on antioxidant defense in rat and rabbit liver. *Toxicol Sci* 2000; **53**: 145-149

S- Editor Tian L **L- Editor** Roberts SE **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6154

World J Gastroenterol 2008 October 28; 14(40): 6154-6162
World Journal of Gastroenterology ISSN 1007-9327

VIRAL HEPATITIS

Liver stiffness in the hepatitis B virus carrier: A non-invasive marker of liver disease influenced by the pattern of transaminases

Filippo Oliveri, Barbara Coco, Pietro Ciccorossi, Piero Colombatto, Veronica Romagnoli, Beatrice Cherubini, Ferruccio Bonino, Maurizia Rossana Brunetto

Filippo Oliveri, Barbara Coco, Pietro Ciccorossi, Piero Colombatto, Veronica Romagnoli, Beatrice Cherubini, Maurizia Rossana Brunetto, UO Epatologia, Azienda Ospedaliero-Universitaria Pisana, Pisa 56124, Italy
Ferruccio Bonino, Università di Pisa, Pisa, and Direzione Scientifica della Fondazione IRCCS Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Milano 20122, Italy
Author contributions: Oliveri F, Coco B, Ciccorossi P, Colombatto P, Romagnoli V, Cherubini B and Brunetto MR performed research (clinical and elastographic assessment); Oliveri F, Coco B, Bonino F and Brunetto MR designed the study, performed the analysis and wrote the paper.
Supported by Educational grants from the Italian Ministry of Health, "ERASMO" 2005
Correspondence to: Dr. Maurizia Rossana Brunetto, UO Epatologia, Azienda Ospedaliero-Universitaria Pisana, Via Paradisa n. 2-Cisanello, Pisa 56124, Italy. brunetto@med-club.com
Telephone: +39-50-996857 Fax: +39-50-995457
Received: May 27, 2008 Revised: July 21, 2008
Accepted: July 28, 2008
Published online: October 28, 2008

Abstract

AIM: To investigate the usefulness of transient elastography by Fibroscan (FS), a rapid non-invasive technique to evaluate liver fibrosis, in the management of chronic hepatitis B virus (HBV) carriers.

METHODS: In 297 consecutive HBV carriers, we studied the correlation between liver stiffness (LS), stage of liver disease and other factors potentially influencing FS measurements. In 87 chronic hepatitis B (CHB) patients, we monitored the FS variations according to the spontaneous or treatment-induced variations of biochemical activity during follow-up.

RESULTS: FS values were 12.3 ± 3.3 kPa in acute hepatitis, 10.3 ± 8.8 kPa in chronic hepatitis, 4.3 ± 1.0 kPa in inactive carriers and 4.6 ± 1.2 kPa in blood donors. We identified the cut-offs of 7.5 and 11.8 kPa for the diagnosis of fibrosis ⩾ S3 and cirrhosis respectively, showing 93.9% and 86.5% sensitivity, 88.5% and 96.3% specificity, 76.7% and 86.7% positive predictive value (PPV), 97.3% and 96.3% negative predictive value (NPV) and 90.1% and 94.2% diagnostic accuracy. At multivariate analysis in 171 untreated carriers, fibrosis stage (t = 13.187, P < 0.001), active *vs* inactive HBV infection (t = 6.437, P < 0.001), alanine aminotransferase (ALT) (t = 4.740, P < 0.001) and HBV-DNA levels (t = -2.046, P = 0.042) were independently associated with FS. Necroinflammation score (t = 2.158, > 10/18 *vs* ⩽ 10/18, P = 0.035) and ALT levels (t = 3.566, P = 0.001) were independently associated with LS in 83 untreated patients without cirrhosis and long-term biochemical remission (t = 4.662, P < 0.001) in 80 treated patients. During FS monitoring (mean follow-up 19.9 ± 7.1 mo) FS values paralleled those of ALT in patients with hepatitis exacerbation (with 1.2 to 4.4-fold increases in CHB patients) and showed a progressive decrease during antiviral therapy.

CONCLUSION: FS is a non-invasive tool to monitor liver disease in chronic HBV carriers, provided that the pattern of biochemical activity is taken into account. In the inactive carrier, it identifies non-HBV-related causes of liver damage and transient reactivations. In CHB patients, it may warrant a more appropriate timing of control liver biopsies.

Key words: Liver elastography; Liver fibrosis; Cirrhosis; Hepatitis B virus; Chronic hepatitis B

Peer reviewer: Juan Ramón Larrubia, PhD, Gastroenterology Unit and Liver Research Unit, Guadalajara University Hospital, University of Alcalá, Guadalajara 19002, Spain

Oliveri F, Coco B, Ciccorossi P, Colombatto P, Romagnoli V, Cherubini B, Bonino F, Brunetto MR. Liver stiffness in the hepatitis B virus carrier: A non-invasive marker of liver disease influenced by the pattern of transaminases. *World J Gastroenterol* 2008; 14(40): 6154-6162 Available from: URL: http://www.wjgnet.com/1007-9327/14/6154.asp DOI: http://dx.doi.org/10.3748/wjg.14.6154

INTRODUCTION

Transient elastography by Fibroscan (FS)[1] has been proposed as a rapid, non-invasive technique to detect liver fibrosis[2], and many studies have confirmed its clinical usefulness, demonstrating good reproducibility

and high correlation between FS and liver fibrosis at histology[3-7]. Nevertheless, liver stiffness (LS) is influenced by factors other than fibrosis, such as major variations of alanine aminotransferase (ALT) levels[8]. We showed that during hepatitis exacerbations, LS increased, paralleling the kinetics of ALT, whereas FS values were lower than expected according to the histological stage in patients with long-lasting (≥ 12 mo) ALT normalization[8]. Similar LS profiles have been reported in patients with acute viral hepatitis[9,10].

Thus, the biochemical status (ALT levels) of the patient has to be taken into account for an accurate interpretation of LS values in clinical practice. This might be highly relevant in chronic hepatitis B virus (HBV) infection where intervening phases of disease activity and remission and asymptomatic hepatitis reactivations are observed[11-14].

In order to assess the usefulness of FS in the clinical management of chronic HBV carriers, we studied prospectively LS and evaluated its variations according to the changes of the virological, biochemical and histological profiles of liver disease.

MATERIALS AND METHODS

Patients

We studied 288 consecutive chronic HBV carriers (192 males, mean age 48.4 years, range 20-78 year) and nine patients with acute hepatitis B followed-up at the Hepatology Unit of the University Hospital of Pisa, Regional Reference Center for Chronic Liver Disease and Hepatocellular Carcinoma. The study was approved by the Ethical Committee of the hospital and patients gave their written informed consent.

HBV carriers were classified, after a monthly follow-up of at least 12 mo, as inactive or active according to their virological profile. Inactive carriers had serum HBV DNA persistently < 10^5 copies/mL (by COBAS Amplicor HBV Monitor, Roche, Basel, Switzerland) and IgM anti-HBc levels < 0.200 (by Core-M™ Axsym System, Abbott, Sligo, Ireland). Chronic hepatitis patients showed the presence of active viral replication (serum HBV-DNA levels persistently or intermittently ≥ 10^5 copies/mL during the follow-up), IgM anti-HBc ≥ 0.200 and liver histology consistent with chronic hepatitis. Exclusion criteria: hepatitis D virus (HDV) or hepatitis C virus (HCV) coinfections, Child B or C cirrhosis.

Cross-sectional study

We studied the correlation between LS and the stage of liver disease with single point FS measurements in 297 HBV carriers (288 with chronic infection: 208 untreated and 80 treated; nine with acute hepatitis B) and 50 blood donors as controls. Transient elastography was performed within 6 mo (median 3 mo, 75% of cases between 0 and 4.6 mo) from liver biopsy in 157 patients with biochemical and/or virological signs of liver disease and 21 inactive carriers. In 47 HBV carriers with inactive infection, 63 patients with cirrhosis (with previous histological diagnosis and actual ultrasonographic signs of cirrhosis) and nine patients with acute hepatitis, who did not undergo liver biopsies, FS was performed within 1 wk from US and Doppler examinations of the liver.

Prospective study

To study the correlation between LS and spontaneous or treatment-induced variations of biochemical activity, we enrolled 87 patients who underwent monthly blood controls and FS measurements at least every 6 mo. In case of ALT flares (ALT values ≥ 300 IU/L with increments of at least 2 SD above previous values), patients were monitored with blood and FS test every 2 wk for the first month and monthly thereafter until flare resolution. Transaminases and virological markers (HBV DNA and IgM anti-HBc) were tested on the same day of FS measurements. In treated patients, FS was monitored every 3 mo.

LS

Transient elastography was measured by Fibroscan (EchoSens, Paris, France). All measures were performed by trained physicians on the right liver lobe through intercostal spaces in the patient lying on his back, with right arm in maximal abduction. The US guide was used to identify a target liver area, at least 6 cm thick, without major vascular structures. The procedure was considered valid if at least 10 validated measurements were performed, with a success rate (ratio between numbers of validated and total measurements) ≥ 60% and interquartile range (IQR) < 20%. LS was recorded in kPa as the median value of all measurements.

Liver histology

Liver biopsies were obtained using 16 G disposable needles (Hepafix B; Braun, Melsungen, Germany). Liver specimens (median 27 mm, range 11-50 mm) were stained with hematoxylin and eosin. Necro-inflammatory activity and liver fibrosis were scored according to Ishak[15]. Steatosis was graded semiquantitatively, as reported previously[4]. Patients in whom liver biopsy yielded specimens shorter than 15 mm and/or with less than 11 portal tracts were excluded from the analysis.

Database

The included variables were sex, age, virological profile (HBeAg/anti-HBe status), liver disease co-factors [alcohol intake (≤ 60 or > 60 g/d), iron overload (present, in case of staining at histology and serum iron > 150 g/L and/or ferritin > 400 μg/L), hyperlipemia (cholesterol > 240 mg/dL and/or triglycerides > 250 mg/dL), diabetes (fasting plasma glucose > 140 mg/dL), overweight [body mass index (BMI) > 25 kg/m^2]. The biochemical profiles were defined as: (1) persistently elevated ALT; (2) biochemical remission (persistently normal ALT for at least 12 mo, at monthly controls); (3) ALT flares (when ALT values increased ≥ 300 IU/L, with increments of at least 2 SD above previous values). Virological profiles included HBV-DNA and IgM anti-HBc levels. Liver biopsy features were: length; number

of fragments; portal tracts number; necro-inflammation, fibrosis and steatosis scores. Cirrhosis at ultrasound (US cirrhosis) was defined when enlargement of left/caudate lobes, nodular liver boundaries, and micro-macronodular liver structure were present. We recorded in addition: the signs of portal hypertension (portal vein diameter > 12 mm; spleen volume > 45 cm^2; esophagus or gastric varices); the transient elastography performance (values, rate of successful measurements and IQRs); the characteristics of therapy (schedule, dose, duration and response).

Statistical analysis

Data are expressed as mean ± SD. The logarithmic transformation was used for quantitative data when their distributions were not normal. The Pearson's correlation coefficient was used to analyze the correlations between values of liver elastometry and fibrosis. Differences between subgroups were analysed using one-way ANOVA, Mann-Whitney rank sum test or Kruskal-Wallis test when appropriate. To identify factors independently correlated with LS, variables with statistical associations ($P < 0.05$) or trends ($P < 0.10$) at univariate analysis were included in multiple regression analyses. The diagnostic performance of transient elastography was evaluated by receiver operating characteristic (ROC) curve. By using the cut-off values with the highest sensitivity + specificity sum, we defined two different cut-off values of liver transient elastography to identify patients with significant fibrosis (Ishak score ⩾ 3/6) or cirrhosis. Statistical analysis was performed by SPSS (version 10.0, SPSS Inc., Chicago, IL, USA) software package.

RESULTS

Cross-sectional study

Overall 277 of 297 (93.3%) HBV carriers were suitable for the analysis: nine had acute hepatitis, 68 inactive infection, and the remaining 200 had chronic hepatitis. Six patients (2.1%) were excluded because their liver biopsies were < 1.5 cm and 14 (4.9%) because their elastographic measures failed (seven cases had BMI > 28). Eighty patients were under treatment [61 nucleos(t)ides, NA; 19 interferon, IFN]. Demographic and clinical characteristics of the 268 chronic carriers are reported in Table 1.

FS values were 4.6 ± 1.2 kPa in 50 blood donors, 12.3 ± 3.3 kPa in nine patients with acute hepatitis and 10.3 ± 8.8 kPa in 268 chronic HBV carriers ($P < 0.001$) (Table 2).

In 68 inactive carriers, the mean FS value was 5.0 ± 1.8 kPa. Seventeen of them had abnormal ALT and at histology showed steatohepatitis or steatosis. Their mean LS values were significantly higher as compared to HBV carriers with normal ALT and without dysmetabolic profile (6.9 ± 2.3 kPa *vs* 4.3 ± 1.0 kPa, $P < 0.001$) (Figure 1). As a result of chronic liver damage caused by factors other than HBV, these 17 inactive carriers were excluded from further analysis.

In the 171 untreated chronic HBV carriers, LS correlated significantly with fibrosis stage ($r = 0.706$, $P < 0.001$). At univariate analysis, in the 171 untreated HBV carriers, LS significantly correlated with age, sex, phase of infection (inactive *vs* active), BMI, ALT levels, biochemical remission and fibrosis stage, showing a correlation trend for HBV-DNA levels, alcohol intake and hyperlipemia (Table 3). At multivariate analysis, the phase of HBV infection ($P < 0.001$), ALT levels ($P < 0.001$), HBV-DNA levels ($P = 0.042$) and fibrosis stage ($P < 0.001$) were independently associated with LS (Table 3). In the separate analysis of the 83 untreated patients with chronic hepatitis, but without cirrhosis, the factors independently associated with LS were ALT levels ($P = 0.001$), fibrosis stage (S3-S4 *vs* S0-S2, $P = 0.001$) and necroinflammation score (⩾ 10/18 *vs* < 10/18; $P = 0.035$) (Table 4).

In 80 treated patients, LS correlated with fibrosis stage ($r = 0.453$, $P < 0.001$), but the mean values were lower than untreated patients with a comparable stage of

Table 1 Clinico-demographic characteristics of 268 chronic HBV carriers *n* (%)

	Chronic HBV carriers (*n* = 268)	Untreated HBV carriers (*n* = 188)	Treated HBV carriers (*n* = 80)
Age (yr)	48.2 ± 12.2	46.0 ± 11.8	53.3 ± 11.8
Male/Female	180/88	120/68	60/20
HBeAg/anti-HBe	31/237	22/166	9/71
Alcohol intake > 60 g/d	23 (8.6)	14 (7.4)	9 (11.3)
Diabetes	12 (4.5)	7 (3.7)	5 (6.3)
Hyperlipaemia	38 (14.2)	34 (18.1)	4 (5.0)
BMI			
25-30 kg/m^2	95 (35.4)	67 (35.6)	28 (35.0)
> 30 kg/m^2	7 (2.6)	6 (3.2)	1 (1.3)
ALT > 300 IU/L	11 (4.1)	9 (4.8)	3 (3.8)
HBV-DNA (Log_{10} IU/mL)	4.70 ± 2.17	4.97 ± 2.17	4.06 ± 2.05
Inactive carriers	68 (25.4)	68 (36.2)	-
CHB Ishak score			
S0-S2	85 (31.7)	71 (37.8)	14 (17.5)
S3-S4	19 (7.1)	12 (6.4)	7 (8.7)
S5-S6	30 (11.2)	14 (7.4)	16 (20.0)
US cirrhosis	66 (24.6)	23 (12.2)	43 (53.8)

US cirrhosis: Ultrasound signs of cirrhosis.

Table 2 Correlation between phase of infection, stage of liver disease and liver stiffness values

	n	Fibroscan values (kPa)
Blood donors	50	4.6 ± 1.2
Acute Hepatitis[1]	9	12.3 ± 3.3
Untreated HBsAg carriers overall[1]	188	8.9 ± 8.0
Inactive carriers whithout LD[2]	51	4.3 ± 1.0
Inactive carriers whit LD[2]	17	6.9 ± 2.3
CHB S0-S2	71	6.4 ± 2.4
CHB S3-S4	12	10.1 ± 3.8
CHB S5-S6	14	15.7 ± 9.0
US Cirrhosis[3]	23	23.6 ± 11.8
Treated CHB overall[1]	80	13.4 ± 9.7
CHB S0-S2	14	6.1 ± 1.7
CHB S3-S4	7	8.5 ± 2.8
CHB S5-S6	16	11.7 ± 5.2
US Cirrhosis[3]	43	17.2 ± 11.4

LD: Liver disease. [1]$P < 0.001$; [2]$P < 0.001$; [3]$P = 0.035$.

Table 3 Factors associated with FS values at uni and multivariate analysis in 171 chronic HBV carriers

Variable		Univariate analysis	Multivariate analysis		
		P	*B*	95% CI	*P*
Age	yr	0.006			NS
Sex	Male	0.002			NS
Phase of infection[1]	CHB	< 0.001	-9.939	-12.989-6.889	< 0.001
Alcohol introduction	> 60 g/d	0.076			NS
Diabetes	Present	NS			
Hyperlipemia	Present	0.089			NS
BMI	> 25 kg/m^2	< 0.001			NS
ALT	Log_{10} IU/mL	< 0.001	5.713	3.333-8.094	< 0.001
Biochemical remission[2]	Present	< 0.001			NS
HBV-DNA serum levels	Log_{10} IU/mL	0.051	-0.470	-0.924/-0.016	0.042
Disease stage[3]	Class	< 0.001	5.021	4.269-5.773	< 0.001

[1]CHB patients *vs* inactive carriers; [2]Biochemical remission means normal ALT ⩾ 12 mo; [3]Inactive carriers, CHB S0-S2, S3-S4, S5-S6, US cirrhosis. NS: No significance.

Table 4 Factors associated with FS values at uni and multivariate analysis in 83 untreated non-cirrhotic CHB patients

Variable		Univariate analysis	Multivariate analysis		
		P	*B*	95% CI	*P*
Age	yr	NS			
Sex	Male	0.062			NS
Alcohol introduction	>60 g/d	NS			
Diabetes	Present	NS			
Hyperlipemia	Present	NS			
BMI	> 25 kg/m^2	NS			
ALT	Log_{10} IU/mL	< 0.001	3.028	1.408-5.008	0.001
Biochemical remission	Present	0.017			NS
HBV-DNA serum levels	Log_{10} IU/mL	0.075			NS
Necroinflammation score	⩾ 10/18	< 0.001	1.611	0.117-3.104	0.035
Disease stage[1]	S3-S4	< 0.001	3.054	1.318-4.789	0.001

[1]CHB S0-S2 *vs* S3-S4; NS: No significance.

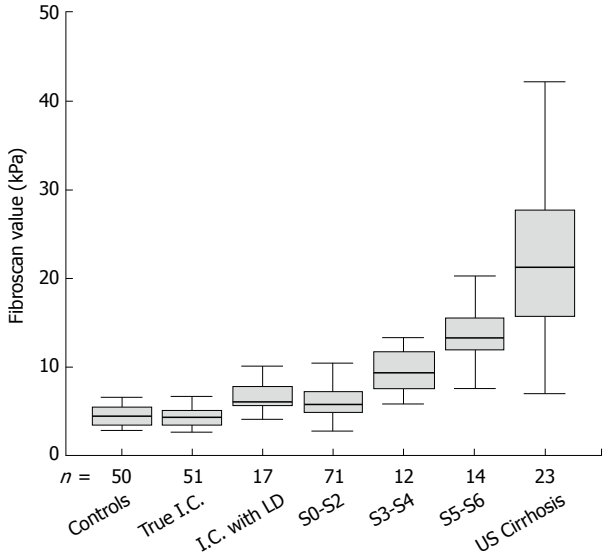


Figure 1 Distribution of Fibroscan values in Blood donors (Controls), Inactive carriers without (True I.C.) or with dysmetabolic liver disease (I.C. with LD) and in CHB patients by fibrosis stage (S0-S2, S3-S4, S5-S6, US Cirrhosis).

fibrosis (6.1 *vs* 6.4 kPa in S0-S2 patients; 8.5 *vs* 10.1 kPa in S3-S4 patients; 11.7 *vs* 15.7 kPa in S5-S6 patients; 17.2 *vs* 23.6 kPa in US cirrhosis patients) (Table 2), and the difference reached the statistical significance in patients with US cirrhosis only ($P = 0.035$). Fifty of them were under long-term NA treatment and in long-term biochemical remission, which was independently associated with FS values ($P < 0.001$, Table 5).

Diagnostic accuracy for identification of fibrosis ⩾ S3 and cirrhosis

To identify the FS cut-offs for fibrosis ⩾ S3 and cirrhosis, we analyzed untreated patients only. Area under ROC curve (AUROCs) for fibrosis ⩾ S3 and cirrhosis were 0.966 and 0.973 (95% CI 0.942-0.989 and 0.952-0.994) (Figure 2) and their cut-off values were 7.5 and 11.8 kPa, respectively.

Fibrosis ⩾ S3: The diagnostic performance of 7.5 kPa cut-off is reported in Table 6. Overall, 46 of 60 patients with elastography ⩾ 7.5 kPa had fibrosis ⩾ S3 (76.7% PPV) and 108 of 111 patients with FS < 7.5 kPa had S0-S2 fibrosis (97.3% NPV). Among the 14 patients with FS ⩾ 7.5 kPa, but a fibrosis stage < S3, five patients had ALT levels > 300 UI/L at the time of FS measurement. None of the three patients with FS < 7.5 but fibrosis ⩾ S3 had cirrhosis: one had S3 and two had S4 fibrosis at liver histology.

Table 5 Factors associated with FS values at uni and multivariate analysis in 80 treated CHB patients

Variable		Univariate analysis	Multivariate analysis		
		P	*B*	95% CI	*P*
Age	yr	NS			
Sex	Male	NS			
Alcohol introduction	> 60 g/d	NS			
Diabetes	Present	NS			
Hyperlipaemia	Present	NS			
BMI	> 25 kg/m^2	NS			
ALT	Log_{10} IU/mL	NS			
Biochemical remission	Present	0.001	8.705	5.277-12.133	< 0.001
HBV-DNA serum levels	Log_{10} IU/mL	NS			
Disease stage[1]	Class	< 0.001	4.374	2.982-5.766	< 0.001

[1]CHB S0-S2, S3-S4, S5-S6, US cirrhosis. NS: No significance.

Table 6 Diagnostic performance of FS for identification of fibrosis ⩾ S3 and cirrhosis by using the cut-offs of 7.5 kPa and 11.8 kPa

	Fibrosis stage ⩾ S3		Fibrosis S5-S6/US cirrhosis	
	IC + UT CHB	T CHB	IC + UT CHB	T CHB
Sensitivity (%)	93.9	78.8	86.5	54.2
Specificity (%)	88.5	71.4	96.3	90.5
Positive predictive value (%)	76.7	92.9	86.5	94.1
Negative predictive value (%)	97.3	41.7	96.3	41.3
Diagnostic accuracy (%)	90.1	77.5	94.2	63.8
Likelihood ratio for pos. test	8.18	2.76	23.18	5.69
Likelihood ratio for neg. test	0.07	0.30	0.14	0.51

IC + UT CHB: Inactive carriers + untreated CHB (171 patients); T CHB: Treated chronic hepatitis B (80 patients).

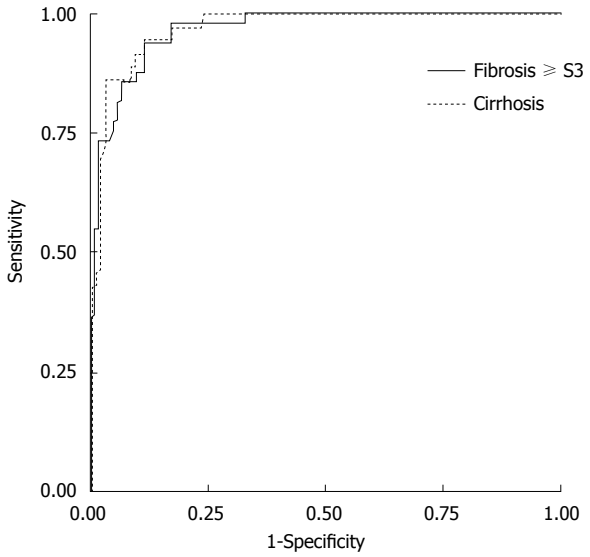


Figure 2 FS diagnostic performance: AUROCs for fibrosis S3 and cirrhosis were 0.966 and 0.973 (95% CI 0.942-0.989 and 0.952-0.994).

Cirrhosis: The diagnostic performance of 11.8 kPa cut-off is shown in Table 6. Thirty-two of 37 patients with elasticity ⩾ 11.8 kPa had histological or US cirrhosis (86.5% PPV); 129 of 134 patients with FS values < 11.8 kPa did not have cirrhosis (96.3% NPV). All but one of the five non-cirrhotic (two with S3 and three with S4 fibrosis stage) patients with FS values ⩾ 11.8 showed LS values ranging from 11.8 and 13.3 kPa; the remaining patient with 20 kPa FS value had S4 fibrosis and ALT levels > 300 UI/L at the time of FS measurement. Two of five cirrhotic patients with low FS (7.0 and 7.6 kPa respectively) were in prolonged spontaneous remission; the remaining three had elastometry values ranging between 8.9 kPa and 11.3 kPa.

Prospective study

In 87 patients, LS was monitored for a mean period of 19.9 ± 7.1 mo (range 6-36 mo): Seventy eight patients had chronic hepatitis (43 untreated and 35 treated) and nine had acute hepatitis. All patients underwent at least three FS measurements (mean 5.6, range 3-10).

Untreated patients: Thirty patients showed stable biochemical and virological profiles without disease progression: their LS did not change, showing minor fluctuations (12 mo/baseline FS mean ratio 1.00 ± 0.20; 24 mo/baseline FS mean ratio 0.99 ± 0.26). The remaining 13 patients experienced hepatitis flares. During flares, FS values increased 1.2 to 4.4-fold as compared to baseline values (mean variation 2.1 ± 1.0-fold), mean FS value during flares being 20.7 ± 12.3 kPa (range 8.6-42 kPa). LS variations paralleled the dynamic profiles of ALT: FS values reached the peak simultaneously with ALT in eight patients (61.5%), later, with 15-30 d of delay, in the remaining five (38.5%). Thereafter, FS values decreased with a latency of 30 d from the initial ALT decrease and returned to baseline values within 3 to 6 mo (Figure 3A). Patients with disease profiles characterised by ALT flares intervened by complete biochemical remission showed major variations of FS values during their hepatitis exacerbations, as compared to patients with persistent ALT elevations between flares (FS variation ranging from 1.4 to 4.4 in the former and from 1.2 to 1.6-fold in the letters, $P = 0.019$).

Acute hepatitis: In nine patients with acute hepatitis B, FS values at presentation ranged from 8.2 to 16.6 (mean

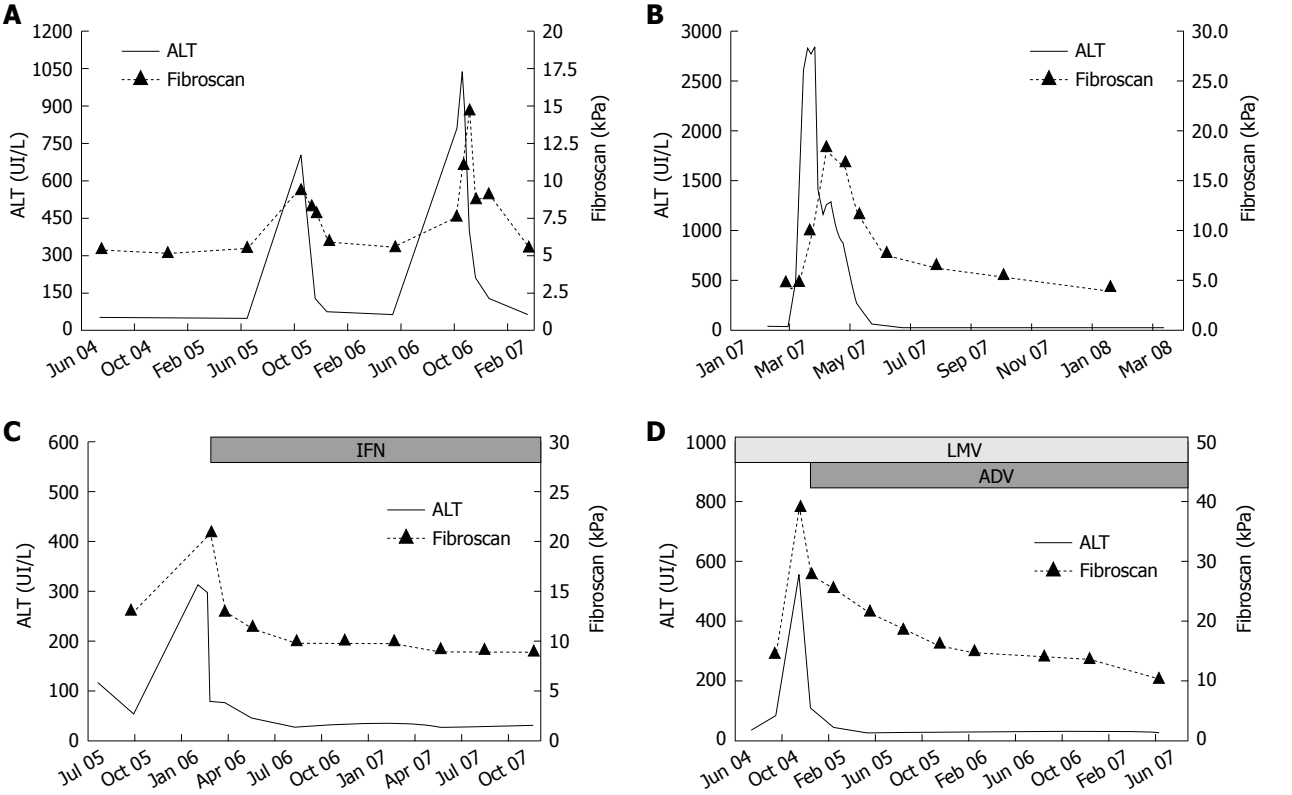


Figure 3 FS and ALT kinetics in four patients. A: CHB with S2 fibrosis stage and recurrent hepatitis flare; B: Acute hepatitis B; C: CHB with S6 fibrosis stage responding to IFN treatment; D: Cirrhosis with biochemical break-through due to lamivudine resistance and response to rescue therapy with adefovir.

12.3 ± 3.3) kPa and reached a peak of 11.8 to 45.7 kPa (20.0 ± 11.6 kPa) at the time of ALT peak. They then declined progressively to 5.6 ± 1.1 (range 4.4-6.9) kPa, in association with the reduction of ALT levels (Figure 3B).

Treated patients: The FS monitoring started with treatment in 18 patients, when treatment was already ongoing in the remaining 13. Overall, FS values showed progressive declines during therapy, with a mean on-treatment/baseline ratio of 0.9 ± 0.4 at 6 mo and 0.7 ± 0.2 at 12 mo. In patients with persistent response to long-term nucleoside/nucleotide analogues treatment, FS values decreased progressively during their follow up, with mean yearly reduction (ratio between two consecutive FS values registered at 12 mo intervals) of 0.8 ± 0.2 at 24 mo, 0.8 ± 0.1 at 36 mo and 0.7 ± 0.1 at 48 mo from the beginning of therapy.

All responders showed decreased FS values during therapy (Figure 3C and D): 0.8 ± 0.2 at 6 mo and 0.6 ± 0.2 at 12 mo, as compared to baseline values, respectively. FS value declines were similar in responders to IFN as compared to responders to NA: 0.7 ± 0.2 *vs* 0.8 ± 0.2 6 mo/baseline ratio and 0.6 ± 0.1 *vs* 0.7 ± 0.2 12 mo/baseline ratio, respectively. Two non-responder HBeAg-positive patients showed an increase of 1.1 and 2.4 times the FS values between 3 and 6 mo, during hepatitis flares, followed by a progressive decline that reached baseline values after 12 mo.

DISCUSSION

Transient elastography[1] is an easy to perform, reproducible method for the rapid and objective evaluation of LS in clinical practice[2,16] and it is proposed as a reliable, non-invasive, surrogate marker of fibrosis[3-7,17,18]. In fact, LS is a physical parameter that correlates primarily with fibrosis, but it is influenced also by other factors that modify the elasticity of the liver, such as significant variations of inflammatory infiltrate, edema and vascular congestion of the liver[8-10,19]. Accordingly, we showed that LS variations parallel ALT values during hepatitis exacerbations in the setting of both acute and chronic liver damage[8]. This evidence has important implications in clinical practice since the interpretation of the LS measure has to take into account the concurrent biochemical profile of the patient[8]. Thus, the interpretation of LS might be more difficult in the setting of CHB when major fluctuations of necrosis and inflammatory activity occur in a significant proportion of patients[11,12,18,19]. On the other hand, the availability of an easy to perform, non-invasive measure for fibrosis might improve the management of the HBV carrier. In the HBV carrier, the repeated measures of LS might help to identify the candidates for liver biopsy and to define both the phase of HBV infection and stage of liver disease that are mandatory to warrant the most appropriate treatment strategy, and to monitor liver disease progression in the single patient[20,21].

Addressing the issue of the clinical usefulness of

LS in the management of the HBV carrier we found a highly significant correlation between transient elastography and fibrosis stages ($P < 0.001$). Using 7.5 and 11.8 kPa as cut-off values for fibrosis ⩾ S3 and cirrhosis, the FS specificities were 88.5% and 96.3%, sensitivities 93.9% and 86.5%, and diagnostic accuracies 90.1% and 94.2%. These results confirm that FS is a reliable method to assess fibrosis in carriers with chronic HBV infection and disease[8].

Additional factors were independently associated with FS, such as active HBV infection ($P < 0.001$), HBV-DNA ($P = 0.042$) and ALT ($P < 0.001$) levels. In inactive HBV carriers, mean FS values were similar to normal controls and significantly lower than in CHB patients (4.3 ± 1.0 *vs* 4.6 ± 1.2 *vs* 11.2 ± 9 kPa; $P < 0.001$). These findings qualify LS as a promising tool to provide an important diagnostic assessment of the HBV carrier with inactive viral profile when the increased FS values suggest the presence of liver damage caused by factors other than HBV. In such cases, liver biopsy can be proposed for the precise characterization of liver disease. Indeed, in our study, 17 inactive carriers with metabolic liver disease had FS values higher (6.9 ± 2.3 kPa) than inactive carriers without liver disease.

In addition to the phase of HBV infection, only two other parameters, namely HBV DNA and ALT, were independently correlated with LS. Since both these parameters are linked with the extent of liver disease activity in the immune competent HBV carrier, our results further support the hypothesis that the extent of necrosis and inflammation influence LS significantly[20,22-24]. Accordingly, in the 83 untreated patients without cirrhosis, multivariate analysis showed that intra-hepatic necrosis and inflammation scores and ALT values were the only factors influencing FS ($P = 0.035$ and $P < 0.001$ respectively), in addition to the stage of liver disease.

The LS values identified as cut-offs for histological stage ⩾ S3 and cirrhosis are lower than those proposed for chronic hepatitis C. A slight variable difference between cut-offs would not be surprising when different cohorts of patients are compared, but FS values in CHB patients with cirrhosis (11.8 kPa) are consistently and persistently lower than in chronic hepatitis C (CHC) cirrhosis[4-6,8,16]. Accordingly, lower values of LS cut-offs have been proposed in preliminary reports[25,26]. These findings are consistent with the specific features of histopathology of hepatitis C, in which the combination of portal lymphoid follicles, bile duct damage, lobular activity and steatosis may contribute to the different LS, as compared to hepatitis B histopathology[27].

In this prospective study of CHB patients, we observed 1.2 to 4.4-fold increases of FS values with ALT flares, and similarly, LS values fluctuated in parallel with ALT values in nine patients with acute hepatitis. Interestingly, the extent of FS fluctuations during the hepatitis exacerbations differed according to the biochemical patterns of CHB. The range of LS variations were significantly wider in patients with ALT flares intervened by complete biochemical remissions, as compared to patients with persistent ALT elevations between flares (FS variations ranged from 1.4 to 4.4-fold in the former and 1.2 to 1.6-fold in the latter group, $P = 0.019$). Altogether these findings confirm our original observation and other more recent reports on the major influence of the biochemical profile on LS in the setting of both acute and chronic liver damage[8-10]. Finally, we found that prolonged biochemical remissions were associated with progressive reductions of FS values. LS declined yearly at about 0.2-fold in treated patients followed-up prospectively for 48 mo, and a proportion of patients who maintained evidence of cirrhosis at US achieved values of FS < 11.8 kPa. This was responsible for the worse diagnostic performance of FS in treated patients in whom the sensitivity for detecting cirrhosis fell from 86.5% to 54.2% in untreated *vs* treated patients with fibrosis ⩾ S5 (Table 6). Altogether, these data suggest a non-linear correlation between the overall kinetics of LS and histological staging during antiviral treatment. Future studies should be addressed to understand the relations among the reductions of LS, necrosis, inflammation and fibrosis in the separate settings of different fibrosis stages (i.e. ⩾ S3/< S3 and presence/absence of cirrhosis) and liver disease etiology (i.e. HBV and HCV). In fact, much of the LS changes depend on the different elastic relations among fine blocks of the liver structure. Thus, the interplay between the extent and structure of the collagen septa within the fine liver block, and the different type and extent of liver inflammatory infiltrate within them, might account for both the different FS cut-offs between CHB and CHC patients and for the different kinetics of FS and fibrosis decline during antiviral therapy.

In conclusion, our study suggests that the LS provides a useful non-invasive tool to monitor liver disease in the chronic HBV carrier. In the inactive carrier, it helps to identify non-HBV-related causes of liver damage and transient reactivation of HBV liver disease. In the CHB patient, provided that the pattern of biochemical activity is taken into account, LS values < 7.5 exclude the presence of significant fibrosis (⩾ S3) with a high NPV (97.3%) and low negative likelihood ratio (0.07). FS values ⩾ 11.8 kPa are highly specific (96.3%) for cirrhosis and show good PPV (86.5%) and positive likelihood ratio (23.18). In the HBV carrier with LS values ranging from 7.5 to 11.8 kPa, which are indicative of significant liver disease, liver biopsy remains the gold standard for an accurate grading and staging of liver disease. Finally, in CHB patients the monitoring of LS appears useful to highlight major changes in intrahepatic liver disease and warrants a more appropriate timing for control liver biopsies.

COMMENTS

Background

The old measure of liver stiffness (LS) by hand palpation has had a new appraisal after the recent introduction of the objective measure of the speed of transmission of an elastic wave across the liver (transient elastography) registered by the new instrument Fibroscan® (EchoSens, Paris, France). Originally, the new technique was proposed in clinical practice as a non-

invasive, surrogate marker of fibrosis and many studies demonstrated good reproducibility and a high correlation between LS and liver fibrosis at histology. However, liver elasticity is influenced not only by fibrosis, but also by the presence and extent of liquid, lipid and inflammatory infiltrates within the liver. The evidence that Fibroscan (FS) is significantly influenced by major variations of liver inflammation (as we previously showed), in addition to variations of staging, prompted the new frontier of testing FS values in the management of patients with chronic hepatitis.

Research frontiers

The course of liver disease in a significant proportion of chronic hepatitis B (CHB) patients is characterized by hepatitis exacerbations, intervened by prolonged remissions whose biochemical and virologic patterns can be mistaken with those of chronic inactive carriers. Thus, measuring LS might be useful to distinguish active from inactive HBV carriers. We addressed this question and present here the results of the cross-sectional and prospective studies of a large cohort of pedigreed hepatitis B virus (HBV) carriers (68 inactive carriers, 200 CHB and nine acute hepatitis B patients).

Innovations and breakthroughs

FS correlates with fibrosis in CHB patients and FS provides a reliable method to assess the overall status of liver disease in the carrier with chronic HBV infection. The mean FS values of HBV-inactive carriers were comparable to those of normal controls and significantly lower than those of CHB patients. Interestingly, in HBV inactive carriers with metabolic liver disease FS values were significantly higher than in HBV-inactive carriers without liver disease. All factors stemming for activity of liver disease, namely the phase of infection (active or inactive), HBV-DNA and ALT levels influenced LS at multivariate analysis. Accordingly, in untreated patients without cirrhosis, histological necrosis and inflammation and ALT were the only factors influencing FS in addition to fibrosis. Thus both necrosis and inflammation influence LS that qualifies as a very promising tool for the non-invasive diagnostic assessment of the liver in the HBV carrier. The best cut-off values for fibrosis and cirrhosis were significantly lower than in chronic hepatitis C (CHC) patients, studied in identical conditions (same center, instrument, operators and test timing), suggesting that FS is influenced also by the different histopathology features of CHB and CHC. This prospective study on patients with hepatitis B exacerbations confirmed 1.2 to 4.4-fold increases of FS values at the time of ALT flares. Similarly, LS paralleled ALT fluctuations in patients with acute hepatitis B. Finally, in treated patients followed up for 48 mo, LS declined yearly at about 0.2-folds, reaching values below the cirrhosis cut-off (11.8 kPa) in a proportion of patients who maintained evidence of cirrhosis. This observation may explain the worse diagnostic performance of FS in treated versus untreated patients. Altogether, these data indicate the non-linear correlation between the kinetics of LS and histological staging during antiviral treatment.

Applications

This study suggests that LS provides a useful non-invasive tool to monitor not only fibrosis, but overall liver disease in the chronic HBV carrier. In monitoring CHB patients, LS appears useful to highlight major changes of liver disease and to warrant a more appropriate timing for control liver biopsies.

Terminology

HBV-inactive carriers mean chronic HBV infection without liver damage caused by HBV, characterized by low HBV-DNA serum levels, persistently normal ALT and undetectable levels of IgM anti-HBc, a marker of HBV-induced liver disease (below the cut-off for CHB). Biochemical remission means transient ALT normalization (spontaneous or induced by antiviral treatment) in patients with CHB.

Peer review

In this study, authors perform a cross-sectional and longitudinal analysis of LS in HBV carriers, correlating this variable with stage of disease, liver inflammation and other factors that could influence FS measurements. They found a good diagnostic accuracy to detect cirrhosis and fibrosis higher than S3. The work is well performed and conclusions are correctly sustained.

REFERENCES

1 **Sandrin L**, Tanter M, Gennisson JL, Catheline S, Fink M. Shear elasticity probe for soft tissues with 1-D transient elastography. *IEEE Trans Ultrason Ferroelectr Freq Control* 2002; **49**: 436-446

2 **Sandrin L**, Fourquet B, Hasquenoph JM, Yon S, Fournier C, Mal F, Christidis C, Ziol M, Poulet B, Kazemi F, Beaugrand M, Palau R. Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. *Ultrasound Med Biol* 2003; **29**: 1705-1713

3 **Ziol M**, Handra-Luca A, Kettaneh A, Christidis C, Mal F, Kazemi F, de Ledinghen V, Marcellin P, Dhumeaux D, Trinchet JC, Beaugrand M. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. *Hepatology* 2005; **41**: 48-54

4 **Castera L**, Vergniol J, Foucher J, Le Bail B, Chanteloup E, Haaser M, Darriet M, Couzigou P, De Ledinghen V. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. *Gastroenterology* 2005; **128**: 343-350

5 **Foucher J**, Chanteloup E, Vergniol J, Castera L, Le Bail B, Adhoute X, Bertet J, Couzigou P, de Ledinghen V. Diagnosis of cirrhosis by transient elastography (FibroScan): a prospective study. *Gut* 2006; **55**: 403-408

6 **Colletta C**, Smirne C, Fabris C, Toniutto P, Rapetti R, Minisini R, Pirisi M. Value of two noninvasive methods to detect progression of fibrosis among HCV carriers with normal aminotransferases. *Hepatology* 2005; **42**: 838-845

7 **Ganne-Carrie N**, Ziol M, de Ledinghen V, Douvin C, Marcellin P, Castera L, Dhumeaux D, Trinchet JC, Beaugrand M. Accuracy of liver stiffness measurement for the diagnosis of cirrhosis in patients with chronic liver diseases. *Hepatology* 2006; **44**: 1511-1517

8 **Coco B**, Oliveri F, Maina AM, Ciccorossi P, Sacco R, Colombatto P, Bonino F, Brunetto MR. Transient elastography: a new surrogate marker of liver fibrosis influenced by major changes of transaminases. *J Viral Hepat* 2007; **14**: 360-369

9 **Arena U**, Vizzutti F, Corti G, Ambu S, Stasi C, Bresci S, Moscarella S, Boddi V, Petrarca A, Laffi G, Marra F, Pinzani M. Acute viral hepatitis increases liver stiffness values measured by transient elastography. *Hepatology* 2008; **47**: 380-384

10 **Sagir A**, Erhardt A, Schmitt M, Haussinger D. Transient elastography is unreliable for detection of cirrhosis in patients with acute liver damage. *Hepatology* 2008; **47**: 592-595

11 **Brunetto MR**, Oliveri F, Coco B, Leandro G, Colombatto P, Gorin JM, Bonino F. Outcome of anti-HBe positive chronic hepatitis B in alpha-interferon treated and untreated patients: a long term cohort study. *J Hepatol* 2002; **36**: 263-270

12 **Hadziyannis SJ**, Papatheodoridis GV. Hepatitis B e antigen-negative chronic hepatitis B: natural history and treatment. *Semin Liver Dis* 2006; **26**: 130-141

13 **Yuen MF**, Yuan HJ, Hui CK, Wong DK, Wong WM, Chan AO, Wong BC, Lai CL. A large population study of spontaneous HBeAg seroconversion and acute exacerbation of chronic hepatitis B infection: implications for antiviral therapy. *Gut* 2003; **52**: 416-419

14 **Liaw YF**. Hepatitis flares and hepatitis B e antigen seroconversion: implication in anti-hepatitis B virus therapy. *J Gastroenterol Hepatol* 2003; **18**: 246-252

15 **Ishak K**, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, Denk H, Desmet V, Korb G, MacSween RN. Histological grading and staging of chronic hepatitis. *J Hepatol* 1995; **22**: 696-699

16 **Fraquelli M**, Rigamonti C, Casazza G, Conte D, Donato MF, Ronchi G, Colombo M. Reproducibility of transient elastography in the evaluation of liver fibrosis in patients with chronic liver disease. *Gut* 2007; **56**: 968-973

17 **de Ledinghen V**, Douvin C, Kettaneh A, Ziol M, Roulot D, Marcellin P, Dhumeaux D, Beaugrand M. Diagnosis of hepatic fibrosis and cirrhosis by transient elastography in HIV/hepatitis C virus-coinfected patients. *J Acquir Immune Defic Syndr* 2006; **41**: 175-179

18 **Castera L**, Forns X, Alberti A. Non-invasive evaluation of liver fibrosis using transient elastography. *J Hepatol* 2008; **48**: 835-847

19 **Roulot D**, Czernichow S, Le Clesiau H, Costes JL, Vergnaud

AC, Beaugrand M. Liver stiffness values in apparently healthy subjects: influence of gender and metabolic syndrome. *J Hepatol* 2008; **48**: 606-613

20 **Lok AS**, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507-539

21 **The EASL Jury**. EASL International Consensus Conference on Hepatitis B: 13-14 September, 2002 Geneva, Switzerland. Consensus statement (Long version). *J Hepatol* 2003; **39**: 3-25

22 **van Nunen AB**, Hansen BE, Suh DJ, Lohr HF, Chemello L, Fontaine H, Heathcote J, Song BC, Janssen HL, de Man RA, Schalm SW. Durability of HBeAg seroconversion following antiviral therapy for chronic hepatitis B: relation to type of therapy and pretreatment serum hepatitis B virus DNA and alanine aminotransferase. *Gut* 2003; **52**: 420-424

23 **Manesis EK**, Papatheodoridis GV, Sevastianos V, Cholongitas E, Papaioannou C, Hadziyannis SJ. Significance of hepatitis B viremia levels determined by a quantitative polymerase chain reaction assay in patients with hepatitis B e antigen-negative chronic hepatitis B virus infection. *Am J Gastroenterol* 2003; **98**: 2261-2267

24 **Feld JJ**, Ayers M, El-Ashry D, Mazzulli T, Tellier R, Heathcote EJ. Hepatitis B virus DNA prediction rules for hepatitis B e antigen-negative chronic hepatitis B. *Hepatology* 2007; **46**: 1057-1070

25 **Marcellin P**, de Ledinghen V, Dhumeaux D, Poupon R, Ziol M, Bedossa P. Non-invasive assessment of liver fibrosis in chronic hepatitis B using FibroScan (Abstract). *Hepatology* 2005; **42** : 715A

26 **Castera L**, Foucher J, Bernard P, Bertet J, Couzigou P, de Ledinghen V. Transient elastography (FibroScan) and FibroTest to assess liver fibrosis in inactive hepatitis B carriers: a prospective controlled study (Abstract). *Hepatology* 2006; **44**: 489A

27 **Scheuer PJ**, Davies SE, Dhillon AP. Histopathological aspects of viral hepatitis. *J Viral Hepat* 1996; **3**: 277-283

S- Editor Li DL **L- Editor** Roberts SE **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6163

World J Gastroenterol 2008 October 28; 14(40): 6163-6170
World Journal of Gastroenterology ISSN 1007-9327

Ataxia telangiectasia-mutated-Rad3-related DNA damage checkpoint signaling pathway triggered by hepatitis B virus infection

Fan Zhao, Ning-Bo Hou, Xiao-Li Yang, Xiang He, Yu Liu, Yan-Hong Zhang, Cong-Wen Wei, Ting Song, Li Li, Qing-Jun Ma, Hui Zhong

Fan Zhao, Ning-Bo Hou, Xiang He, Yan-Hong Zhang, Cong-Wen Wei, Ting Song, Li Li, Qing-Jun Ma, Hui Zhong, Molecular Genetics Department, Beijing Institute of Biotechnology, Beijing 100850, China
Xiao-Li Yang, Yu Liu, Biochemical and Immunology Analyze Center, The General Hospital of Chinese People's Armed Police Forces, Beijing 100039, China
Author contributions: Zhao F and Hou NB contributed equally to this work; Ma QJ, Li L, Zhang YH and Zhong H designed research; Yang XL, He X, Liu Y, Zhang YH, Wei CW, Song T and Li L performed research; Zhong H wrote the paper.
Supported by National Natural Science Foundation of China, No. 30700413
Correspondence to: Hui Zhong, PhD, Molecular Genetics Department Beijing Institute of Biotechnology, Beijing 100850, China. towall@yahoo.com
Telephone: +86-10-66931809 Fax: +86-10-88272563
Received: April 14, 2008 Revised: June 21, 2008
Accepted: June 28, 2008
Published online: October 28, 2008

Abstract

AIM: To explore whether acute cellular DNA damage response is induced upon hepatitis B virus (HBV) infection and the effects of the HBV infection.

METHODS: We incubated HL7702 hepatocytes with HBV-positive serum, mimicking a natural HBV infection process. We used immunoblotting to evaluate protein expression levels in HBV-infected cells or in non-infected cells; immunofluorescence to show ATR foci ands Chk1 phosphorylation foci formation; flow cytometry to analyze the cell cycle and apoptosis; ultraviolet (UV) radiation and ionizing radiation (IR)-treated cells to mimic DNA damage; and Trypan blue staining to count the viable cells.

RESULTS: We found that HBV infection induced an increased steady state of ATR protein and increased phosphorylation of multiple downstream targets including Chk1, p53 and H2AX. In contrast to ATR and its target, the phosphorylated form of ATM at Ser-1981 and its downstream substrate Chk2 phosphorylation at Thr-68 did not visibly increase upon infection. However, the level of Mre11 and p21 were reduced beginning at 0.5 h after HBV-positive serum addition. Also, HBV infection led to transient cell cycle arrest in the S and the G2 phases without accompanying increased apoptosis. Research on cell survival changes upon radiation following HBV infection showed that survival of UV-treated host cells was greatly increased by HBV infection, owing to the reduced apoptosis. Meanwhile, survival of IR-treated host cells was reduced by HBV infection.

CONCLUSION: HBV infection activates ATR DNA damage response to replication stress and abrogates the checkpoint signaling controlled by DNA damage response.

Key words: Hepatitis B virus; DNA damage response; Cell cycle; p21; Mre11

Peer reviewer: Dr. Michael Torbenson, Department of Pathology, Johns Hopkins University School of Medicine, 1503 E Jefferson, Room B314, Baltimore 21231, United States

Zhao F, Hou NB, Yang XL, He X, Liu Y, Zhang YH, Wei CW, Song T, Li L, Ma QJ, Zhong H. Ataxia telangiectasia-mutated-Rad3-related DNA damage checkpoint signaling pathway triggered by hepatitis B virus infection. *World J Gastroenterol* 2008; 14(40): 6163-6170 Available from: URL: http://www.wjgnet.com/1007-9327/14/6163.asp DOI: http://dx.doi.org/10.3748/wjg.14.6163

INTRODUCTION

Eukaryotic cells employ multiple strategies of checkpoint signaling and DNA repair mechanisms to monitor and repair damaged DNA[1-5]. There are two branches of the checkpoint response pathway, ataxia telangiectasia-mutated (ATM) pathway and ATM-Rad3-related (ATR) pathway. The major difference between ATM and ATR is the type of DNA damage to which each responds. For example, ATM responds to ionizing radiation (IR) and other agents that cause double-strand breaks (DSBs) in DNA. ATR responds to ultraviolet radiation (UV) radiation and other agents that induce the accumulation of stalled replication forks and subsequent single-stranded breaks (SSBs) in DNA. The DSBs are recognized by the Mre11-Rad50-Nbs1 complex, which recruits and activates ATM kinase[6]. The SSBs are coated

by replication protein A, and it recruits a complex of ATR kinases and ATR-interacting protein, which is then activated by the Rad9-Rad1-Hus1 complex and other factors. Accumulating evidence suggests that checkpoint signaling through ATR is intimately linked to the process of DNA replication[7,8].

A variety of checkpoint and DNA repair proteins have been identified as substrates for ATM and ATR kinases, including the checkpoint kinases Chk1 and Chk2, as well as p53, Smc1 and H2AX. Chk1 was first identified in *Schizosaccharomyces pombe* because of its role in the checkpoint arrest at G2/M, and it is mainly phosphorylated by ATR in response to UV, hydroxyurea and aphidicolin[9-11]. p21 was initially identified as a component of quaternary complex containing cdk-cyclin kinases and PCNA. Previous study has shown that p21 protein is degraded after low doses of UV; this degradation is essential for optimal DNA repair and is ATR-dependent[12]. The MRN complex consisting of Mre11, Rad50, and Nbs1 is a target of both ATM and ATR and is involved in both pathways[13-15].

Virus replication presents the host cells with large amounts of exogenous genetic material, including DNA ends and unusual structures. Therefore, infected cells recognize viral replication as a DNA damage stress and elicit DNA damage signal transduction, which ultimately induces apoptosis as part of host immune surveillance. However, recent reports have shown that viruses evolve a variety of mechanisms to manipulate DNA damage signaling for their replication and propagation. For example, Epstein-Barr Virus (EBV)[16] abrogates the p53 checkpoint signaling pathway through the interaction of the BZLF1 protein and p53 to avoid apoptosis. Other viruses such as human immunodeficiency virus type 1 (HIV-1)[17-20], herpes simplex virus type 1 (HSV-1)[21,22] and human cytomegalovirus[23] can activate and exploit a cellular DNA damage response, which aids viral replication. Adenovirus blocks ATM signaling and concatemer formation through targeting the DNA repair complex of MRN for degradation and mislocalization[24,25]. Thus, under some circumstances, viruses have co-opted endogenous checkpoint regulators to ensure their own efficient replication[26,27].

Hepatitis B virus (HBV) is a partially enveloped double-stranded DNA virus with a genome of 3.2 kb. Upon infection, the viral genome is transported into the cell, where it is converted into a covalently closed circular DNA (cccDNA). The cccDNA serves as a template for transcription by host cell RNA polymerase Ⅱ. The pregenomic RNA is then reverse transcribed into DNA replicative intermediates in the cytoplasm within immature viral core particles, by the virally encoded polymerase. Integration into host chromosome may happen during its replication.

There is no evidence thus far that the ATM/ATR kinases or their downstream pathways are triggered by HBV infection. The present study was undertaken by culturing normal hepatocyte cell line HL7702 and primary hepatocytes from a healthy liver donor with HBV-positive serum, mimicking natural HBV infection. We showed here, perhaps for the first time, that HBV infection elicited acute cellular DNA damage response dependent on ATR. However, the ATR checkpoint signaling was blocked downstream of p53-dependent and p53-independent pathways to evade apoptosis.

MATERIALS AND METHODS

Chemicals

Mimosine and aphidicolin were obtained from Sigma. The stock concentration of mimosine was 100 mmol/L, the stock concentration of aphidicolin was 10 mmol/L; both were dissolved in Dimethyl Sulphoxide (DMSO).

Cell culture, synchronization and infection

The human hepatocyte cell line HL7702, which was isolated from a HBV-seronegative individual, was obtained from Shanghai Biochemistry Institute. HL7702 were cultured in RPMI-1640 with 10% heat-inactivated FBS (Gibco). Serum samples from HBV carriers were analyzed. The patient was anti-HBsAg-positive, as detected by ELISA (SIIC Ke-Hua, Shanghai), and HBV DNA in the serum sample was quantified using FQ-PCR (Da-An Gene Corp). The patient had received no antiviral therapy prior to the study and was not infected with HCV or HIV. The number of serum HBV viral particles was 7×10^9 copies/mL, as quantified by FQ-PCR. Normal serum was obtained from healthy non-infected individuals as a control. The sera were stored at -80℃ until use.

When synchronized, the HL7702 cells were cultured in RPMI-1640 containing 0.1% FBS for 2 d; the culture medium was then replaced with fresh RPMI-1640 including 10% FBS and 200 μmol/L mimosine for 24 h, in order to arrest the cells at G1-S phase. The arrested cells were then washed twice, and the culture medium was replaced with RPMI-1640 containing normal or HBV-positive serum. The cells were then harvested at different times after mimosine release. All procedures were performed under level P2 biosafety conditions to minimize the possibility of cross-contamination.

Primary culture of human hepatocytes

Hepatocytes were prepared from a 35-year-old healthy male liver donor according to previously described procedures[28]. Briefly, the liver tissues were cut with scissors into 0.1-0.5 mm^3 pieces and were shattered with a 5-mL syringe into single cells or cell aggregates. Cells were seeded into 12-well culture dishes and incubated with 1 mL of 10% FBS in RPMI-1640 at 37℃ under 50 mL/L CO_2. The medium was changed after the first 48 h with serum-free medium. The serum-free medium was composed of DMEM/F12 (1:1) and 0.01 nmol/L nicotinamide, 0.02 ng/L epidermal growth factor (EGF), 0.02 ng/L basic fibroblast growth factor (bFGF), 0.365 ng/L glutamate, B27 (1:50, Sigma), 0.1 U/L penicillin, 0.1 ng/L streptomycin, and 0.1 ng/L fluconazole. HBV-positive serum was added to the culture medium 2 wk after hepatocyte phenotype cell development, and cells were harvested for immunoblotting assay 3 h later.

Immunoblotting assay

Cell extracts were lysed in ice-cold Tris buffer (50 mmol/L, pH 7.5) containing 5 mmol/L EDTA, 300 mmol/L NaCl, 0.1% Igepal, 0.5 mmol/L NaF, 0.5 mmol/L Na_3VO_4, 0.5 mmol/L PMSF, and antiprotease mixture (Roche Molecular Biochemicals) for 30 min on ice and centrifuged at 13 000 *g* for 10 min. The supernatant protein concentration was determined by the Bradford procedure (BioRad). The proteins were resolved on 15% SDS-PAGE and transferred onto nitrocellulose membranes. Blots were blocked in TBST containing 5% non-fat dried milk (NFDM) and incubated with primary antibodies as follows: antibodies against p21, Mre11, ATR, (Santa Cruz) and tubulin (Sigma) were incubated at room temperature for 1 h. Antibodies against ATM phosphoserine 1981 (ATMp), Chk2 phosphothreonine 68 (Chk2p), Chk1 phosphoserine 345 (Chk1p), p53 phosphoserine 15 (p53p) and H2AX phosphoserine 139 (H2AXp) (Cell Signaling) were incubated at 4℃ overnight. Secondary antibodies were from Jackson Laboratories. Horseradish-peroxidase-based detection was performed using a chemiluminescence reagent (Amersham Biosciences), according to the manufacturer's instructions.

DNA damage sensitivity assays

For radiation sensitivity assays, cells were irradiated with the indicated doses of γ rays from a ^{137}Cs source for indicated times, or 254 nm UV light with complete medium plated in triplicate. HBV-positive serum was added to the cultures before indicated doses of UV or IR radiation. Cells were washed extensively to remove viral inputs 24 h after HBV-positive serum addition, and then treated with different dose of UV or IR radiation. After 48 h of UV and 4 d of IR treatment, cells were collected and surviving cells were counted with Trypan blue staining. The percentage survival was determined by quantization of the relative viable number of treated cells divided by the viable number of untreated cells.

Flow cytometry

For propidium iodide staining, cells were harvested by trypsinization, fixed with ice-cold 70% ethanol, and resuspended in a solution containing 50 mg/L propidium iodide, 0.1% Triton X-100, 50 mg/L RNase A, and 5 mmol/L EDTA at room temperature (RT) for 1 h. Cells were then diluted 1:1 in 1% BSA PBS for cytometric analysis.

Immunofluorescence assay

HL7702 cells were grown on glass coverslips for 12 to 18 h prior to infection. At 0.5 h post-infection, cells were washed briefly in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) in PBS for 10 min, then permeabilized for 10 min in 1% Triton X-100 in PBS. For the visualization of detergent-resistant or chromatin-associated nuclear proteins, an *in situ* extraction method that removed the cytoplasm and nucleosolic proteins was used. Cells were pre-extracted for 5 min on ice with 0.5% Triton X-100 in cytoskeletal buffer as described previously[29] then fixed in 4% PFA for 10 min. After washing with PBS and blocking in 2% fetal bovine serum (FBS) in PBS, cells were incubated with primary antibodies diluted in 2% FBS overnight at 4℃. Anti-Chk1p was used at a concentration of 1:100, while anti-ATR was used at 1:400. After washing with PBS three times, cells were incubated with secondary antibodies diluted 1:200 in 2% FBS for 30 min, then washed again three times with PBS. Next, the nuclei were stained with DAPI diluted in PBS for 10 min. After a final wash in PBS, samples were preserved in glycerol and images were captured by using a Zeiss LSM510 confocal microscope.

RESULTS

HBV infection induced a cellular DNA damage response dependent on ATR

To explore whether acute cellular DNA damage response was induced upon HBV infection, we incubated HL7702 hepatocytes with HBV-positive serum to examine the phosphorylation status of DNA damage response proteins. 10^5 HL7702 monolayer cells in a 6-cm plate were infected with HBV-positive serum containing 10^6 HBV at 37℃ under 50 mL/L CO_2. Serum from uninfected individuals was used as a non-infected control. Cells were washed eight times to remove excess viral inputs before harvesting. Whole-cell lysates from the HBV-infected and non-infected cultures were examined for the status of the DNA damage response proteins. Figure 1A reveals that HBV infection induced an increase in the steady state levels of the ATR protein and in the phosphorylation levels of its downstream substrates Chk1, p53 and H2AX. An increase in Chk1 phosphorylation at Ser-345 was evident at 3 h from the start of HBV-positive serum addition, with further increase from 6 h of infection (hoi) to 48 hoi. The phosphorylation of p53 Ser-15 was elevated beginning at 24 hoi and increased greatly at 48 hoi. Finally, there was a sharp increase in the amount of phosphorylated H2AX Ser-139 beginning at 24 hoi. To confirm these results, primary hepatocytes from a healthy male liver donor were obtained and incubated with HBV-positive serum for 3 h; obvious Chk1 phosphorylation was detected in HBV-infected cells (Figure 1B). We then used immunofluorescence to examine the localization of these proteins early after infection. Figure 1C shows that ATR foci as well as the Chk1 phosphorylation foci in non-infected cells were very faint, as opposed to the HBV infected cells where the foci were larger, more numerous and much brighter.

In contrast to ATR and its targets, the phosphorylated form of ATM at Ser-1981 was not visibly increased upon infection, and phosphorylation of its downstream substrate Chk2 at Thr-68 began to decrease starting from 3 hoi (Figure 1A), with slight recovery at later time points, indicating that the p53-independent pathway was blocked. Phosphorylation of p53 at Ser-15 in response to DNA damage usually correlates with the ability of p53 to trans-activate downstream target genes. Therefore, we examined expression levels of p53 transcriptional targets $p21^{cip1/waf1}$, a cyclin-dependent kinase inhibitory protein. Figure 1A

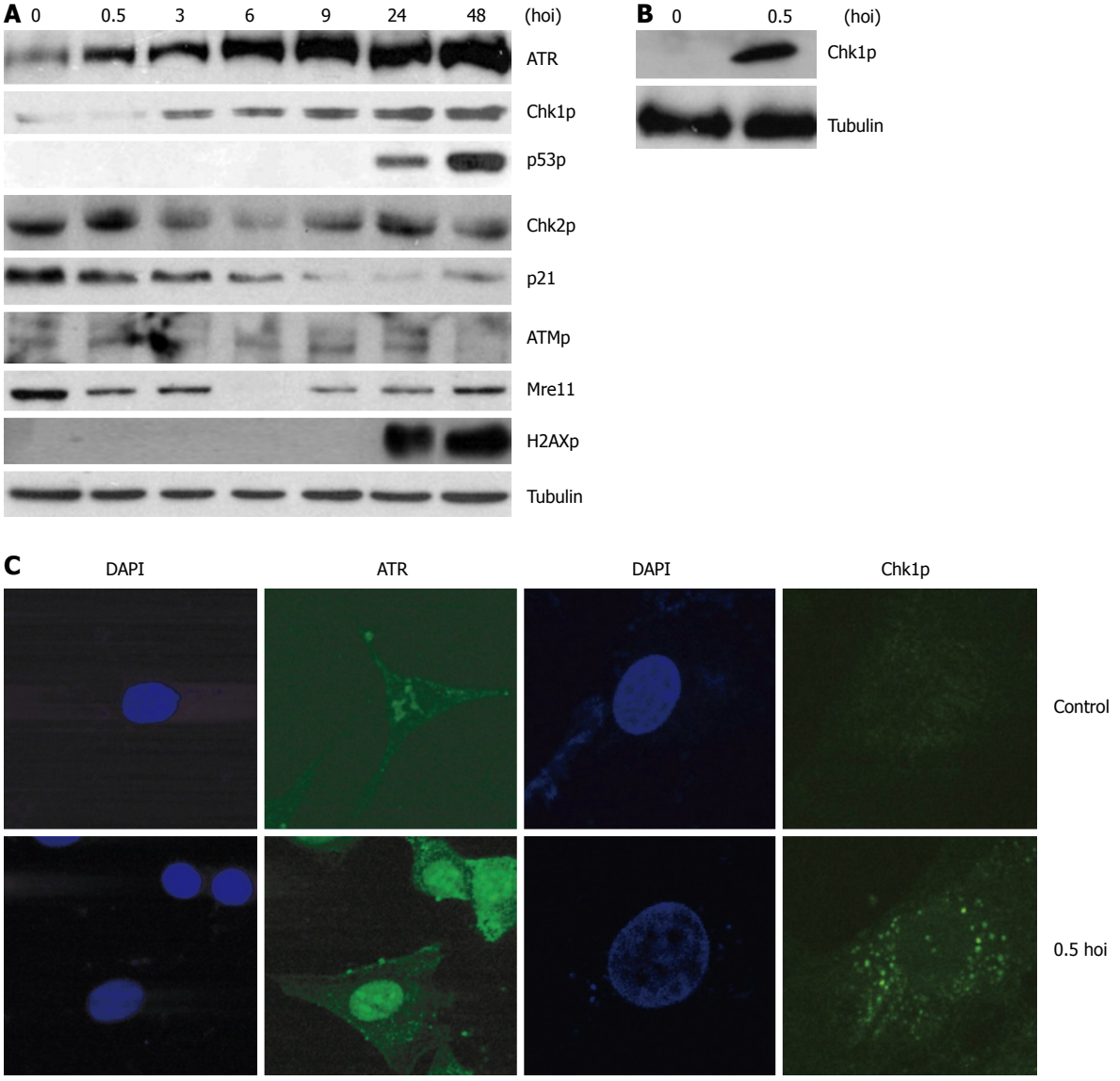


Figure 1 HBV infection activates a cellular checkpoint response dependent on ATR. A: 105 human hepatocyte HL7702 monolayer cells in a 6-cm plate were infected with 106 virus particles from HBV-positive patients at 37℃ under 50 mL/L CO_2; normal serum from healthy individuals was used as a non-infected control. Prior to cell harvesting, the cells were washed eight times thoroughly to remove excess viral input. Whole-cell lysates were prepared at various times of infection (hoi) and subjected to an immunoblotting assay by using antibodies against the indicated proteins. Tubulin was used as the equal loading control; B: Primarily cultured hepatocyte cells were prepared and were harvested 3 h after HBV-positive serum addition. Whole cell lysates were prepared and subjected to immunoblotting assay by using Chk1 phosphorylation antibody; tubulin was used as the equal loading control; C: HL7702 cells were infected with HBV-positive serum for 0.5 h. Normal serum from healthy individuals was used as a non-infected control. Immunofluorescence with antibodies to Chk1 Ser-345 and ATR (green) were monitored. DNA was stained with DAPI (blue).

shows that the amount of p21 decreased substantially with time after infection, suggesting that p53-dependent downstream signaling was blocked during HBV infection, despite the appearance of phosphorylated p53. By 0.5 h after HBV-positive serum addition, downregulation of Mre11 began to be detected. This reduction in Mre11 protein occurs shortly after HBV infection, implying that an incoming virion protein may lead to this degradation. Further investigation is required to explain this phenomenon.

Since HBV infection activated DNA damage checkpoint pathway that responded to replication stress, we asked if HBV infection would effect cell cycle progression and cell death. Cells were synchronized in G1 phase by mimosine, an inhibitor of DNA synthesis initiation, for 24 h. Arrested cells were then washed twice and media replaced with RPMI-1640 containing HBV serum or normal serum. Cells were harvested at different times after mimosine release. The results of fluorescence-activated cell sorting (FACS) analysis showed that approximately 5% of the cells were in early S phase with 95% in G1 after synchronization (Figure 2A), indicating that the synchronization was fulfilled. Control cells treated with normal serum had approximately 44.4% of cells in S phase after 12 h release from mimosine, whereas cells treated with HBV-positive serum still had 56.6% of cells in S phase (Figure 2A and B). The percentage of total cells in S phase indicates the effect of virus in extending the period of DNA synthesis. Toward the end of the cell cycle, there was an accumulation of cells

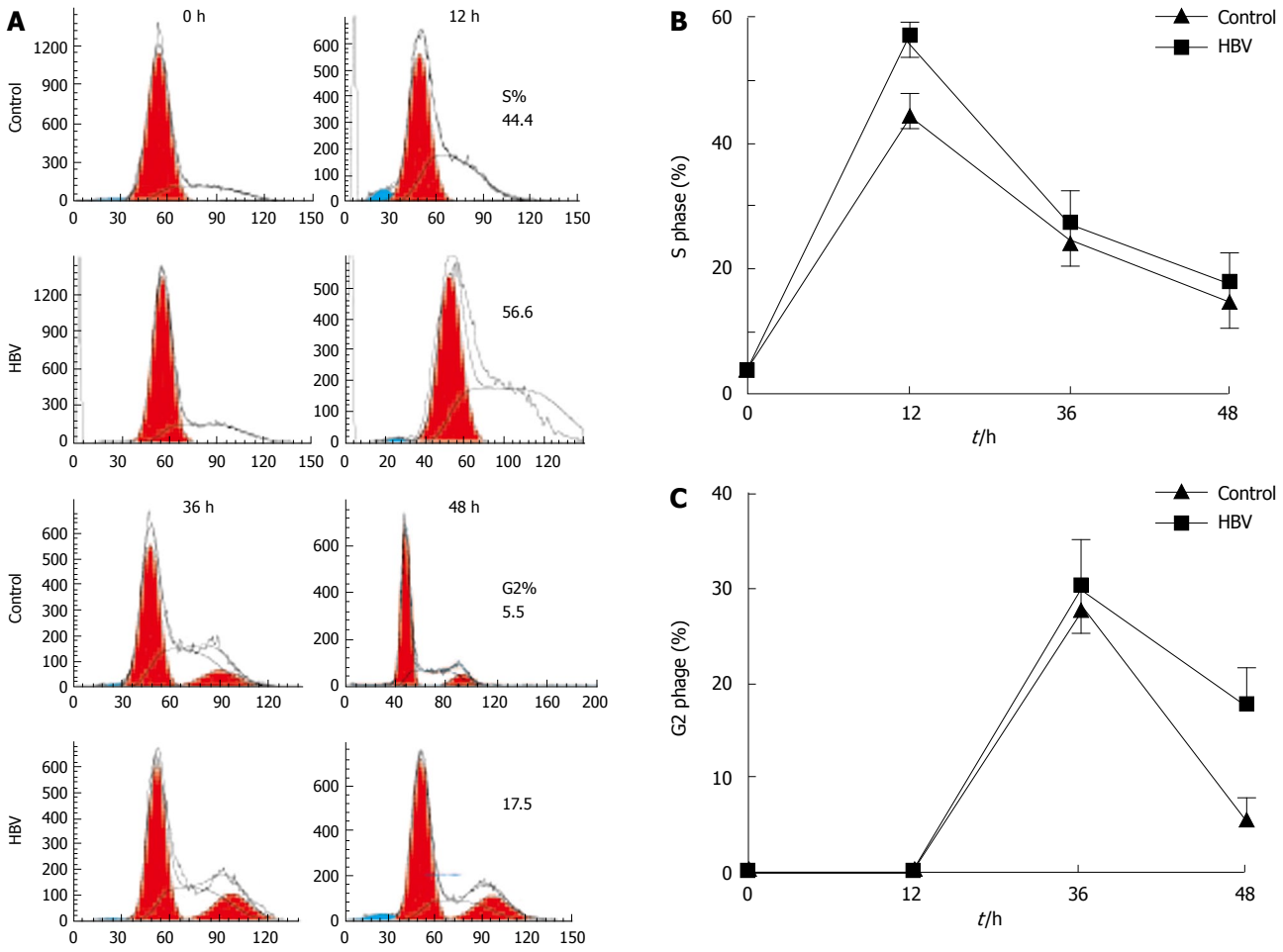


Figure 2 HBV infection caused transient cell cycle arrest in the S and the G2 phase. The duration of the S and G2 phase were measured in HBV-infected or non-infected cells. Cells were synchronized with mimosine for 24 h, and then media were replaced with RPMI-1640 containing 10% HBV serum or normal serum. At indicated times, cells were harvested and examined for cell cycle profile using propidium iodide staining and flow cytometry. A: Cell cycle profile in HBV-infected cells and in non-infected cells; B: Percentages of cells in the S phase; C: Percentages of cells in the G2 phase. Mean and standard error are presented for three independent experiments.

in G2 phase in HBV-infected cells (5.5% for control cells versus 17.5% for HBV infection cells) without the appearance of fragments with less than 2N DNA (Figure 2A and C). These findings indicate that DNA damage pathway responding to replication stress induced by HBV infection will lead to transient cell cycle arrest without accompanying increased apoptosis.

Checkpoint signaling pathway was compromised in HBV-infected cells

Previous results show that HBV infection did not show increased apoptosis even though a cellular DNA damage response dependent on ATR was activated (Figure 2A). To better understand the consequences of the ATR signaling pathway triggered by HBV infection, we examined if HBV infection had an impact on host cell survival after IR and UV radiation. Cells were washed extensively to remove viral inputs 24 h after HBV-positive serum addition and then treated with different doses of UV or IR radiation. After 48 h of UV and 4 d of IR treatment, cells were collected and surviving cells were counted with Trypan blue staining. Figure 3A and B showed that the survival rate of UV-irradiated cells was enhanced by HBV infection, while the survival rate of IR-irradiated cells was reduced by HBV infection. Figure 3C shows that while the cell survival rate after 8 s of UV or 4 Gy of IR was about 30%, the survival rate of HBV-infected cells after 8 s of UV radiation increased from 30% to 49%. By contrast, HBV infection pre-treatment with 4 Gy of IR radiation reduced the cell survival rate from 31% to 21%, suggesting that survival of UV-treated cells was enhanced by HBV infection (Figure 3C). Based on this result, we analyzed the difference in apoptosis between UV radiation only and combined treatment of UV with HBV infection. Decreased apoptosis was seen in combined treatment cells (Figure 4A and B). Twenty-four percent apoptosis was seen in UV-radiation-only cells, while about 8% apoptosis was detected in cells treated with UV radiation followed by HBV infection. It is known that cell cycle checkpoint triggered by DNA damage response induces cell apoptosis if damaged DNA cannot be repaired. The inhibitory effect of HBV on cell survival was reduced in cells treated with UV, indicating that checkpoint signaling controlled by the ATR DNA damage pathway was at least partially compromised in HBV-infected cells; therefore, the ATR signal pathway did not function completely.

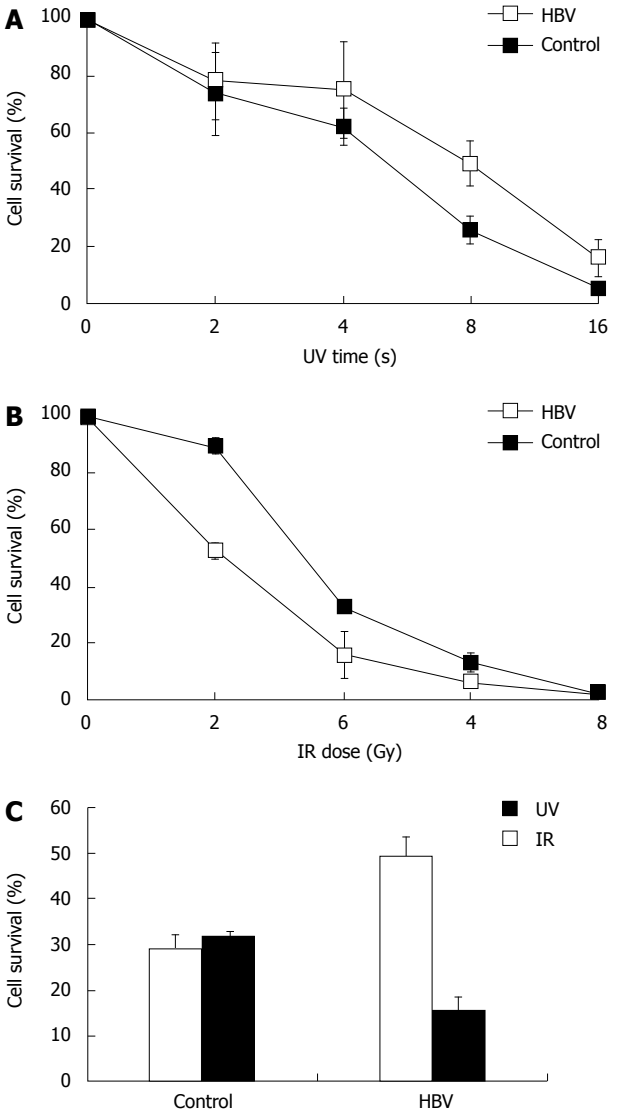


Figure 3 HBV infection hypersensitizes host cells to IR and causes hyper-resistance of host cells to UV. A: HL7702 cells were treated with indicated doses of IR followed HBV positive serum addition for 24 h, and continued in culture for another 4 d, trypan blue staining was used for viable cell counting; B: HL7702 cells were treated with indicated doses of UV followed by HBV-positive serum addition for 24 h, and continued culture for another 48 h, trypan blue staining was used for viable cell counting; C: HL7702 cells were treated with 8 s of UV or 4 Gy of IR followed HBV infection, and viable cells were counted. Percentage survival was determined by the number of treated cells normalized to untreated cells. Mean and standard error are presented for three independent experiments.

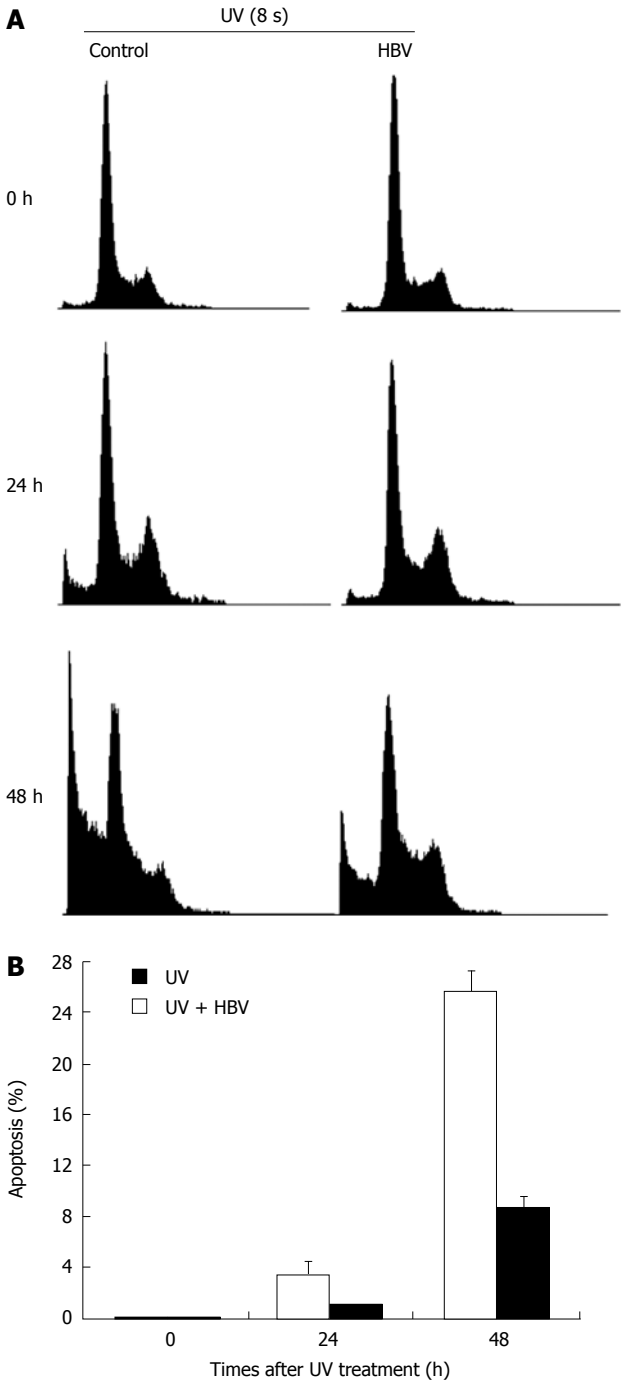


Figure 4 HBV infection followed by UV radiation led to decreased cell apoptosis. A: HBV-positive serum was added to the culture medium before UV radiation treatment. Cells were washed extensively 24 h after HBV-positive serum addition and then treated with 8 s of UV radiation. Cell cycle profile was examined by propidium iodide staining and flow cytometry at indicated times after UV treatment; B: Apoptosis percentage after UV radiation followed HBV infection. Mean and standard error are presented for three independent experiments.

DISCUSSION

The induction of cell cycle checkpoints and activation of the ATM/ATR-dependent pathway have been reported to accompany infection by a number of different viruses. Recent reports have shown that viruses have evolved a variety of mechanisms to manipulate DNA damage signaling for their replication and propagation. In this study, HL7702 cells and primarily cultured hepatocytes were inoculated with HBV-positive serum (10^6 particles per 10^5 cells), mimicking the HBV infection process. Serum from healthy individuals was used as a non-infected control. We propose that HBV infection induces acute cellular DNA damage response dependent on ATR, as demonstrated by ATR protein and increased phosphorylation of Chk1, p53 and H2AX. Since Chk1 phosphorylation, ATR foci formation, and Mre11 and p21 degradation happened shortly after HBV-positive serum addition, we propose that incoming virion protein and genetic materials triggered this response. p53 and

H2AX phosphorylation did not begin to accumulate until 24 h after HBV serum addition, implying HBV replication inside the infected cells may be responsible for these phenomena. Interesting questions are raised and need further investigation.

Although ATM/ATR kinase phosphorylates Chk2 at Thr-68 and p53 at Ser-15, the ATR kinase predominantly targets Chk1 at Ser-345, leading to increased Chk1 activity. Our results indicate that HBV infection preferentially activates ATR DNA damage response signaling, as is the case with human adeno-associated-virus type 2[30-36].

As a latent virus, HBV abrogates checkpoint signaling controlled by ATR, to prevent triggering of signals for apoptosis in multiple ways. The mechanism of regulation of apoptosis by HBV was *via* both p53-dependent and p53-independent pathways. p53-dependent cell cycle checkpoint features p21-mediated inactivation of cdk2/cyclinE; HBV abrogates p53 dependent checkpoint activation by p21 degradation. Chk2 inhibition inhibits cdk2/cyclin E activity by phosphorylation of cdk2 at Tyr-15 in a p53-independent fashion, and the virus-decreased phosphorylation of Chk2 by Mre11 degradation inhibits the p53-independent DNA damage signaling pathway. It is known that ATM and MRN complex function in a common pathway, and the MRN complex can function to activate ATM kinase activity, so degradation of Mre11 protein by virus would inhibit ATM kinase activity, and thus affect phosphorylation of its downstream target Chk2. Therefore, like adenovirus[24,25], HBV appears to have evolved double check mechanisms to block cell cycle checkpoint signaling pathways. Consistent with this, HBV has been reported to express an additional anti-apoptotic gene HBV X that mislocates p53 to the cytoplasm, in order to evade host cellular DNA damage response and modulate apoptosis. HBX has also been reported to sensitize liver cells to environmental carcinogens, including diethylnitrosamine and aflatoxin B and UV. We observed that HBV virus enhanced cell survival upon UV radiation but hypersensitized host cells to IR; this discrepancy may be due to the complex interactions between the virus as a whole and DNA repair machinery, indicating that the virus mainly blocks cellular signaling checkpoint dependent on ATR. The risk of acquiring mutations would be enhanced by compromised cellular DNA repair caused by HBV infection. Accordingly, exposure to other environmental risk factors should act synergistically to favor the carcinogenesis process[37-39].

In summary, HBV induces cellular DNA damage response dependent on ATR, but escapes the consequences of activation of the DNA damage checkpoint by degradation of checkpoint proteins at different levels. The implication of this is that with time, persistent HBV infection may lead to the accumulation of a variety of mutations which would ultimately give rise to hepatocellular carcinoma.

COMMENTS

Background

Eukaryotic cells employ multiple strategies of checkpoint signaling and DNA repair mechanisms to monitor and repair damaged DNA. There are two branches of the checkpoint response pathway, ataxia telangiectasia-mutated (ATM) pathway and ATM-Rad3-related (ATR) pathway. Virus replication presents the host cells with large amounts of exogenous genetic material, including DNA ends and unusual structures. Thus, infected cells recognize viral replication as a DNA damage stress and elicit DNA damage signal transduction, which ultimately induces apoptosis as part of host immune surveillance. However, recent reports have shown that viruses evolve a variety of mechanisms to manipulate DNA damage signaling for their replication and propagation.

Research frontiers

Epstein-Barr virus (EBV) abrogates p53 checkpoint signaling pathway through the interaction of the BZLF1 protein and p53 to avoid apoptosis. Other viruses such as human immunodeficiency virus type 1 (HIV-1), herpes simplex virus type 1 (HSV-1) and human cytomegalovirus can activate and exploit a cellular DNA damage response, which aids viral replication. Adenovirus blocks ATM signaling and concatemer formation through targeting the DNA repair complex of MRN for degradation and mislocalization. Thus, under some circumstances, viruses have co-opted endogenous checkpoint regulators to ensure their own efficient replication.

Innovations and breakthroughs

There was no evidence indicating that the ATM/ATR kinase or their downstream pathways were triggered by hepatitis B virus (HBV) infection. The present study was undertaken by culturing normal hepatocyte cell line HL7702 and primary hepatocytes from a healthy liver donor with HBV-positive serum, mimicking natural HBV infection. We showed, perhaps for the first time, that HBV infection elicited acute cellular DNA damage response dependent on ATR. However, the ATR checkpoint signaling was blocked downstream of p53-dependent and p53-independent pathways to evade apoptosis.

Applications

Since DNA damage response is an acute response that happens quickly after virus infection, we assume that early intervention of the DNA damage pathway will function more efficiently and can be used clinically as HBV infection therapy during its early infectious stage or fulminant HBV infection.

Peer review

In this interesting study the authors investigated whether exposure to HBV infection will upregulate DNA damage checkpoint signaling pathways. They show that ATM-Rad3 is upregulated as well as several downstream targets.

REFERENCES

1 **Shiloh Y**. ATM and related protein kinases: safeguarding genome integrity. *Nat Rev Cancer* 2003; **3**: 155-168
2 **Sancar A**, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annu Rev Biochem* 2004; **73**: 39-85
3 **Bakkenist CJ**, Kastan MB. Initiating cellular stress responses. *Cell* 2004; **118**: 9-17
4 **Lim DS**, Kim ST, Xu B, Maser RS, Lin J, Petrini JH, Kastan MB. ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway. *Nature* 2000; **404**: 613-617
5 **Brown EJ**, Baltimore D. Essential and dispensable roles of ATR in cell cycle arrest and genome maintenance. *Genes Dev* 2003; **17**: 615-628
6 **Lee JH**, Paull TT. ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. *Science* 2005; **308**: 551-554
7 **Limoli CL**, Giedzinski E, Bonner WM, Cleaver JE. UV-induced replication arrest in the xeroderma pigmentosum variant leads to DNA double-strand breaks, gamma -H2AX formation, and Mre11 relocalization. *Proc Natl Acad Sci USA* 2002; **99**: 233-238
8 **Helt CE**, Cliby WA, Keng PC, Bambara RA, O'Reilly MA. Ataxia telangiectasia mutated (ATM) and ATM and Rad3-related protein exhibit selective target specificities in response to different forms of DNA damage. *J Biol Chem* 2005; **280**: 1186-1192
9 **Zhao H**, Piwnica-Worms H. ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. *Mol Cell Biol* 2001; **21**: 4129-4139
10 **Guo Z**, Kumagai A, Wang SX, Dunphy WG. Requirement for Atr in phosphorylation of Chk1 and cell cycle regulation

in response to DNA replication blocks and UV-damaged DNA in Xenopus egg extracts. *Genes Dev* 2000; **14**: 2745-2756
11 **Liu Q**, Guntuku S, Cui XS, Matsuoka S, Cortez D, Tamai K, Luo G, Carattini-Rivera S, DeMayo F, Bradley A, Donehower LA, Elledge SJ. Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. *Genes Dev* 2000; **14**: 1448-1459
12 **Bendjennat M**, Boulaire J, Jascur T, Brickner H, Barbier V, Sarasin A, Fotedar A, Fotedar R. UV irradiation triggers ubiquitin-dependent degradation of p21(WAF1) to promote DNA repair. *Cell* 2003; **114**: 599-610
13 **Uziel T**, Lerenthal Y, Moyal L, Andegeko Y, Mittelman L, Shiloh Y. Requirement of the MRN complex for ATM activation by DNA damage. *EMBO J* 2003; **22**: 5612-5621
14 **Carson CT**, Schwartz RA, Stracker TH, Lilley CE, Lee DV, Weitzman MD. The Mre11 complex is required for ATM activation and the G2/M checkpoint. *EMBO J* 2003; **22**: 6610-6620
15 **Stiff T**, Reis C, Alderton GK, Woodbine L, O'Driscoll M, Jeggo PA. Nbs1 is required for ATR-dependent phosphorylation events. *EMBO J* 2005; **24**: 199-208
16 **Kudoh A**, Fujita M, Zhang L, Shirata N, Daikoku T, Sugaya Y, Isomura H, Nishiyama Y, Tsurumi T. Epstein-Barr virus lytic replication elicits ATM checkpoint signal transduction while providing an S-phase-like cellular environment. *J Biol Chem* 2005; **280**: 8156-8163
17 **Daniel R**, Kao G, Taganov K, Greger JG, Favorova O, Merkel G, Yen TJ, Katz RA, Skalka AM. Evidence that the retroviral DNA integration process triggers an ATR-dependent DNA damage response. *Proc Natl Acad Sci USA* 2003; **100**: 4778-4783
18 **Zimmerman ES**, Sherman MP, Blackett JL, Neidleman JA, Kreis C, Mundt P, Williams SA, Warmerdam M, Kahn J, Hecht FM, Grant RM, de Noronha CM, Weyrich AS, Greene WC, Planelles V. Human immunodeficiency virus type 1 Vpr induces DNA replication stress in vitro and in vivo. *J Virol* 2006; **80**: 10407-10418
19 **Roshal M**, Kim B, Zhu Y, Nghiem P, Planelles V. Activation of the ATR-mediated DNA damage response by the HIV-1 viral protein R. *J Biol Chem* 2003; **278**: 25879-25886
20 **Lau A**, Swinbank KM, Ahmed PS, Taylor DL, Jackson SP, Smith GC, O'Connor MJ. Suppression of HIV-1 infection by a small molecule inhibitor of the ATM kinase. *Nat Cell Biol* 2005; **7**: 493-500
21 **Wilkinson DE**, Weller SK. Herpes simplex virus type I disrupts the ATR-dependent DNA-damage response during lytic infection. *J Cell Sci* 2006; **119**: 2695-2703
22 **Lilley CE**, Carson CT, Muotri AR, Gage FH, Weitzman MD. DNA repair proteins affect the lifecycle of herpes simplex virus 1. *Proc Natl Acad Sci USA* 2005; **102**: 5844-5849
23 **Gaspar M**, Shenk T. Human cytomegalovirus inhibits a DNA damage response by mislocalizing checkpoint proteins. *Proc Natl Acad Sci USA* 2006; **103**: 2821-2826
24 **Liu Y**, Shevchenko A, Shevchenko A, Berk AJ. Adenovirus exploits the cellular aggresome response to accelerate inactivation of the MRN complex. *J Virol* 2005; **79**: 14004-14016
25 **Stracker TH**, Carson CT, Weitzman MD. Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. *Nature* 2002; **418**: 348-352
26 **Shin YC**, Nakamura H, Liang X, Feng P, Chang H, Kowalik TF, Jung JU. Inhibition of the ATM/p53 signal transduction pathway by Kaposi's sarcoma-associated herpesvirus interferon regulatory factor 1. *J Virol* 2006; **80**: 2257-2266
27 **Liang X**, Pickering MT, Cho NH, Chang H, Volkert MR, Kowalik TF, Jung JU. Deregulation of DNA damage signal transduction by herpesvirus latency-associated M2. *J Virol* 2006; **80**: 5862-5874
28 **Lin M**, Chen Q, Yang LY, Li WY, Cao XB, Wu JR, Peng YP, Chen MR. Hepatitis B virus infection and replication in primarily cultured human fetal hepatocytes. *World J Gastroenterol* 2007; **13**: 1027-1031
29 **Dimitrova DS**, Gilbert DM. Stability and nuclear distribution of mammalian replication protein A heterotrimeric complex. *Exp Cell Res* 2000; **254**: 321-327
30 **Nunnari G**, Argyris E, Fang J, Mehlman KE, Pomerantz RJ, Daniel R. Inhibition of HIV-1 replication by caffeine and caffeine-related methylxanthines. *Virology* 2005; **335**: 177-184
31 **Ward IM**, Chen J. Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. *J Biol Chem* 2001; **276**: 47759-47762
32 **Weitzman MD**, Carson CT, Schwartz RA, Lilley CE. Interactions of viruses with the cellular DNA repair machinery. *DNA Repair* (Amst) 2004; **3**: 1165-1173
33 **Everett RD**. Interactions between DNA viruses, ND10 and the DNA damage response. *Cell Microbiol* 2006; **8**: 365-374
34 **Lilley CE**, Schwartz RA, Weitzman MD. Using or abusing: viruses and the cellular DNA damage response. *Trends Microbiol* 2007; **15**: 119-126
35 **Jurvansuu J**, Raj K, Stasiak A, Beard P. Viral transport of DNA damage that mimics a stalled replication fork. *J Virol* 2005; **79**: 569-580
36 **Hoeijmakers JH**. Genome maintenance mechanisms for preventing cancer. *Nature* 2001; **411**: 366-374
37 **Lee AT**, Ren J, Wong ET, Ban KH, Lee LA, Lee CG. The hepatitis B virus X protein sensitizes HepG2 cells to UV light-induced DNA damage. *J Biol Chem* 2005; **280**: 33525-33535
38 **Leupin O**, Bontron S, Schaeffer C, Strubin M. Hepatitis B virus X protein stimulates viral genome replication via a DDB1-dependent pathway distinct from that leading to cell death. *J Virol* 2005; **79**: 4238-4245
39 **White E**. Mechanisms of apoptosis regulation by viral oncogenes in infection and tumorigenesis. *Cell Death Differ* 2006; **13**: 1371-1377

S- Editor Zhong XY **L- Editor** Li M **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6171

World J Gastroenterol 2008 October 28; 14(40): 6171-6179
World Journal of Gastroenterology ISSN 1007-9327

CLINICAL RESEARCH

Does an association exist between chronic pancreatitis and liver cirrhosis in alcoholic subjects?

Luis Aparisi, Luis Sabater, Juan Del-Olmo, Juan Sastre, Miguel-Angel Serra, Ricardo Campello, Daniel Bautista, Abdalla Wassel, José-Manuel Rodrigo

Luis Aparisi, Juan Del-Olmo, Miguel-Angel Serra, Abdalla Wassel, José-Manuel Rodrigo, Liver Unit, Universitary Clinic Hospital, Valencia 46010, Spain
Luis Sabater, Department of Surgery, Universitary Clinic Hospital, Valencia 46010, Spain
Juan Sastre, Department of Physiology, University of Valencia, Valencia 46010, Spain
Ricardo Campello, Unit of Psiquiatry, Servicio Valenciano de Salud, Valencia 46010, Spain
Daniel Bautista, Department of Preventive Medicine, Hospital Dr. Peset, Valencia 46010, Spain
Author contributions: Aparisi L, Sabater L and Sastre J contributed to the research design and preparation of the manuscript; Aparisi L, Del-Olmo J, Serra MA, Campello R, Wassel A and Rodrigo JM did patient evaluation and data collection; Bautista D made statistical analysis.
Supported by Grants SAF2006-06963 and CSD2007-00020 from Ministerio de Educación y Ciencia
Correspondence to: Dr. Luis Aparisi, Liver Unit, Hospital Clínico Universitario, Avda. Blasco Ibañez 17, Valencia 46010, Spain. aparisi_lui@gva.es
Telephone: +34-963862600 Fax: +34-963862644
Received: April 15, 2008 Revised: June 23, 2008
Accepted: June 30, 2008
Published online: October 28, 2008

Abstract

AIM: To study the possible association between chronic pancreatitis (CP) and liver cirrhosis (LC) of alcoholic etiology, after excluding any other causes.

METHODS: One hundred and forty consecutive alcoholic patients were subdivided into three groups: CP (n = 53), LC (n = 57), and asymptomatic alcoholic (n = 30). Clinical, biochemical and morphological characteristics, Child-Pugh index, indocyanine green test, and fecal pancreatic elastase-1 test were assessed.

RESULTS: In patients with cirrhosis, major clinical manifestations of CP such as pancreatic pain and steatorrhea, as well as imaging alterations of CP such as calcifications, duct dilation and pseudocysts were absent; insulin-dependent diabetes was present in 5.3% of cases, and elastase-1 test was altered in only 7%, and severely altered in none. In patients with CP, clinical characteristics of cirrhosis such as ascites, encephalopathy and gastrointestinal hemorrhage were present in one case, Child-Pugh grade > A in 5.7%, and altered indocyanine green test in 1.9% cases. In asymptomatic alcoholism, there was only a non-coincident alteration of elastase-1 test and indocyanine test in 14.8% and 10%, respectively, but other characteristics of cirrhosis or CP were absent. An inverse correlation (r = -0.746) between elastase-1 test and indocyanine test was found in alcoholic patients.

CONCLUSION: There is a scarce coincidence in clinical and morphological alterations among patients with CP or LC of alcoholic etiology, but an inverse correlation between pancreatic and liver function tests. These findings support that these alcoholic diseases evolve in a different manner and have different etiopathogenesis.

Key words: Alcoholic chronic pancreatitis; Alcoholic liver cirrhosis; Alcoholism; Pancreatic function; Hepatic function

Peer reviewer: Katsutoshi Yoshizato, PhD, Phoenixbio Co. Ltd., 3-4-1, Kagamiyama, Higashihiroshima 739-0046, Japan

Aparisi L, Sabater L, Del-Olmo J, Sastre J, Serra MA, Campello R, Bautista D, Wassel A, Rodrigo JM. Does an association exist between chronic pancreatitis and liver cirrhosis in alcoholic subjects? *World J Gastroenterol* 2008; 14(40): 6171-6179 Available from: URL: http://www.wjgnet.com/1007-9327/14/6171.asp DOI: http://dx.doi.org/10.3748/wjg.14.6171

INTRODUCTION

Chronic alcoholism is a well-known etiologic factor associated with chronic and irreversible pancreatic and liver disorders. Excessive alcohol consumption is the most frequent cause of chronic pancreatitis (CP) in Western countries[1]. There is a correlation between increased ethanol consumption over many years and the risk of developing CP[2,3]. Nevertheless, it has recently been estimated that < 5% of alcoholic subjects develop CP[1,4,5]. This low percentage, together with the absence of adequate experimental models of alcoholic CP, suggest that ethanol is only a cofactor in the development of CP, and therefore other predisposing

factors may be involved[6].

An excessive consumption of alcohol is also associated with liver cirrhosis (LC), again with a correlation between increased ethanol consumption and the risk of LC[7,8]. The development of LC also requires chronic alcoholism over several years[8,9]. For decades, it was considered that around 10%-35% of subjects with chronic alcoholism developed alcoholic LC[10]. However, more recent prospective studies have shown that the prevalence in alcoholics is indeed much lower, around 2%[7,8], once having excluded the confusing cases of hepatitis B or C[11,12]. Experimental models of chronic alcoholism have not provided insights into the pathophysiological mechanisms responsible for the different outcome in CP and LC[13].

There is still controversy about the frequency of coincidence between CP and LC in alcoholic patients. This lack of coincidence may rely on differences in methodology, retrospective *versus* prospective studies[14], and evaluated parameters such as clinical[15], functional[16], imaging[17] or histopathological[18,19]. In addition, there are diagnostic difficulties in the initial stages[20], as well as several confounders hindering this question, such as the presence of hepatitis B or C virus[14,19], and age-related pancreatic alterations[21,22].

It is worth noting that pancreatic function is often increased in patients with alcoholic LC, leading to the hypersecretory status of the pancreas[16,23]. Thus, Hayakawa *et al*[24] have reported that pancreatic secretion, measured with the pancreozymine-secretine test, increases with severity of liver damage in alcoholic liver disease. It has been suggested that this hypersecretory status diminishes protein and calcium concentration in pancreatic juice, protecting against formation of protein plugs and pancreatic stones[16].

In clinical practice, the coincidence of both diseases, LC and CP, is rare[15,24]. Furthermore, these two diseases do not share risk factors apart from alcohol consumption[15]. In alcoholic CP, the duration of chronic alcoholism is shorter than in LC, and liver disease is found at older ages[14]. Nakamura *et al*[15] have recently assessed the genotypes of alcohol dehydrogenase (ADH), ductal anatomy by endoscopic retrograde cholangiopancreatography (ERCP) and the Child-Pugh classification in Japanese alcoholics, and reported a lack of association between the risk of LC and CP. In addition, no clear genetic predisposition has been found so far in studies of genetic polymorphisms in alcoholic CP[25] and alcoholic LC[26].

To the best of our knowledge, there are no previous studies comparing clinical, functional, morphological and biochemical parameters in alcoholic subjects with a definitive diagnosis of CP or LC. In this study, we therefore aimed to assess the association of CP and LC, both with definitive diagnosis, in subjects with chronic alcoholism, based on clinical, functional and morphological parameters.

MATERIALS AND METHODS

Case-control study with two groups of consecutive patients who attended the University Clinic Hospital of Valencia, Spain for 3 years included: (1) alcoholic CP (n = 53); and (2) alcoholic LC (n = 57). Controls were 30 asymptomatic alcoholic (ASA) patients. The diagnosis of CP was based on the Cambridge and Marseille criteria[27,28]. The diagnosis of cirrhosis was histologically proven in 27 patients and based on compatible clinical, laboratory and ultrasonographic finding in the others[29]. Daily ethanol intake was over 40 g for more than 5 years in alcoholic subjects[7,8] and before entering the study a period of 60 d of alcohol abstinence was required. Exclusion criteria for alcoholic LC were the presence of antigens or antibodies against B or C virus, as well as liver diseases not related to chronic alcoholism such as hemochromatosis or any other causes. Exclusion criteria for CP were the presence of toxic-metabolic (other than alcohol), genetic, autoimmune, and obstructive factors considered as confounders[6]. Other exclusion criteria for the study were gastric or liver surgery, pancreatic surgery prior to diagnosis, and gastrointestinal neoplasia. Smoking was considered when more than 10 cigarettes were consumed daily[30,31].

Characteristic manifestations of LC[13] were assessed in all patients, including hemorrhage in the digestive tract secondary to portal hypertension, ascites, hepatic encephalopathy, alterations of the Quick index, and Child-Pugh index (PI)[32]. PI was classified according to the following scoring system: A (5-6 score), B (7-9 score), and C (10-15 score)[32]. The following characteristic manifestations of CP[27,28] were also assessed in all patients: pancreatic pain, non-insulin-dependent diabetes mellitus (NIDDM), insulin-dependent diabetes mellitus (IDDM), and chronic diarrhea/steatorrhea. Liver function was assessed by indocyanine green clearance test (ICG test). Pancreatic function was assessed by determination of fecal pancreatic elastase-1 test (E1 test) and oral glucose tolerance test (OGTT). The presence of esophageal varices, splenomegaly, portal hypertension, pancreatic calcifications, pseudocysts or alterations of the pancreatic duct were assessed by ultrasonography (US), computer tomography (CT), magnetic resonance cholangiopancreatography (MRCP), or ERCP. Splenomegaly was considered when the size of the spleen was $\geqslant$ 135 mm. Portal hypertension was established according to the following criteria: Portal vein diameter > 12 mm and/or presence of esophageal varices and/or splenomegaly. Alterations in the Quick index were established when the international normalized ratio (INR) was higher than 1.2[33].

The ICG test was performed by pulsidensitometry (Limon PC5000, Pusion Medical Systems, Munich RFA). Briefly, ICG was intravenously administered at a dose of 0.5 mg/kg, monitoring the blood concentration of ICG to determine the plasma disappearance rate (PDR, %/min). It was considered that the ICG clearance was altered when PDR was < 15%, and severely altered when PDR was < 10%[34,35].

Pancreatic fecal elastase-E1 activity was determined by ELISA (Pancreatic Elastase stool test, Schebo-Biotech, Giessen RFA). E1 activity was considered altered when it was < 200 μg/g, and severely altered when it was <

Table 1 General characteristic of patients with ACP, ALC or ASA

	ACP (n = 53)	ALC (n = 57)	ASA (n = 30)	P
Age at the beginning of the study	51.8 ± 9.7	56.1 ± 9.9	49.9 ± 7.9	CP *vs* LC, CP *vs* ASA, NS; LC *vs* ASA, P = 0.010
Men (%)	98	84	70	CP *vs* LC, P = 0.011; CP *vs* ASA, P = 0.001; LC *vs* ASA, NS
Age at diagnosis	39 ± 9.2	51.5 ± 9.6	-	P = 0.001
Alcohol intake (g/d)	120 ± (75-250)	118 (70-255)	121 (70-400)	NS
Years of alcoholism	20 (10-35)	25 (10-52)	18.5 (9-36)	CP *vs* LC, P = 0.01; LC *vs* ASA, P = 0.006; CP *vs* ASA, NS
Ethanol, kg of total intake	876 (273-2920)	1095 (481-3832)	965 (229-3358)	NS
Smoking	94.3%	80.7%	96.7%	CP *vs* LC, P = 0.030; CP *vs* ASA, NS; LC *vs* ASA, P = 0.035
BMI	23.4 ± 3.6	26.5 ± 4.4	24.8 ± 4.6	CP *vs* LC, P = 0.001

Data are expressed as mean ± SD, percentage or median (minimum-maximum). NS: Not significant.

100 μg/g[36] in at least two consecutive determinations. Fecal fat was determined by the van de Kamer method[37].

The body mass index (BMI) was measured in all subjects as index of nutritional status. BMI values < 20 were considered malnutrition, whereas values > 28 were considered as indicative of being overweight[38].

Statistical analysis

Proportions for qualitative variables and mean ± SD for quantitative variables were calculated. Means were compared by t test or ANOVA and post-hoc Scheffe test. According to the Kolmogorov-Smirnov test, when quantitative variables did not fit a normal distribution, medians and ranges and consequently Kruskal-Wallis test were used. Qualitative variables were analyzed by χ^2 test or Fisher's exact test when appropriate. Odds ratios (ORs) with 95% confidence intervals (95% CI) were also calculated. Spearman's rank correlation coefficient between E1 test and indocyanine green test (ICG test) or PI was calculated. $P < 0.05$ was considered statistically significant.

RESULTS

General characteristics of patients: Alcohol consumption, smoking, and nutritional status

Table 1 shows the general characteristics of each group of subjects. Although the age of patients when entering the study was not different between CP and LC, the age at the moment of diagnosis was significantly lower in CP than LC patients. Most patients were men in all groups, especially in the CP group.

Regarding the alcohol consumption, the daily alcohol intake did not differ among groups. The percentages of heavy drinkers (daily consumption > 150 g) were 30.2%, 33.3%, and 45.0% for CP, LC and ASA groups, respectively, without significant differences among the groups. The duration of alcohol consumption was lower in CP than in LC patients. However, there was no significant difference between CP and LC in the percentage of patients with more than 20 years of alcohol consumption (41.6% and 52.6% for CP and LC subjects, respectively). The percentage of patients with more than 20 years of alcohol consumption was significantly higher in LC than in ASA patients (52.6% *vs* 33.7%). The total amount of ethanol consumed before diagnosis did not differ among groups (Table 1).

Table 2 Potential risk factors for CP or LC: Distribution of frequencies and OR in patients with ACP or ALC n (%)

	ACP 53	ALC 57	OR (95% CI)	P
Gender (female)	1 (1.9)	9 (15.8)	9.7 (1.1-79.8)	0.017
BMI > 28	4 (7.5)	15 (26.8)	4.5 (1.4-14.5)	0.008
BMI < 20	7 (13.2)	4 (7)	0.49 (0.13-1.8)	0.280
Age > 45 yr at diagnosis	14 (26.4)	41 (71.9)	7.1 (3.1-16.6)	0.001
Smoking	50 (94.3)	46 (80.7)	0.25 (0.06-0.95)	0.044
Alcohol > 150 g/d	16 (30.2)	19 (33.3)	1.12 (0.5-2.5)	0.775
Years alcoholism > 20	22 (41.5)	30 (52.6)	1.56 (0.73-3.32)	0.243

The percentage of cigarette smokers was slightly higher in CP than in LC patients. The BMI was higher in LC than in CP patients. The percentage of patients with overweight (BMI > 28) was also higher in LC than in CP group, but it was no different when compared to ASA patients (7.5%, 26.8% and 9% for CP, LC and ASA subjects, respectively) (Table 2). The percentage of patients with malnutrition (BMI < 20) was low in all groups, without significant differences among them (13.3%, 7.0% and 13.3% for CP, LC and ASA subjects, respectively).

Features of alcoholic CP in CP, LC and ASA subjects

It is worth noting that there was absence of the two major clinical manifestations of CP, pancreatic pain and diarrhea/steatorrhea in patients with LC (Table 3). IDDM was frequent in CP (35 cases, 68%), but was found only in three LC cases (5.3%) and in none with ASA. Among these three LC patients with IDDM, one exhibited reduced E1 test (164 μg/g) as the unique altered pancreatic parameter. However, the percentage of patients with NIDDM was higher in the LC group (19.3%) than in the CP group. Diarrhea/steatorrhea was present in many patients with CP (54.7%), but absent in the other two groups (Table 3).

The major morphological alterations such as calcification, dilatation of the pancreatic duct, and pseudocysts were found only in CP patients.

Features of alcoholic LC in CP, LC and ASA subjects

Clinical manifestations characteristic of LC such as ascites, encephalopathy, or upper gastrointestinal

Table 3 Clinical manifestations, functional parameters and imaging characteristics of CP in patients with ACP, ALC or ASA

	ACP (n = 53)	ALC (n = 57)	ASA (n = 30)	P
Pancreatic pain	85%	0%	0%	< 0.001
IDDM	68%	5.3%	0%	< 0.001
NIDDM	5.7%	19.3%	10%	CP *vs* LC, P = 0.03; CP *vs* ASA, LC *vs* ASA, NS
Diarrhoea/ steatorrhoea	54.7%	0%	0%	< 0.001
Calcifications	77.4%	0%	0%	< 0.001
Dilatation of pancreatic duct	67.9%	0%	0%	< 0.001
Pancreatic pseudocysts	58.5%	0%	0%	< 0.001

NS: Not significant.

Table 4 Clinical manifestations, functional parameters and imaging characteristics of LC in patients with ACP, ALC or ASA

	ACP (n = 53)	ALC (n = 57)	ASA (n = 30)	P
Ascites	0%	66.7%	0%	
Haemorrhage in upper digestive tract	1.9%	45.6%	0%	< 0.001
Child-Pugh index > 5	5.7%	98.2%	0%	< 0.001
INR > 1.2	0%	73.7%	0%	< 0.001
Esophageal varices	1.9%	77.2%	0%	< 0.001
Splenomegaly	1.9%	77.2%	0%	< 0.001
Portal hypertension	3.8%	96.5%	0%	< 0.001

hemorrhage were found in most patients with LC, in one case (1/53) with CP and in none with ASA (Table 4). This particular case with CP exhibited gastrointestinal hemorrhage and portal vein thrombosis secondary to pancreopathy, splenomegaly, a PI of 6, with serum albumin level of 2.9 g/dL, severe steatorrhea, severe diabetes mellitus and malnutrition. Nevertheless, his ICG test was normal (23.8%/min).

The PI was > 5 in almost all cases (56/57) with LC, in three (3/53) with CP and in none (0/30) with ASA (Table 4). According to Child-Pugh classification, 35% (20/57) of LC patients were ascribed to class A, 35% (20/57) belonged to class B, and 30% (17/57) belonged to class C. Three cases of CP had a PI of 6. One was the previously described patient with gastrointestinal hemorrhage. In another case, a liver biopsy was available and ruled out the diagnosis of cirrhosis; however, the patient had an elevated Quick index (1.5 INR) at the moment of inclusion which could be attributed to severe steatorrhea because it normalized after parenteral treatment with vitamin K. The third CP case had a CPI of 6, and at the moment of inclusion exhibited cholestasis (total serum bilirubin = 2.6 mg/dL) due to compression of the common bile duct secondary to pancreopathy. This patient had a normal ICG test (28.9% min) and did not exhibit signs of hepatopathy.

Esophageal varices were found in 77% of LC patients, in one case (1/53) with CP and in none with ASA (Table 4). In this CP patient, there were no manifestations of hepatopathy, and varices were secondary to pancreopathy involving portal vein thrombosis. This patient had a large pseudocyst (50 mm × 50 mm) in the head of the pancreas, which caused obstructive jaundice that required surgical treatment, but the ICG test later at the moment of inclusion was normal (PDR = 28.9%/min).

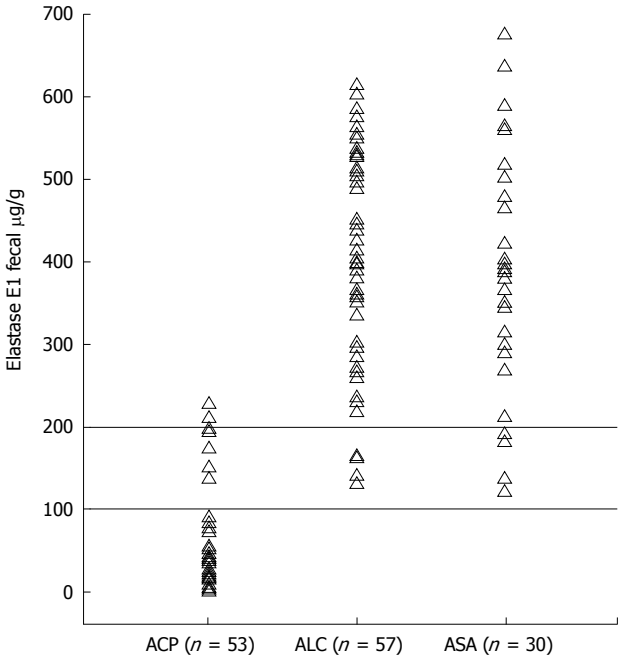


Figure 1 Fecal E1 test (μg/g feces) in patients with ACP, ALC or ASA. Reference lines for altered E1: < 200 μg/g, and severely altered E1: < 100 μg/g. ACP *vs* ALC, P < 0.001; ACP *vs* ASA, P < 0.001; ALC *vs* ASA, P = 0.747.

Splenomegaly was also found in 72.2% of LC patients, in one case with CP and in none with ASA (Table 4). This CP case was the one with upper gastrointestinal hemorrhage, which has been previously described.

Portal hypertension was present in almost all LC patients (96.5%), in two patients with CP and in none with ASA (Table 4). In these two CP cases, portal hypertension was secondary to pancreatic disease, as previously described, with normal ICG test.

Pancreatic fecal elastase E1 in CP, LC and ASA subjects

Fecal E1 test was significantly lower (P < 0.001) in CP patients [9 (0.1-228) μg/g feces] than in LC patients [400.0 (130-614) μg /g feces] and ASA subjects [388 (121-675) μg/g feces] (Figure 1). Moreover, 94.3% of CP patients (50/53) exhibited E1 test lower than the normal limit of 200 μg/g feces, whereas 84.9% (45/53) had very low levels (i.e. < 100 μg/g feces), and among them 29, (64.4%) with diarrhea/steatorrhea. All these percentages were markedly higher (P < 0.001) than those in the LC and ASA groups.

In the LC group, E1 test was < 200 μg/g only in four of 57 cases (7%), and in none of them was it < 100 μg/g. As mentioned before, none of the LC patients exhibited diarrhea/steatorrhea or pancreatic alterations assessed

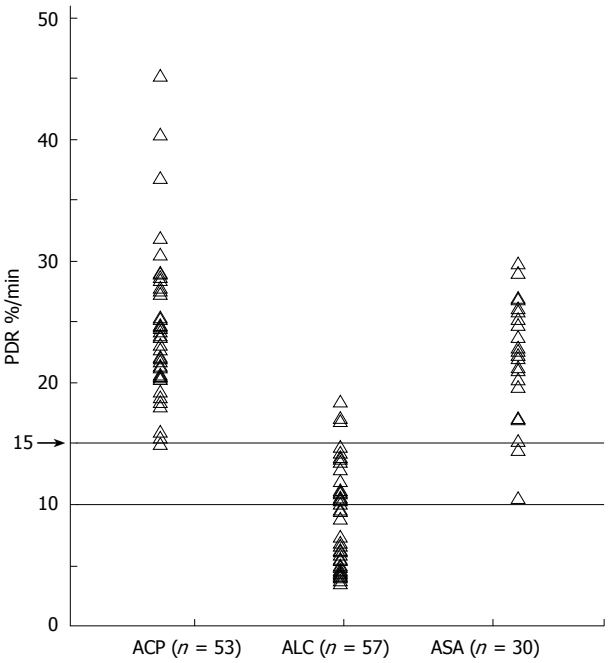


Figure 2 PDR, %/min of ICG in patients with ACP, ALC or ASA. Reference lines for altered PDR: < 15%/min, and severely altered PDR: < 10%/min. ACP *vs* ALC, $P < 0.001$; ACP *vs* ASA, $P = 0.048$; ALC *vs* ASA, $P < 0.001$.

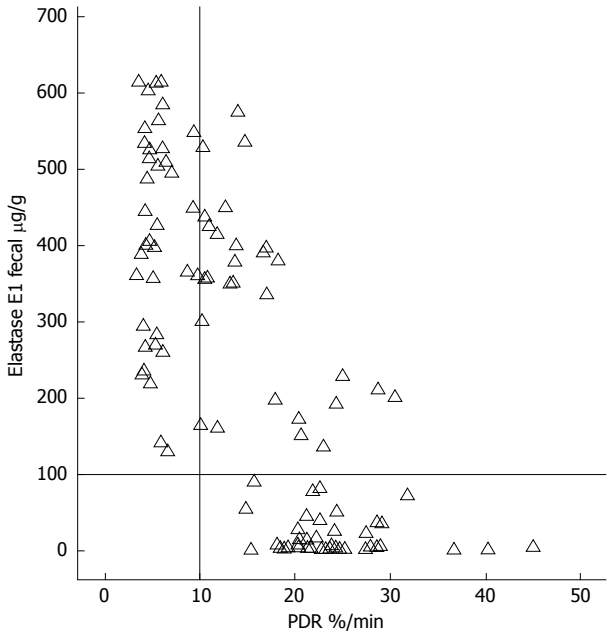


Figure 3 An inverse correlation ($r = -0.746$, $P < 0.001$) between indocyanine clearance (PDR, %/min) and fecal E1 test (μg/g feces) in patients with ACP, ALC or ASA. Reference lines: PDR (%/min) = 15; E1 test = 100. Vertical line for PDR (%/min) = 10, and horizontal line for E1 test = 100 are marked in the figure.

by imaging studies.

Among the 27 ASA subjects, E1 test was reduced only in four (14.8%), but it was never < 100 μg/g. As mentioned, none of the ASA subjects exhibited diarrhea/steatorrhea or pancreatic alterations assessed by imaging studies.

ICG clearance test in CP, LC and ASA subjects

PDR values of the ICG test were markedly more reduced in LC patients [6.1 (3.4-18.2)%/min] than in CP patients [22.6 (14.8-45)%/min] and ASA subjects [22.1 (10.4-30.2)%/min] (Figure 2). Ninety-three percent of LC patients (53/57) had a PDR lower than the normal limit (< 15%/min), and 64% of them exhibited a severe PDR reduction (< 10%/min). However, only one patient with CP (1.9%) showed PDR < 15%/min and none had PDR < 10%/min.

In the ASA group, three of 29 subjects (10%) had reduced PDR (< 15%/min) and none had PDR < 10%/min. These ASA subjects had a normal E1 test, and had no pancreatic or hepatic alterations assessed by imaging studies compatible with CP or LC; all had PI of 5.

It is worth noting that a significant inverse correlation ($r = -0.746$; $P < 0.001$) was found between E1 test levels and PDR values in patients with CP or LC (Figure 3). A significant correlation was also found between E1 test levels and PI ($r = 0.759$; $P < 0.001$). Therefore, in general, the reduction of functional hepatic reserve was associated with normal pancreatic function, and vice versa.

The analysis of potential risks of developing CP or LC showed significantly different values for gender, overweight (BMI > 28), age > 45 years at the time of diagnosis, and smoking (Table 2). Nevertheless, these differences were too low to explain the different evolution towards CP or LC. No significant differences were found regarding malnutrition (BMI < 20), elevated alcohol consumption (> 150 g/d) or long-term chronic alcohol consumption (> 20 years).

DISCUSSION

Chronic ethanol consumption is a common and frequent etiological factor in both CP and LC, and their risk correlates very well with the amount of ethanol daily consumed[2,7]. However, only a minority of alcoholics develop these diseases[1,4,5,7,8]; this together with the requirement of several years of ethanol intake, racial factors[39], and the difficulty of developing adequate experimental models have led to the consideration of other associated etiological factors[26].

The presence of a common etiological factor, i.e. alcoholism, for CP and LC could lead to the assumption that both diseases may coincide in some patients. The study of the presence and coincidence of these two diseases and their risk factors may provide new insights into their etiopathogenesis. Nevertheless, there is controversy about the frequencies of the coincidence between CP and LC in the studies published so far[15-19]. Differences in methodology, and particularly, the presence of hepatitis B or C virus are important confounders which hinder an accurate and reliable interpretation of the data. In fact, more than one-third of patients with alcoholic LC exhibit antibodies against hepatitis C virus[11,12].

In the present study, the frequency of the association between alcoholic CP and LC has been assessed after exclusion of main confounders (hepatitis B or C virus,

non-alcoholic liver or pancreatic diseases, or surgery) and taken into account the major clinical manifestations, the morphological alterations detected by imaging studies, and the alterations of pancreatic and hepatic functions assessed by specific tests.

Our results clearly showed no overlapping between patients with alcoholic CP and alcoholic LC, not only regarding clinical manifestations, but also in imaging studies and functional tests. Furthermore, an inverse relationship was found between pancreatic and liver function in patients with CP or LC.

In our clinical study, the median age of patients at the moment of diagnosis was around a decade higher in LC than in CP (Table 1), due to the different natural history of these diseases[1,2,6,40]. Nevertheless, the age of patients at the time of inclusion in the study was not significantly different. As previously reported in other studies, the percentage of men was higher than that of women in all groups of alcoholics[21,41].

Regarding ethanol consumption, only the duration of ethanol intake was different among groups (20, 25 and 18.5 years for CP, LC and ASA, respectively) (Table 1). However, the mean daily ethanol intake and the total amount of ethanol consumed before diagnosis were not significantly different among groups, which is in agreement with those reported by other authors[2,8,19,42]. Moreover, the percentage of patients with ethanol intake > 150 g/d and the percentage of drinkers for more than 20 years were not significantly different. Therefore, the populations of the three groups in this study were homogeneous regarding ethanol intake.

The percentage of smokers was higher in CP (94.3%) and ASA (96.7%) than in LC (80.7%) (Table 1). Previous studies have reported that smoking is a risk factor for CP in alcoholics, which promotes the progression of the disease[31,43-45]. In addition, smoking has also been considered a risk factor in LC induced by hepatitis C virus[46]. The present study demonstrates that smoking is a risk factor not only for alcoholic CP but also for LC.

The BMI was slightly but significantly lower in CP than in LC, but not when compared with ASA. This slight difference may be ascribed to the presence of steatorrhea and diabetes mellitus in CP. In addition, the percentage of patients with BMI > 28 was significantly higher in LC than in CP and ASA, and these differences may by ascribed to retention of liquids in advanced LC.

The diagnosis of alcoholic CP is hindered in the initial stages of the disease due to the lack of specificity of clinical manifestations. Because of the difficulty in obtaining pancreatic biopsy, the diagnosis is established by combination of clinical manifestations, morphological alterations and impairment of the pancreatic exocrine and endocrine function[27,28].

In our study, 94.3% CP patients showed low E1 levels together with a high percentage of steatorrhea and IDDM, indicating that most patients were at an advanced stage of the disease. However, only four LC patients (7%) had E1 levels between 100 and 200 μg/g (Figure 1), and they neither exhibited steatorrhea nor any clinical or morphological manifestations of CP. In a similar fashion, only three LC patients (5.3%) exhibited IDDM, without the other clinical and morphological manifestations of CP. Only one of these patients exhibited 164 μg/g E-1 level; hence, the presence of CP in this patient could not be ruled out. Eleven LC patients exhibited NIDDM, which is common in LC[47] with normal E-1 level, but not a characteristic manifestation of CP[48]. In the ASA group, three subjects exhibited NIDDM, without symptoms of pancreopathy and normal E-1 and ICG tests.

The former studies that assessed the frequency of pancreatic alterations in patients with LC showed contradictory results. The frequency of pancreatic fibrosis compatible with CP in autopsies of LC patients was between 2% and 20% when the presence or absence of hepatitis B or C was not taken into account[14,16,18,19]. In addition, the age-related changes in morphofunctional parameters of the pancreas may be another confounder when the diagnosis of CP is based only on these parameters[49-53]. In a study using endoscopic ultrasonography and ERCP, Hastier *et al*[17] reported only moderate pancreatic alterations in 5.5% patients with alcoholic LC, without evolution to CP.

Some previous studies[51,54,55] on necropsies of patients with alcoholic LC showed very low frequency of the fibrotic pancreatic alteration characteristic of CP, which is predominantly perilobular fibrosis with heterogeneous distribution. In contrast, these patients exhibited diffuse and intralobular pancreatic fibrosis without calcifications or pseudocysts. Accordingly, these authors suggested that pancreatic morphological lesions in alcoholic LC correspond to a pathological process different from that of alcoholic CP.

It is worth noting that many studies on the association of CP and LC involve patients older than 50 years. It is well known that normal subjects at this age exhibit some morphofunctional pancreatic alterations[21,22,49-51,53] that could be taken for those characteristic of CP, but without evolution towards this disease. Consequently, morphofunctional pancreatic alterations with age may be another confounding factor. These findings might explain some of the discrepancies reported by different authors.

On the other hand, regarding the hepatic alterations, most LC patients in our study were at an advanced stage of the disease and showed ascites, encephalopathy and/or gastrointestinal hemorrhage as well as Child-Pugh B or C and markedly reduced PDR in the ICG test. However, only a few CP patients exhibited these manifestations, which were not ascribed to any hepatic disorder (Table 4).

There is also controversy concerning the frequency of LC in CP patients. Previous studies have reported that the presence of LC in patients with CP was variable, ranging between 5% and 30%[56]. Later, Angelini *et al*[19] found 12.5% of LC cases in CP patients. Although, in this study, cases with hepatitis C virus were not excluded, the authors did not find any relationship between the grade of hepatic histological alteration and the degree of pancreatic functional severity. Recently, Nakamura

et al[15] found no association between liver and pancreatic disease in a population of alcoholics with abdominal pain, after exclusion of patients with viral hepatitis[15]. Accordingly, only 5% of patients who had altered ERCP also exhibited Child-Pugh class B or C[15].

It should be taken into account that many hepatic alterations in patients with alcoholic CP are ascribed to cholestasis secondary to pancreatitis[57,58], and indeed, they are not characteristic of advanced alcoholic liver disease[19,55]. Moreover, most of these cases exhibit regression of liver fibrosis after biliary drainage[59].

In our study, there was no coincidence at all between severe alterations of the pancreatic function assessed by fecal E-1 test and severe alterations of the liver function assessed by ICG test. Furthermore, we found a strong inverse correlation ($r = -0.752$; $P < 0.001$) between these functions (Figure 3). Similarly, we found a significant correlation between PI and fecal E-1 test ($r = 0.759$; $P < 0.001$). These findings support the hypothesis of a different and independent etiopathogenesis of CP and LC associated with alcoholism[25]. Hayakawa *et al*[24] reported a negative correlation between pancreatic and liver functions, but only in patients with alcoholic liver disease. In this regard, a general increase in exocrine pancreatic secretion in LC patients has been reported[19,60]. Thus, Dreiling *et al*[23] have suggested a hypersecretory state of the pancreas in alcoholic LC, with increased volume output[16] and maintaining either normal or elevated bicarbonate and enzyme outputs[16,19,23,24]. This has been considered a washout phenomenon, resulting in a decreased tendency of ductal protein and calcium precipitation in these patients[16].

Hayakawa *et al*[24] have reported that pancreatic secretion increases in patients with different degrees of alcoholic liver disease, assessed by the cholcystokinin-secretin test, ICG test and liver histopathology. Moreover, these authors have found a significant inverse correlation between bicarbonate secretion and ICG clearance[24].

The hypersecretory state of the pancreas in alcoholics with LC has been ascribed to a reduced inactivation of secretin[60] or even to the increased portal pressure[24]. Recently, it has been confirmed by an experimental result that chronic alcoholism impairs the neurohormonal control of the pancreas, both at the central nervous system and acinar levels, promoting the secretory response to feeding or other stimuli[61]. All these findings regarding the inverse correlation between the alterations of liver and pancreatic function tests may be a reflection of some correlation in the etiology of both diseases.

In summary, the present study demonstrates the scarce coincidence in clinical manifestations, morphological alterations and organ function between CP and LC in alcoholic subjects. This finding, together with the inverse correlation between indexes of pancreatic and liver functions in these patients, supports the hypothesis that alcoholic CP and LC evolve in a different manner and have different etiopathogenesis. Accordingly, chronic alcoholism, although a necessary factor, is not a sufficient cause in their etiopathogenesis. Further studies should provide new insights into the different risk factors-other than alcoholism-involved in the etiopathogenesis of alcoholic CP and LC.

ACKNOWLEDGMENTS

We thank Mrs. Remedios Gonzalez for her technical help and Mrs. Landy Menzies for her assistance in preparing the English version of the manuscript.

COMMENTS

Background

Despite sharing similar risk factors, medical literature regarding the simultaneous occurrence of chronic pancreatitis (CP) and liver cirrhosis (LC) in patients with chronic alcoholism is scarce and the results are uncertain. This lack of coincidence may rely on differences in methodology such as retrospective *versus* prospective studies, and the evaluated parameters such as clinical, functional, imaging or histopathological. However, it may also reflect the hypothesis that alcoholism is not a sufficient cause, but a cofactor, for the development of such diseases.

Research frontiers

This study has been carefully designed to investigate the coincidence in clinical manifestations, morphological alterations and organ function between CP and LC in alcoholic subjects. The results support the hypothesis that alcoholic CP and alcoholic LC evolve in a different manner and have different etiopathogenesis, despite sharing a common risk factor.

Innovations and breakthroughs

There is a lack of coincidence between CP and LC in alcoholic subjects. There is an inverse correlation between indexes of pancreatic and liver functions in these patients, supporting the hypothesis that chronic alcoholism, although a necessary factor, is not a sufficient cause in their etiopathogenesis.

Applications

This paper suggests the necessity for researching other factors together with chronic alcoholism involved in the development of CP or LC.

Peer review

Authors studied the possible association between CP and LC of alcoholic etiology, after excluding any other causes. This is an interesting study. It was undertaken according to a carefully designed plan and with adequate statistical considerations. The authors successfully provided the evidence, suggesting that CP and LC evolve in a different manner, which will be useful for clinical researchers of these diseases.

REFERENCES

1 **Worning H**. Alcoholic chronic pancreatitis. In: Beger HG, Warshaw AL, Buchler MW, Carr-Locke DL, Neoptolemos JP, Russell Ch, Sarr MG, eds. The pancreas. Oxford: Blackwell Science, 1998: 672-682

2 **Durbec JP**, Sarles H. Multicenter survey of the etiology of pancreatic diseases. Relationship between the relative risk of developing chronic pancreaitis and alcohol, protein and lipid consumption. *Digestion* 1978; **18**: 337-350

3 **Almela P**, Aparisi L, Grau F, Sempere J, Rodrigo JM. Influence of alcohol consumption on the initial development of chronic pancreatitis. *Rev Esp Enferm Dig* 1997; **89**: 741-746, 747-752

4 **Lankisch PG**, Lowenfels AB, Maisonneuve P. What is the risk of alcoholic pancreatitis in heavy drinkers? *Pancreas* 2002; **25**: 411-412

5 **Aparisi L**, Navarro S, Perez Mateo M, Bautista D. [Prevalence of malnutrition and morphofunctional alterations of the pancreas in asymptomatic chronic alcoholic patients] *Med Clin* (Barc) 2000; **114**: 444-448

6 **Etemad B**, Whitcomb DC. Chronic pancreatitis: diagnosis, classification, and new genetic developments. *Gastroenterology* 2001; **120**: 682-707

7 **Becker U**, Deis A, Sorensen TI, Gronbaek M, Borch-Johnsen K, Muller CF, Schnohr P, Jensen G. Prediction of risk of liver disease by alcohol intake, sex, and age: a prospective population study. *Hepatology* 1996; **23**: 1025-1029

8 **Bellentani S**, Saccoccio G, Costa G, Tiribelli C, Manenti F, Sodde M, Saveria Croce L, Sasso F, Pozzato G, Cristianini G, Brandi G. Drinking habits as cofactors of risk for alcohol induced liver damage. The Dionysos Study Group. *Gut* 1997; **41**: 845-850

9 **Grant BF**, Dufour MC, Harford TC. Epidemiology of alcoholic liver disease. *Semin Liver Dis* 1988; **8**: 12-25

10 **Lelbach WK**. Cirrhosis in the alcoholic and its relation to the volume of alcohol abuse. *Ann N Y Acad Sci* 1975; **252**: 85-105

11 **Pares A**, Caballeria J, Bruguera M, Torres M, Rodes J. Histological course of alcoholic hepatitis. Influence of abstinence, sex and extent of hepatic damage. *J Hepatol* 1986; **2**: 33-42

12 **Serra MA**, Escudero A, Rodriguez F, del Olmo JA, Rodrigo JM. Effect of hepatitis C virus infection and abstinence from alcohol on survival in patients with alcoholic cirrhosis. *J Clin Gastroenterol* 2003; **36**: 170-174

13 **Nanji AA**. Alcoholic liver disease. In: Zakim D, Boyer T, eds. Hepatology. A Texbook of liver disease. 4th ed. Phyladelphia: WB Sauders, 2003: 839-922

14 **Ichihara S**, Sato M, Kozuka S. Prevalence of pancreatitis in liver diseases of various etiologies: an analysis of 107,754 adult autopsies in Japan. *Digestion* 1992; **51**: 86-94

15 **Nakamura Y**, Kobayashi Y, Ishikawa A, Maruyama K, Higuchi S. Severe chronic pancreatitis and severe liver cirrhosis have different frequencies and are independent risk factors in male Japanese alcoholics. *J Gastroenterol* 2004; **39**: 879-887

16 **Renner IG**, Rinderknecht H, Wisner JR Jr. Pancreatic secretion after secretin and cholecystokinin stimulation in chronic alcoholics with and without cirrhosis. *Dig Dis Sci* 1983; **28**: 1089-1093

17 **Hastier P**, Buckley MJ, Francois E, Peten EP, Dumas R, Caroli-Bosc FX, Delmont JP. A prospective study of pancreatic disease in patients with alcoholic cirrhosis: comparative diagnostic value of ERCP and EUS and long-term significance of isolated parenchymal abnormalities. *Gastrointest Endosc* 1999; **49**: 705-709

18 **Renner IG**, Savage WT 3rd, Stace NH, Pantoja JL, Schultheis WM, Peters RL. Pancreatitis associated with alcoholic liver disease. A review of 1022 autopsy cases. *Dig Dis Sci* 1984; **29**: 593-599

19 **Angelini G**, Merigo F, Degani G, Camplani N, Bovo P, Fratta Pasini A, Cavallini G, Brocco G, Scuro LA. Association of chronic alcoholic liver and pancreatic disease: a prospective study. *Am J Gastroenterol* 1985; **80**: 998-1003

20 **Ammann RW**, Buehler H, Bruehlmann W, Kehl O, Muench R, Stamm B. Acute (nonprogressive) alcoholic pancreatitis: prospective longitudinal study of 144 patients with recurrent alcoholic pancreatitis. *Pancreas* 1986; **1**: 195-203

21 **Anand BS**, Vij JC, Mac HS, Chowdhury V, Kumar A. Effect of aging on the pancreatic ducts: a study based on endoscopic retrograde pancreatography. *Gastrointest Endosc* 1989; **35**: 210-213

22 **Rajan E**, Clain JE, Levy MJ, Norton ID, Wang KK, Wiersema MJ, Vazquez-Sequeiros E, Nelson BJ, Jondal ML, Kendall RK, Harmsen WS, Zinsmeister AR. Age-related changes in the pancreas identified by EUS: a prospective evaluation. *Gastrointest Endosc* 2005; **61**: 401-406

23 **Dreiling DA**, Greenstein AJ, Bordalo O. The hypersecretory states of the pancreas. Implications in the pathophysiology of pancreatic inflammation and the pathogenesis of peptic ulcer diathesis. *Am J Gastroenterol* 1973; **59**: 505-511

24 **Hayakawa T**, Kondo T, Shibata T, Kitagawa M, Sakai Y, Sobajima H, Ishiguro H, Nakae Y, Kato K. Exocrine pancreatic function in chronic liver diseases. *Am J Gastroenterol* 1991; **86**: 201-204

25 **Whitcomb DC**. Genetic predisposition to alcoholic chronic pancreatitis. *Pancreas* 2003; **27**: 321-326

26 **Stickel F**, Osterreicher CH. The role of genetic polymorphisms in alcoholic liver disease. *Alcohol Alcohol* 2006; **41**: 209-224

27 **Sarner M**, Cotton PB. Classification of pancreatitis. *Gut* 1984; **25**: 756-759

28 **Singer MV**, Gyr K, Sarles H. Revised classification of pancreatitis. Report of the Second International Symposium on the Classification of Pancreatitis in Marseille, France, March 28-30, 1984. *Gastroenterology* 1985; **89**: 683-685

29 **Anthony PP**, Ishak KG, Nayak NC, Poulsen HE, Scheuer PJ, Sobin LH. The morphology of cirrhosis. Recommendations on definition, nomenclature, and classification by a working group sponsored by the World Health Organization. *J Clin Pathol* 1978; **31**: 395-414

30 **Talamini G**, Bassi C, Falconi M, Frulloni L, Di Francesco V, Vaona B, Bovo P, Rigo L, Castagnini A, Angelini G, Vantini I, Pederzoli P, Cavallini G. Cigarette smoking: an independent risk factor in alcoholic pancreatitis. *Pancreas* 1996; **12**: 131-137

31 **Klatsky AL**, Armstrong MA. Alcohol, smoking, coffee, and cirrhosis. *Am J Epidemiol* 1992; **136**: 1248-1257

32 **Pugh RN**, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. *Br J Surg* 1973; **60**: 646-649

33 **Tacke F**, Fiedler K, Trautwein C. A simple clinical score predicts high risk for upper gastrointestinal hemorrhages from varices in patients with chronic liver disease. *Scand J Gastroenterol* 2007; **42**: 374-382

34 **Okochi O**, Kaneko T, Sugimoto H, Inoue S, Takeda S, Nakao A. ICG pulse spectrophotometry for perioperative liver function in hepatectomy. *J Surg Res* 2002; **103**: 109-113

35 **Serra MA**, Rodrigo JM, Guix J, Montes F, Wassel A, Aparisi L, Olmo JA, Bixquert M. The discriminant diagnostic power of liver function tests in hepatic cirrhosis. *Acta Med Port* 1981; **3**: 3-10

36 **Loser C**, Mollgaard A, Folsch UR. Faecal elastase 1: a novel, highly sensitive, and specific tubeless pancreatic function test. *Gut* 1996; **39**: 580-586

37 **van de Kamer J**, Huinink B, Woyers H. Rapid method for the determination of fat in feces. *J Biol Chem* 1949; **177**: 347-355

38 **Fernandez-Real JM**, Vayreda M, Casamitjana R, Saez M, Ricart W. [Body mass index (BMI) and percent fat mass. A BMI > 27.5 kg/m2 could be indicative of obesity in the Spanish population] *Med Clin* (Barc) 2001; **117**: 681-684

39 **Lowenfels AB**, Maisonneuve P, Grover H, Gerber E, Korsten MA, Antunes MT, Marques A, Pitchumoni CS. Racial factors and the risk of chronic pancreatitis. *Am J Gastroenterol* 1999; **94**: 790-794

40 **Naveau S**, Perlemuter G, Balian A. [Epidemiology and natural history of cirrhosis] *Rev Prat* 2005; **55**: 1527-1532

41 **del Olmo JA**, Serra MA, Rodriguez F, Escudero A, Gilabert S, Rodrigo JM. Incidence and risk factors for hepatocellular carcinoma in 967 patients with cirrhosis. *J Cancer Res Clin Oncol* 1998; **124**: 560-564

42 **Mezey E**, Kolman CJ, Diehl AM, Mitchell MC, Herlong HF. Alcohol and dietary intake in the development of chronic pancreatitis and liver disease in alcoholism. *Am J Clin Nutr* 1988; **48**: 148-151

43 **Bourliere M**, Barthet M, Berthezene P, Durbec JP, Sarles H. Is tobacco a risk factor for chronic pancreatitis and alcoholic cirrhosis? *Gut* 1991; **32**: 1392-1395

44 **Lowenfels AB**, Zwemer FL, Jhangiani S, Pitchumoni CS. Pancreatitis in a native American Indian population. *Pancreas* 1987; **2**: 694-697

45 **Maisonneuve P**, Lowenfels AB, Mullhaupt B, Cavallini G, Lankisch PG, Andersen JR, Dimagno EP, Andren-Sandberg A, Domellof L, Frulloni L, Ammann RW. Cigarette smoking accelerates progression of alcoholic chronic pancreatitis. *Gut* 2005; **54**: 510-514

46 **Hezode C**, Lonjon I, Roudot-Thoraval F, Mavier JP,

Pawlotsky JM, Zafrani ES, Dhumeaux D. Impact of smoking on histological liver lesions in chronic hepatitis C. *Gut* 2003; **52**: 126-129

47 **Joffe BI**, Bank S, Jackson WP, Keller P, O'Reilly IG, Vinik AI. Insulin reserve in patients with chronic pancreatitis. *Lancet* 1968; **2**: 890-892

48 **Zakim D**, Metabolism of glucose and fatty acids by the liver. In: Zakim D, Bayer T, eds. Hepatology. A textbook of liver disease. 4th ed. Philadelphia: Sauders, 2003: 49-80

49 **Schmitz-Moormann P**, Himmelmann GW, Brandes JW, Folsch UR, Lorenz-Meyer H, Malchow H, Soehendra LN, Wienbeck M. Comparative radiological and morphological study of human pancreas. Pancreatitis like changes in postmortem ductograms and their morphological pattern. Possible implication for ERCP. *Gut* 1985; **26**: 406-414

50 **Kreel L**, Sandin B. Changes in pancreatic morphology associated with aging. *Gut* 1973; **14**: 962-970

51 **Martin E**. Different pathomorphological aspects of pancreatic fibrosis correlated with etiology. Anatomical study of 300 cases. In: Gyr R, Singer M, Sarles H, eds. Pancreatitis. Concepts and classification. Amsterdam: Elsevier, 1984: 77-80

52 **Laugier R**, Bernard JP, Berthezene P, Dupuy P. Changes in pancreatic exocrine secretion with age: pancreatic exocrine secretion does decrease in the elderly. *Digestion* 1991; **50**: 202-211

53 **Ikeda M**, Sato T, Morozumi A, Fujino MA, Yoda Y, Ochiai M, Kobayashi K. Morphologic changes in the pancreas detected by screening ultrasonography in a mass survey, with special reference to main duct dilatation, cyst formation, and calcification. *Pancreas* 1994; **9**: 508-512

54 **Martin E**, Bedossa P. Diffuse fibrosis of the pancreas: a peculiar pattern of pancreatitis in alcoholic cirrhosis. *Gastroenterol Clin Biol* 1989; **13**: 579-584

55 **Suda K**, Takase M, Takei K, Nakamura T, Akai J, Nakamura T. Histopathologic study of coexistent pathologic states in pancreatic fibrosis in patients with chronic alcohol abuse: two distinct pathologic fibrosis entities with different mechanisms. *Pancreas* 1996; **12**: 369-372

56 **Lankisch PG**, Banks PA. Pancreatitis. Berlin: Springer V, 1998: 279-301

57 **Wilson C**, Auld CD, Schlinkert R, Hasan AH, Imrie CW, MacSween RN, Carter DC. Hepatobiliary complications in chronic pancreatitis. *Gut* 1989; **30**: 520-527

58 **Frey C**. The surgical treatment of chronic pancreatitis. In: Go VLW, DiMagno EP, Gardner JD, Lebnthal E, Rever HA, Scheele GA, eds. The pancreas. Biology, Pathobiology and disease. 2nd ed. New York: Raven Press, 1986: 707-740

59 **Hammel P**, Couvelard A, O'Toole D, Ratouis A, Sauvanet A, Flejou JF, Degott C, Belghiti J, Bernades P, Valla D, Ruszniewski P, Levy P. Regression of liver fibrosis after biliary drainage in patients with chronic pancreatitis and stenosis of the common bile duct. *N Engl J Med* 2001; **344**: 418-423

60 **Turnberg LA**, Grahame G. Secretion of water and electrolytes into the duodenum in normal subjects and in patients with cirrhosis: the response to secretin and pancreozymin. *Gut* 1974; **15**: 273-277

61 **Deng X**, Wood PG, Eagon PK, Whitcomb DC. Chronic alcohol-induced alterations in the pancreatic secretory control mechanisms. *Dig Dis Sci* 2004; **49**: 805-819

S- Editor Li DL **L- Editor** Kumar M **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6180

World J Gastroenterol 2008 October 28; 14(40): 6180-6187
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Bravo capsule system optimizes intragastric pH monitoring over prolonged time: Effects of ghrelin on gastric acid and hormone secretion in the rat

Tobias Rudholm, Per Mikael Hellström, Elvar Theodorsson, Colin Allan Campbell, Peter Geoffrey McLean, Erik Näslund

Tobias Rudholm, Per Mikael Hellström, Department of Medicine Solna, Karolinska Institutet, Stockholm, Sweden
Elvar Theodorsson, Department of Neurochemistry, Linköping University Hospital, Sweden
Colin Allan Campbell, Peter Geoffrey McLean, Neurology and GI CEDD, GlaxoSmithKline, Harlow, UK, Essex CM19 5AW, United Kingdom
Erik Näslund, Division of Surgery, Department of Clinical Sciences, Danderyd Hospital, Karolinska Institutet, Stockholm, Sweden
Author contributions: Hellström PM, Näslund E, Campbell CA, and McLean PG contributed equally to this work; Hellström PM, Näslund E, Campbell CA, McLean PG, and Rudholm T designed research; Rudholm T performed research; Campbell CA and McLean PG contributed new reagents; Theodorsson E and Rudholm T analyzed data; Rudholm T wrote the paper.
Correspondence to: Tobias Rudholm, MSc, Gastro Centre Medicine, Karolinska University Hospital Solna, 17176 Stockholm, Sweden. tobias.rudholm@ki.se
Telephone: +46-8-51772054 Fax: +46-8-51771100
Received: March 27, 2008 Revised: June 2, 2008
Accepted: June 9, 2008
Published online: October 28, 2008

Abstract

AIM: To evaluate measurements of intragastric pH with the Bravo capsule system over a prolonged time.
METHODS: A Bravo capsule was placed inside the rat gastric body and pH was studied for periods up to five consecutive days. For comparison, a gastric fistula model was used. Effects of ghrelin and esomeprazole, with or without pentagastrin, on gastric pH were studied. In addition, effects of esomeprazole on plasma ghrelin, gastrin and somatostatin were analyzed.
RESULTS: All rats recovered after surgery. The average 24-h pH during free feeding was 2.3 ± 0.1 (n = 20) with a variation of 18% ± 6% over 5 d. Ghrelin, 2400 pmol/kg, t.i.d. increased pH from 1.7 ± 0.1 to 3.1 ± 0.3 (P < 0.01) as recorded with the Bravo system. After esomeprazole (1 mg/kg, 3 mg/kg and 5 mg/kg) there was a dose-dependent pH increase of maximally 3.4 ± 0.1, with day-to-day variation over the entire period of 8% ± 3%. The fistula and pH studies generated similar results. Acid inhibition with esomeprazole increased plasma ghrelin from 10 ± 2 pmol/L to 65 ± 26 pmol/L (P < 0.001), and somatostatin from 10 ± 2 pmol/L to 67 ± 18 pmol/L (P < 0.001).
CONCLUSION: pH measurements with the Bravo capsule are reliable, and comparable to those of the gastric fistula model. The Bravo system optimizes accurate intragastric pH monitoring over prolonged periods and allows both short- and long-term evaluation of effects of drugs and hormones.

Key words: Gastric acid; Bravo system; Intragastric pH; Ghrelin; Somatostatin

Peer reviewers: Kyoichi Adachi, MD, Department of Gastroenterology and Hepatology, Shimane University, School of Medicine Shimane, 89-1 Enya-cho, Izumo-shi Shimane 693-8501, Japan; Satoshi Osawa, MD, First Department of Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu, 431-3192, Japan

Rudholm T, Hellström PM, Theodorsson E, Campbell CA, McLean PG, Näslund E. Bravo capsule system optimizes intragastric pH monitoring over prolonged time: Effects of ghrelin on gastric acid and hormone secretion in the rat. *World J Gastroenterol* 2008; 14(40): 6180-6187 Available from: URL: http://www.wjgnet.com/1007-9327/14/6180.asp DOI: http://dx.doi.org/10.3748/wjg.14.6180

INTRODUCTION

In the past, different techniques have been employed to study gastric acid secretion in rodents. The main principle for these methods has been collection of gastric juice, and in order to measure acid secretion, pH titration has been carried out. One of the earliest methods was the pylorus ligation technique[1]. The principle of this method is distension of the stomach as a potent stimulus of acid secretion. Later, this method was altered with an esophageal ligature[2,3], after which the stomach of the rat was removed and secretions analyzed. Esophageal ligation in the pylorus-ligated rat has been shown to significantly inhibit acid secretion by inhibition of central vagus function[2]. Since then,

the most reliable method has been the chronic fistula method[4-6] where a gastric fistula is implanted at the greater curvature of the stomach. This technique requires movement restriction of the animal which is in a conscious state during the study. The gastric contents are collected and acid output measured. This technique allows re-use of animals following a recovery period from the experimental procedure. Other methods used today are perfusion of the gastric lumen[7] and isolated perfused, as well as vascularly perfused rat stomach[8-10].

Most of the above studies have the drawback that they do not measure intragastric pH directly and are not very physiological, as the animal is either restrained or anesthetized. The main goal of this study was to test the feasibility of a capsule normally used in the clinical setting in humans to measure gastroesophageal reflux disease (Bravo system) for monitoring intragastric pH in the rat. The Bravo capsule system has primarily been used in humans[11-14], but also in animals[15] for diagnosis of gastroesophageal reflux disease.

The aim of the study was to evaluate the Bravo capsule for pH monitoring in the rat. To validate the method, we compared the data to those of the standard gastric fistula model.

MATERIALS AND METHODS

Animals

Sprague-Dawley male rats (300-350 g) were purchased from Scanbur B&K AB (Sollentuna, Sweden). The rats were housed in wire-meshed cages at 24℃ with constant humidity and 12:12 h light-dark cycle. The animals were fed *ad libitum* with a commercial rat diet consisting of pellet (LABFOR, Lactamin R36, Kimstad, Sweden) and tap water prior to the studies. The experiments were approved by the Animal Ethics Committee in northern Stockholm.

Surgery

Surgery was performed under anesthesia with pentobarbital sodium (50 mg/kg; Apoteket AB, Stockholm, Sweden) intraperitoneally, and Hypnorm (fentanyl citrate, 0.315 mg/kg and fluanisone 10 mg/kg; Janssen, Oxford, USA) intramuscularly. Marcain (bupivacaine hydrochloride, 2.5 mg/kg; AstraZeneca, Södertälje, Sweden) was given subcutaneously after surgery along the abdominal incision.

For the Bravo system studies, a midline incision was performed, and a small opening created in the proximal greater curvature, and gastric contents were evacuated. An externally pre-calibrated (buffers pH 1.07 and 7.1) Bravo capsule (an electronic sensor encapsulated in PVC-plastic, 25 mm × 5 mm × 5 mm; Synmed Medicinteknik AB, Spånga, Sweden) was placed inside the stomach with the pH sensor pointing distally and anchored with a suture. An indwelling silastic catheter (Dow Corning Co., Midland, MI, USA) was inserted into the external jugular vein.

For the gastric acid fistula studies, rats were provided with a plastic gastric fistula placed immediately proximal to the oxyntic gland area near the greater curvature. The fistula was closed between experimental periods. A silastic catheter was implanted into the external jugular vein for drug administration.

Studies of intragastric pH (Bravo system)

Studies of intragastric pH began in the morning 2 d after surgery. The studies were carried out in conscious rats, one experiment for each rat, under normal conditions, or after a 16-h fasting period in wire-bottom cages with free access to water. The animals gained weight (10 ± 3.4 g during 1 wk) and behaved in a normal fashion, with a normal feeding pattern throughout the experiments. At post-mortem examination, no mucosal lesions, obstruction of the pylorus or gastric distension were seen. Drugs were administered through the external jugular vein in all experiments.

The pH recorded by the Bravo capsule was transmitted to the Bravo receiver placed directly outside the cage. The sampling frequency was 6 Hz. The Bravo system was set for a 48-h registration period, after which the data were downloaded, batteries replaced and recording continued. This procedure was then repeated in two more 48-h periods.

All test compounds were dissolved and diluted in isotonic saline solution (sodium chloride, 9 g/L; 300 mosm/kg H_2O, Fresenius Kabi, Halden, Norway).

The effect of ghrelin on pH: The effect of ghrelin on intragastric pH was studied with ghrelin (2400 pmol/kg) given t.i.d (08:00, 12:00 and 16:00) for 5 d in a row (n = 7).

Evaluation of basal pH: Baseline pH was studied over 24 h under fed (n = 20) and fasting (n = 8) conditions.

Effect of esomeprazole on pH: The effect of increasing bolus doses of esomeprazole (AstraZeneca) (1 mg/kg, 3 mg/kg or 5 mg/kg iv, n = 10) or saline (iv, n = 8) was studied for 24 h in fed rats. Furthermore, the effect of esomeprazole (3 mg/kg iv, n = 10) or saline (iv, n = 8) was studied for 24 h in fasting rats.

The effect of pentagastrin and esomeprazole on pH: The effect of esomeprazole (3 mg/kg iv, n = 10) or saline (iv, n = 8) was studied under pentagastrin (NeoMPS, Strasbourg, France) infusion (90 pmol/kg per min, iv) over 6 h in both fed and fasting rats. In these experiments, the rats were restrained in Bollman cages to mimic the gastric fistula studies and for infusion of pentagastrin.

The effect of a 24-h infusion of pentagastrin (90 pmol/kg per min iv, n = 6), of esomeprazole (9 pmol/kg per min, n = 6), or saline (0.154 mol/L, n = 6) on pH was studied.

Plasma levels of gut hormones: The effect of esomeprazole (3 mg/kg iv) on plasma levels of ghrelin, gastrin and somatostatin was studied. A group of animals (n = 10) was divided into two treatment groups (each n = 5). All animals were treated with esomeprazole

daily during 1 wk. The first group of animals was then euthanized, while the other group was followed for another week without esomeprazole and then euthanaized. Blood was drawn and centrifuged, and plasma assayed for concentrations of ghrelin, gastrin and somatostatin.

For ghrelin measurements, the ghrelin (active) radioimmunoassay kit (Linco Research, St. Charles, MI, USA) was used, which utilizes ^{125}I-labeled ghrelin and ghrelin antiserum to determine the level of active ghrelin in plasma. For the analysis, a Gamma Master 1277 (LKB-Wallac, Perkin-Elmer Inc, Massachusetts, NH, USA) was used. The intra- and interassay coefficients of variation were 7% and 14%, respectively.

Somatostatin was analyzed using an EIA kit (EK-060-03) from Phoenix Pharmaceuticals, Burlingame, CA, USA), which reacts 100% to somatostatin-14 and somatostatin-28. The intra- and interassay coefficients of variation were 5% and 14%, respectively.

Gastrin was analyzed using C-terminal-directed CCK/gastrin antiserum 2609/10 (Rehfeld, 1978). Chloramine-T-labeled and HPLC-purified gastrin-17 (NeoMPS) was used as radioligand and gastrin-17 as calibrator/standard. The intra- and interassay coefficients of variation were 6% and 8%, respectively.

Studies of gastric acid secretion (fistula)

Studies of gastric acid secretion began 7 d after surgery. The animals gained weight (8 ± 2.6 g during 1 wk) and had normal behavior during the experimentation periods. Prior to each experiment, food was withheld for 18 h, but with free access to water. At the start of the experiments, the stomach was rinsed with 10-15 mL luke-warm tap water to evacuate remaining food, followed by a 30-min period before the experiments were started. During the experiments, the conscious rats were placed in Bollman cages. Gastric juice was collected at 30-min intervals, and volumes measured to the nearest 0.1 mL. pH was calculated by back-titration using 0.1 mmol/L sodium hydroxide. Acid output was calculated by multiplying the secretion volumes with hydrogen ion concentrations and expressed as μmol per 30-min period.

Baseline acid secretion was studied for 60 min followed by esomeprazole (3 mg/kg iv), after which acid secretion was studied for another 2 h. During the experiment, saline was administered in the same amount as collected from the gastric fistula to compensate for the volume loss during the experiment. Furthermore, baseline acid secretion was studied for 60 min, followed by an infusion of pentagastrin (90 pmol/kg per min) for 4 h. After 1 h of pentagastrin infusion, a bolus of esomeprazole (3 mg/kg iv) was administered and acid secretion studied for another 3 h.

Data and statistical analysis

The data obtained with the Bravo capsule analyzed using (POLYGRAM NET™ pH Testing Application software, Synmed Medicinteknik) in 48-h periods. Results of studies with esomeprazole were analyzed by calculating changes in pH at various timepoints from baseline (defined as 0.5 h prior to onset of studies). For analysis of the fistula studies, the first 30-min collection was discarded and the second collection used as baseline for comparison with esomeprazole and pentagastrin.

All data are mean ± SE. A Kruskal-Wallis test followed by Mann-Whitney *U* test was used for statistical comparisons using specific time points for pH. $P < 0.05$ was considered statistically significant. For comparison of the variability between the fistula and the Bravo system the Bland-Altman analysis was used[16,17]. The Prism software package 4.0 (GraphPad Software Inc., San Diego, CA, USA) was used for the statistical comparisons.

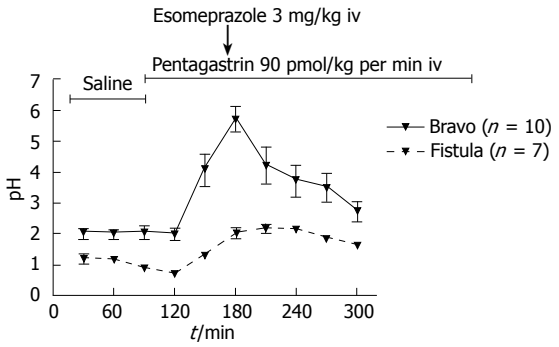


Figure 1 Change in pH ± SE in the Bravo system and gastric fistula model during fasting conditions after iv bolus of esomeprazole (3 mg/kg) and pentagastrin infusion (90 pmol/kg per min) for 2 h.

RESULTS

Comparison between the Bravo system and the fistula model

Pentagastrin resulted in a marked increase, 83 ± 9 mmol/L to 132 ± 8 mmol/L ($P < 0.05$) of acid output in the fistula model, which was not evident as a corresponding decrease in pH with the Bravo system. During esomeprazole treatment, there was a marked increase in pH from 2.0 ± 0.2 to 3.7 ± 0.5, as recorded with the Bravo system and correspondingly, a marked decrease in acid secretion from 105 ± 21 mmol/L to 31 ± 7 mmol/L in the fistula model ($P < 0.05$; Figure 1). Bland-Altman analysis of these conditions showed a high degree of agreement between the Bravo system and the fistula method as shown in Figure 2.

Evaluation of basal pH

A typical 120-h baseline registration including dose of esomeprazole (day 1, 3 and 5) with the Bravo system is shown in Figure 3. The feeding status did not alter the mean pH over 24 h, but increases in pH were observed during afternoon and night-time when animals were fed. The mean 24-h pH was 2.3 ± 0.1 during fed conditions and 2.5 ± 0.3 during fasted conditions, with 18% ± 6% variation during the next four 24-h periods. There was no difference in pH between daytime and night-time (1.4 ± 0.1 and 1.7 ± 0.2, respectively).

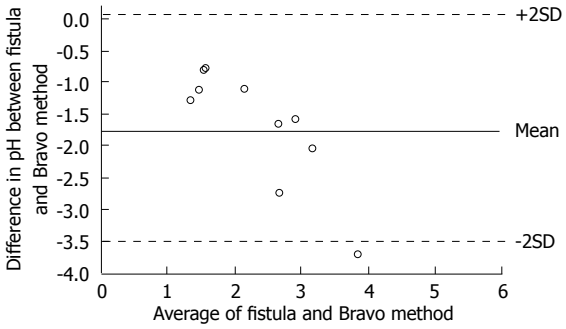


Figure 2 Bland-Altman analysis comparing the Bravo system with the fistula method. Mean value -1.7 with 2SD from -3.5 to 0.12.

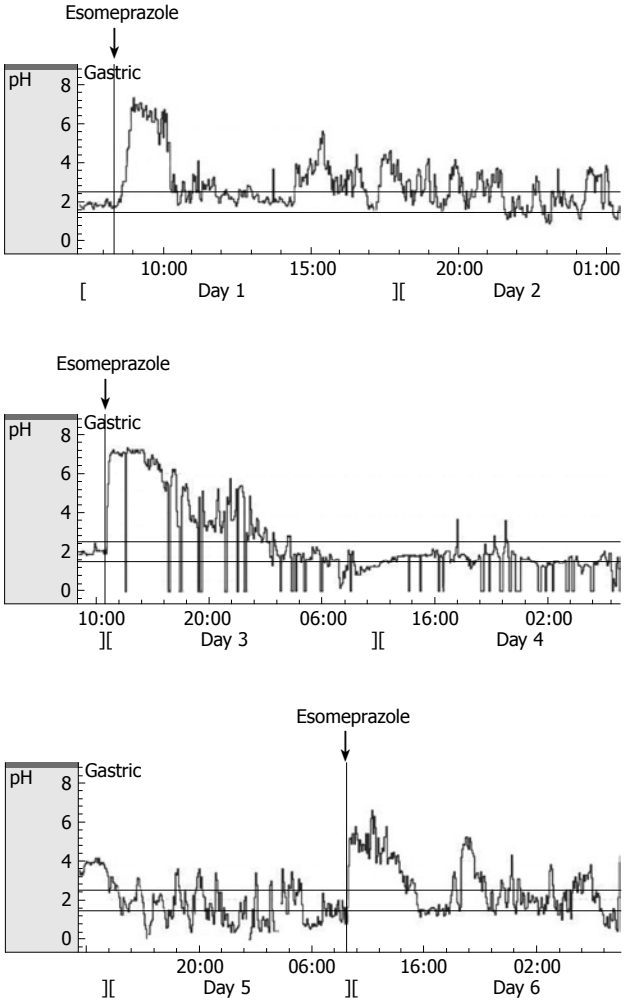


Figure 3 Standard recording with the Bravo system of intragastric pH in a rat during 6 d. The solid line indicates bolus doses of esomeprazole (3 mg/kg) given iv during 3 min under fed conditions.

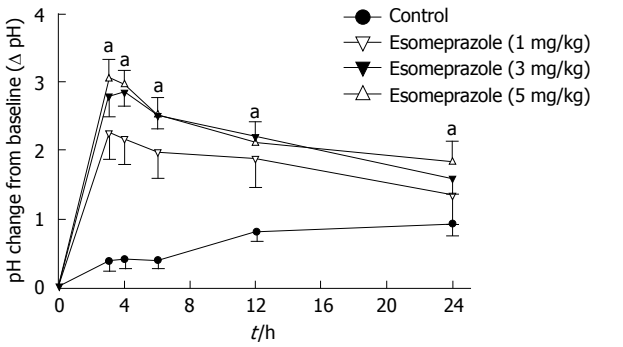


Figure 4 Change from baseline of intragastric pH ± SE after an iv bolus of esomeprazole in three different doses and saline studied for 24 h during fed conditions. Mean for all doses *vs* control and for dose 1 mg/kg vs 5 mg/kg ($^aP < 0.05$).

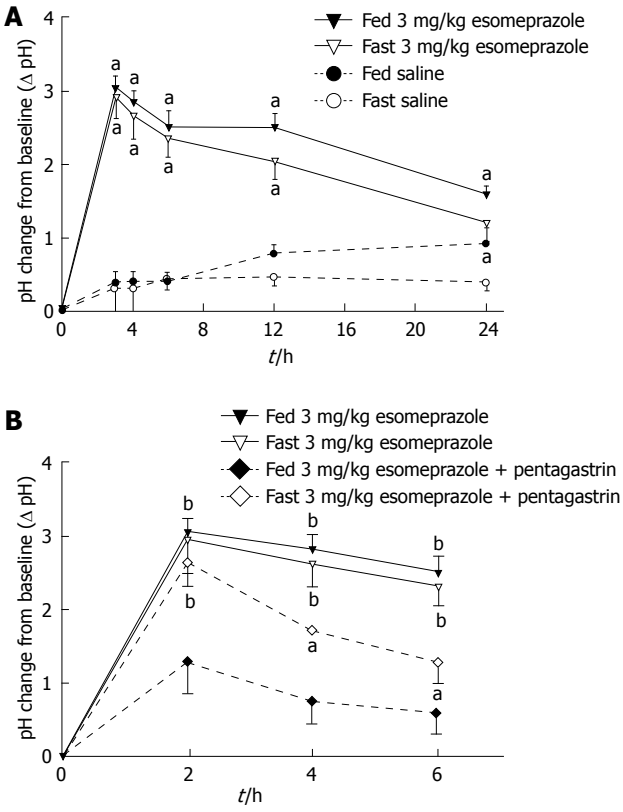


Figure 5 Changes of intragastric after an iv bolus of esomeprazole. A: Change from baseline of intragastric pH ± SE after an iv bolus of esomeprazole (3 mg/kg) or saline during fed or fasting conditions for 24 h ($^aP < 0.05$); B: Change form baseline of intragastric pH ± SE after an iv bolus of esomeprazole (3 mg/kg) or saline during pentagastrin (90 pmol/kg per min) infusion during fed or fasting conditions during 6 h. Mean esomeprazole *vs* esomeprazole and pentagastrin for fed ($^bP < 0.01$). Mean esomeprazole fed *vs* fasting esomeprazole and pentagastrin ($^aP < 0.05$). Mean esomeprazole fed *vs* fasting esomeprazole and pentagastrin ($^bP < 0.01$).

The effect of bolus esomeprazole on pH

As studied over 24 h, there was a dose-dependent increase of pH after esomeprazole, 1 mg/kg, 3 mg/kg, and 5 mg/kg, during free roaming conditions (Figure 4). Already 3 h after administration of esomeprazole, pH was significantly higher with 5 mg/kg, 3.1 ± 0.4, than with 1 mg/kg, 2.2 ± 0.4 ($P < 0.05$). Esomeprazole (3 mg/kg) increased intragastric pH during saline infusion over a 6-h period (2.5 ± 0.2) compared to baseline pH (1.6 ± 0.2), whereas saline did not ($P < 0.01$; Figure 5A).

Esomeprazole was equally effective during fed or fasting conditions (Figure 5A). As a control, saline did not change intragastric pH during either fed (baseline pH 1.4 ± 0.1) or fasting (baseline pH 1.6 ± 0.2) conditions (Figure 4, Figure 5A).

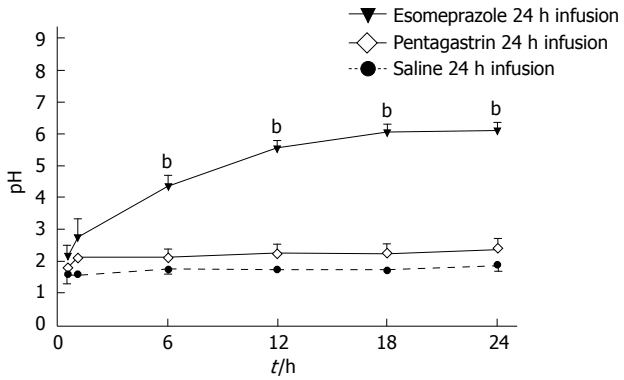


Figure 6 Change in mean pH ± SE over time. Mean esomeprazole (9 pmol/kg per min) 24 h infusion and pentagastrin (90 pmol/kg per min) infusion. ($^bP < 0.01$ *vs* control).

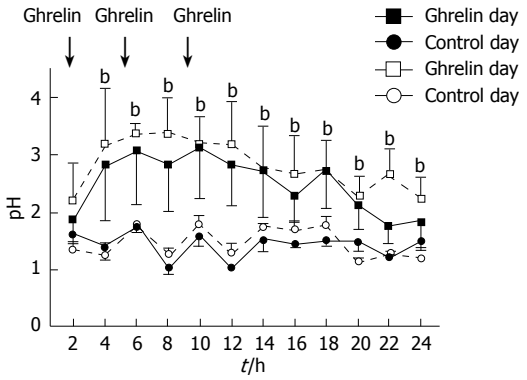


Figure 7 Change in mean pH ± SE during treatment with bolus dose ghrelin t.i.d. (2400 pmol/kg, $^bP < 0.01$ ghrelin *vs* control). There was no day-to-day difference in variation of the ghrelin response during the 5 d.

The effect of pentagastrin on pH

Pentagastrin alone did not change pH over 6 h compared with fed (baseline pH 2.1 ± 0.2) or fasting (baseline pH 2.4 ± 0.2) conditions. After esomeprazole (3 mg/kg), pentagastrin infusion markedly decreased pH from 2.0 ± 0.3 to 1.0 ± 0.2 ($P < 0.05$, Figure 5B). This effect was most marked in fed animals.

The effect of 24-h infusion of esomeprazole on pH

After esomeprazole (9 pmol/kg per min) the average 24-h pH was substantially higher than in the controls, 5.7 ± 0.3 and 2.1 ± 0.2, respectively ($P < 0.01$). Pentagastrin alone did not change pH over the 24-h infusion period as compared to saline (Figure 6).

The effect of ghrelin on pH

Administration of ghrelin, t.i.d markedly increased gastric 24-h pH from day 1 (2.5 ± 0.6) to day 5 (2.8 ± 0.5) compared to control day 1 (1.4 ± 0.1) and day 5 (1.5 ± 0.2) ($P < 0.01$; $n = 7$). There was no significant day-to-day variation of the ghrelin effect during the five days (Figure 7).

Plasma levels of gut hormones

Esomeprazole (3 mg/kg) t.i.d resulted in a marked increase in plasma ghrelin and somatostatin concentrations as shown in Figure 8 ($P < 0.001$). Plasma gastrin, however, remained stable over the same time period (Figure 8).

DISCUSSION

This study demonstrates that the Bravo system can be used for studies of intragastric pH in rats and that the results are comparable to those of a standard fistula model. The system allows for long-term studies during unrestrained living conditions. There are several advantages with the use of the Bravo system. Previous models for studies of gastric acid secretion do not allow measurements of pH over a long time. Furthermore, during these studies, the animals are kept under stressful conditions, which to a certain degree, may influence the responsiveness of the animals to different stimuli. The Bravo system uses a telemetric system that records gastric pH during 24 h for up to five consecutive days. The day-to-day variation was within acceptable limits. The system allows for real-time recordings of intragastric pH with the ability to record from the start of a treatment until a detectable effect is seen. The system is suitable for long-term studies with continuous infusions that are difficult to perform using the fistula model, as the animals do not tolerate being restrained in cages during prolonged studies. The Bravo recording system is also a digital recording system, which means that primary data are logged, and permits detailed measurements as determined by the set sampling frequency.

The data are, however, limited to pH-values as no secretion volumes are obtained. With the gastric fistula model, recordings are made over no less than 15-min periods, which can be a limiting factor as regards rapid changes in pH, i.e. drug effects. However, in the fistula model, secretion volumes are recorded, which permit calculation of a true acid output. The Bravo system has a few drawbacks. It is expensive, the battery life of the capsule is short (5 d) and therefore, the animals can only be used in studies for about a week. This means that experiments must start immediately after the operation (in this case 2 d after the surgical procedure), and the recovery from surgery may influence the results and the comparison with the fistula model. Despite this, the Bravo system seems to be well tolerated, as the stomach of the rats did not show any abnormalities or mucosal lesions upon autopsy. The animals also gain weight and behaved in a normal fashion during the experiments.

From a physiological viewpoint, our results demonstrated expected results; intragastric pH in rodents was stable over time, with a slight increase during the night during fed conditions.

In addition, treatment with esomeprazole and pentagastrin gave expected results. The agreement between the Bravo system and the established fistula method was evaluated employing a Bland-Altman analysis. When the two methods were compared, the pH results obtained with the Bravo system were comparable to those obtained using the fistula model. The differences lie within acceptable limits of agreement approximately 95% of the time, and the variability

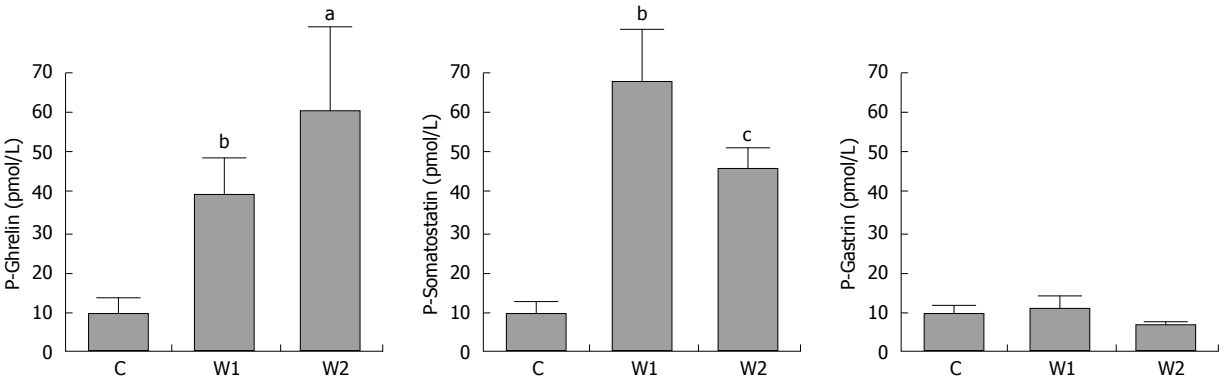


Figure 8 Gut peptide concentrations during treatment week 1 (W1) and week 2 (W2). The two groups of animals (n_{tot} = 10) were treated during one week with esomeprazole (3 mg/kg). After the first treatment week the first group (n = 5) were euthanaized and plasma were taken for peptide measurements. The other group (n = 5) went on for another week without any drug treatment and then euthanaized and plasma taken for analysis of peptides. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$ *vs* control.

was consistent across the graph; the scatter around the baseline (mean) did not increase with increasing means.

During comparative studies, the animals were restrained in Bollman cages for infusion of pentagastrin, so the experimental conditions were the same. During esomeprazole treatment, pH rose and gastric acid output decreased accordingly. There seemed to be a slight delay in response to esomeprazole when studied by the fistula method as compared to the Bravo system. The reason for this is probably related to the fact that the secretory response depends on the physical emptying of gastric contents from the fistula until measurements can be done. As judged from our experiments, this causes a delay of the recorded response of about 30 min. Pentagastrin increased acid output, but no change was seen in intragastric pH with the Bravo system. This is explained by the fact that a change in secretion volume does not affect the pH recorded, even though acid output is changed. The fact that pH does not change when introducing pentagastrin may be due to the constantly low basal pH level in the rat stomach.

The gut hormones assayed in this study, ghrelin, gastrin, and somatostatin, are all found in the mucosa of the stomach[18]. They operate in a coherent inhibitory/stimulatory fashion against one another, i.e. increasing levels of somatostatin stimulates ghrelin, while gastrin is inhibited[19,20]. Pentagastrin acts as an agonist on acid secretion and has a stimulatory effect on somatostatin, which in turn down-regulates the release of gastrin so that excessive amounts of acid are not produced[20]. The fact that basal plasma gastrin levels remained stable with the Bravo system indicates that the Bravo capsule by itself does not distend the stomach to such a degree that gastrin levels are affected[21].

Our results using the Bravo system, with an increase of intragastric pH during 1 wk after three times daily, administration of ghrelin, are in accordance with earlier studies[19,22], but at variance with another[23]. This may be explained by the fact that different methods for studying gastric acid secretion have been employed, some of which are dependent on gastric motility for the emptying of gastric secretions through the fistula. By using the Bravo system, we found no desensitization of the pH response to ghrelin. This is at variance with our previous studies on intestinal motility, in which a loss of the ghrelin response was shown[19,24]. This might be due to the fact that motility was stimulated by a continuous infusion of the hormone, whereas the pH effect was brought about by repeated injections of ghrelin, a form of administration that is considered less liable to desensitization effects. As ghrelin not only increases intragastric pH, but also stimulates gastric emptying in rodents[22,23,25,26]. This may be an erroneous factor in determining acid secretion using the fistula method.

With esomeprazole treatment, plasma concentrations of ghrelin and somatostatin were increased. This effect was maintained for 1 wk after esomeprazole treatment. The underlying mechanism for this increase in plasma ghrelin and somatostatin is not yet fully understood, but may be due to a direct effect of esomeprazole on ghrelin and somatostatin, but also by an indirect effect through changes in gastric pH. The counter-balancing effects between pentagastrin (low pH) and esomeprazole (high pH) as regards ghrelin levels point to a physiological role of ghrelin in the control of gastric acid secretion[27,28]. The rise in somatostatin concentration is likely due to a direct effect of the continuous doses of ghrelin, as pH was not affected. The lack of elevated levels of gastrin for the two groups are probably attributed to the increase in somatostatin[29] or, although less likely, low doses of esomeprazole[30,31].

To conclude, the Bravo capsule system is to be used for prolonged studies of gastric pH in free roaming conscious rats over days and is well tolerated, and could serve as a complement to the gastric fistula model, as shown by acid and gut hormone secretion measurements.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical support by Wiveca Ring-Persson and the invaluable assistance by Berndt Wallin at the Karolinska University Hospital Solna. This study was funded by GlaxoSmithKline and the Karolinska Institute.

COMMENTS

Background

The pharmacological treatment of gastrointestinal acid-related diseases aims at providing ulcer and mucosal healing, symptom relief and improved quality of life. Gastric acid inhibitory compounds are widely used in the clinical setting in order to treat not only benign gastric and duodenal ulcers, but also gastritis and reflux esophagitis. Over the past two decades, there has been a number of reports on the use of proton pump inhibitors (PPIs) such as omeprazole and the following competitors. The PPIs are activated in the acid environment in the stomach and inhibit the final step of gastric acid secretion. They bind in a non-competitive way to the H^+, K^+ -ATPase and inhibit secretion. Even though the PPIs have many good properties compared to other treatment regims, and are considered the treatment of choice in acid-related gastrointestinal diseases, there are drawbacks with PPI treatment. For instance, the onset of action is slow as compared to that of H2-receptor antagonists, which induce an immediate acid inhibition, and the duration of action may be too short giving room for night-time acid breakthrough. So far, treatments have got around this problem by recommending a two-dose regimen. Pharmaceutical development has been directed against finding a compound with profound acid inhibitory action over prolonged periods of time, not permitting night-time acid breakthrough to take place. The development of such drugs, however, require new methods of studying gastric acid secretion over prolonged periods, up to 120 h over or more.

Research frontiers

Research concerning acid-related diseases has been focused on PPIs targeted against the H^+, K^+ -ATPase of the stomach and H2-receptor antagonists. Recent studies have shown that the proton pump is the most likely candidate for a sustainable therapeutic application in the regulation of acid suppression. One of the hurdles in this field is the possibility to perform long-term measurements of acid secretion in the development of pharmacological treatment of acid diseases. Although PPIs are highly effective as a class, differences in their pharmacokinetics, such as bioavailability, metabolism, and elimination half-life, may translate into differences in clinical outcomes.

Innovations and breakthroughs

Over the latest years, new drugs have emerged on the market, such as being PPIs (3rd generation), new potassium channel blocking agents that inhibit gastric secretion (P-CAP), and even combinations of PPIs and H2-receptor antagonists. A second line to this further development is to be expected and with this new method, developed as a tool for evluation of such long-acting drugs, may become a feasible tool in the clinical setting for treatment of acid-dependent diseases.

Applications

Our research demonstrates stable recordings with the Bravo capsule system in the rat. The animals were given PPI and ghrelin and this resulted in an almost immediate response in pH, sustained during approximately 6 h. The capsule model was compared with the fistula model and showed agreement in compliance between the two methods. This indicates that the capsule model could eventually replace the fistula model. It seems better to use the former method because of less strain on the rats, and easier and more gentle handling and experimental procedures. Furthermore, the Bravo system set-up is easy to manage and the information recorded allows many different analysis variables. The system also records over five consecutive days, which previously has not been possible in this setting.

Terminology

Bravo capsule system: A catheter-free system used to measure esophageal pH (acidity) levels in patients who have or are suspected of having gastroesophageal reflux disease, but has now also been used for intragastric titration of pH.

Peer review

The measurement of intragastric pH with the Bravo capsule system is comparable to that of the gastric fistula model, and is useful for prolonged studies of gastric pH, even in free roaming conscious rats over days, as described. Although further studies are required, this study indicates the novel possibility for investigating the acid and gut hormone secretion under more physiological conditions.

REFERENCES

1 **Shay H**, Komarov SA, Gruenstein M. Effects of vagotomy in the rat. *Arch Surg* 1949; **59**: 210-226

2 **Brodie DA**, Knapp PG. The mechanism of the inhibition of gastric secretion produced by esophageal ligation in the pylorus-ligated rat. *Gastroenterology* 1966; **50**: 787-795

3 **Hakanson R**, Hedenbro J, Liedberg G, Sundler F, Vallgren S. Mechanisms of acid secretion after pylorus and oesophagus ligation in the rat. *J Physiol* 1980; **305**: 139-149

4 **Lane A**, Ivy AC, Ivy EK. Response of the chronic gastric fistula rat to histamine. *Am J Physiol* 1957; **190**: 221-228

5 **Emas S**, Nylander G, Wallin B. Comparison of the dose-response curves for acid output to pentagastrin determined by two techniques in chronic gastric fistula rats. *Digestion* 1981; **22**: 94-100

6 **Pascaud XB**, Roger AR, Genton MJ. Comparison of the step-dose and single-dose acid response to histamine and pentagastrin in chronic gastric fistula rats. *Digestion* 1977; **16**: 57-68

7 **Blandizzi C**, Colucci R, Carignani D, Natale G, Lazzeri G, Crema F, Del Tacca M. Role of peripheral GABAB receptors in the regulation of pepsinogen secretion in anaesthetized rats. *Eur J Pharmacol* 1995; **294**: 191-200

8 **Short GM**, Wolfe MM, McGuigan JE. Pentagastrin-stimulated gastric acid secretion by the isolated perfused rat stomach. *Life Sci* 1984; **34**: 2515-2523

9 **Kleveland PM**, Haugen SE, Sandvik S, Waldum HL. The effect of pentagastrin on the gastric secretion by the totally isolated vascularly perfused rat stomach. *Scand J Gastroenterol* 1986; **21**: 379-384

10 **Kleveland PM**, Waldum HL, Larsson H. Gastric acid secretion in the totally isolated, vascularly perfused rat stomach. A selective muscarinic-1 agent does, whereas gastrin does not, augment maximal histamine-stimulated acid secretion. *Scand J Gastroenterol* 1987; **22**: 705-713

11 **Suzuki T**, Yamaguchi T, Odaka T, Kobayashi M, Seza A, Kouzu T, Yokosuka O. Four-day continuous gastric pH monitoring following anti-acid secretory drug administration: cross-over test to assess the early effects. *Aliment Pharmacol Ther* 2008; **27**: 66-71

12 **Kwiatek MA**, Pandolfino JE. The Bravo pH capsule system. *Dig Liver Dis* 2008; **40**: 156-160

13 **Hirano I**. Review article: modern technology in the diagnosis of gastro-oesophageal reflux disease--Bilitec, intraluminal impedance and Bravo capsule pH monitoring. *Aliment Pharmacol Ther* 2006; **23** Suppl 1: 12-24

14 **Bhat YM**, McGrath KM, Bielefeldt K. Wireless esophageal pH monitoring: new technique means new questions. *J Clin Gastroenterol* 2006; **40**: 116-121

15 **Chen EP**, Mahar Doan KM, Portelli S, Coatney R, Vaden V, Shi W. Gastric pH and gastric residence time in fasted and fed conscious cynomolgus monkeys using the Bravo pH system. *Pharm Res* 2008; **25**: 123-134

16 **Bland JM**, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986; **1**: 307-310

17 **Bland JM**, Altman DG. Measuring agreement in method comparison studies. *Stat Methods Med Res* 1999; **8**: 135-160

18 **Schubert ML**. Gastric secretion. *Curr Opin Gastroenterol* 2007; **23**: 595-601

19 **Levin F**, Edholm T, Ehrstrom M, Wallin B, Schmidt PT, Kirchgessner AM, Hilsted LM, Hellstrom PM, Naslund E. Effect of peripherally administered ghrelin on gastric emptying and acid secretion in the rat. *Regul Pept* 2005; **131**: 59-65

20 **Kolivas S**, Shulkes A. Regulation of expression of the receptors controlling gastric acidity. *Regul Pept* 2004; **121**: 1-9

21 **Lloyd KC**, Raybould HE, Tache Y, Walsh JH. Role of gastrin, histamine, and acetylcholine in the gastric phase of acid secretion in anesthetized rats. *Am J Physiol* 1992; **262**: G747-G755

22 **Murakami N**, Hayashida T, Kuroiwa T, Nakahara K, Ida T, Mondal MS, Nakazato M, Kojima M, Kangawa K. Role for central ghrelin in food intake and secretion profile of stomach ghrelin in rats. *J Endocrinol* 2002; **174**: 283-288

23 **Dornonville de la Cour C**, Lindstrom E, Norlen P, Hakanson R. Ghrelin stimulates gastric emptying but is without effect on acid secretion and gastric endocrine cells. *Regul Pept* 2004; **120**: 23-32
24 **Bassil AK**, Haglund Y, Brown J, Rudholm T, Hellstrom PM, Naslund E, Lee K, Sanger GJ. Little or no ability of obestatin to interact with ghrelin or modify motility in the rat gastrointestinal tract. *Br J Pharmacol* 2007; **150**: 58-64
25 **Fujino K**, Inui A, Asakawa A, Kihara N, Fujimura M, Fujimiya M. Ghrelin induces fasted motor activity of the gastrointestinal tract in conscious fed rats. *J Physiol* 2003; **550**: 227-240
26 **Depoortere I**, De Winter B, Thijs T, De Man J, Pelckmans P, Peeters T. Comparison of the gastroprokinetic effects of ghrelin, GHRP-6 and motilin in rats in vivo and in vitro. *Eur J Pharmacol* 2005; **515**: 160-168
27 **Scarpignato C**, Pelosini I, Di Mario F. Acid suppression therapy: where do we go from here? *Dig Dis* 2006; **24**: 11-46
28 **Hagiwara T**, Mukaisho K, Ling ZQ, Sugihara H, Hattori T. Development of pancreatic acinar cell metaplasia after successful administration of omeprazole for 6 months in rats. *Dig Dis Sci* 2007; **52**: 1219-1224
29 **Schubert ML**. Regulation of gastric acid secretion. *Curr Opin Gastroenterol* 1999; **15**: 457-462
30 **Hagiwara T**, Mukaisho K, Ling ZQ, Sakano T, Sugihara H, Hattori T. Rebamipide contributes to reducing adverse effects of long-term administration of omeprazole in rats. *Dig Dis Sci* 2007; **52**: 988-994
31 **Ryberg B**, Bishop AE, Bloom SR, Carlsson E, Hakanson R, Larsson H, Mattsson H, Polak JM, Sundler F. Omeprazole and ranitidine, antisecretagogues with different modes of action, are equally effective in causing hyperplasia of enterochromaffin-like cells in rat stomach. *Regul Pept* 1989; **25**: 235-246

S- Editor Xiao LL **L- Editor** Rippe RA **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6188

World J Gastroenterol 2008 October 28; 14(40): 6188-6194
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Gardenia jasminoides protects against cerulein-induced acute pancreatitis

Won-Seok Jung, Young-Seok Chae, Do-Yun Kim, Sang-Wan Seo, Hee-Je Park, Gi-Sang Bae, Tae-Hyeon Kim, Hyo-Jeong Oh, Ki-Jung Yun, Rae-Kil Park, Jong-Suk Kim, Eun-Cheol Kim, Sung-Yeon Hwang, Sung-Joo Park, Ho-Joon Song

Won-Seok Jung, Jong-Suk Kim, Department of Biochemistry, Institute of Medical Science, Chonbuk National University Medical School, Jeonju, 570-749 Jeonbuk, South Korea
Won-Seok Jung, Young-Seok Chae, Sang-Wan Seo, Hee-Je Park, Gi-Sang Bae, Do-Yun Kim, Sung-Yeon Hwang, Sung-Joo Park, Ho-Joon Song, Department of Herbology, School of Oriental Medicine, Wonkwang University, Iksan, 570-749 Jeonbuk, South Korea
Sang-Wan Seo, Kohwang Medical Institute, College of Medicine, Kyung Hee University, 130-701 Seoul, South Korea
Tae-Hyeon Kim, Hyo-Jeong Oh, Department of internal medicine, College of Medicine, Wonkwng University, Iksan, 570-749 Jeonbuk, South Korea
Ki-Jung Yun, Department of Pathology, College of Medicine, Wonkwng University, Iksan, 570-749 Jeonbuk, South Korea
Rae-Kil Park, Vestibulocochlear Research Center & Department of Microbiology, College of Medicine, Wonkwng University, Iksan, 570-749 Jeonbuk, South Korea
Eun-Cheol Kim, Department of Oral & Maxillofacial Pathology, Dental College, Wonkwang University, Iksan, 570-749 Jeonbuk, South Korea
Author contributions: Jung WS and Chae YS contributed equally to the research; Kim DY, Seo SW, Park HJ and Bae GS performed in vivo experiments; Kim TH and Oh HJ perfomed MPO; Yun KJ and Park RK performed immunohistochemistry; Kim JS, Kim EC, Hwang SY and Park SJ performed ELISA and RT-PCR: Jung WS and Song HJ wrote the paper.
Supported by The Ministry of Science & Technology (MoST)/ Korea Science & Engineering Foundation (KOSEF) through the Vestibulocochlear Research Center (VCRC) at Wonkwang University (R13-2002-055-00000-0)
Correspondence to: Ho-Joon Song, Department of Herbology, School of Oriental Medicine, Wonkwang University, Iksan, Jeonbuk 570-749, South Korea. parksj08@wku.ac.kr
Telephone: +82-63-8506450 Fax: +82-63-8562283
Received: April 4, 2008 Revised: July 17, 2008
Accepted: July 24, 2008
Published online: October 28, 2008

Abstract

AIM: To investigate the effect of *Gardenia jasminoides* (GJ) on cerulein-induced acute pancreatitis (AP) in mice.
METHODS: C57BL/6 mice weighing 18-20 g were divided into three groups. (1) Normal saline-treated group, (2) treatment with GJ at a dose of 0.1 g/kg, (3) treatment with GJ at a dose of 1 g/kg. GJ was administered orally (n = 6 per group) for 1 wk. Three hours later, the mice were given an intraperitoneal injection of cerulein (50 μg/kg), a stable cholecystokinin (CCK) analogue, every hour for a total of 6 h as described previously. The mice were sacrificed at 6 h after completion of cerulein injections. Blood samples were obtained to determine serum amylase, lipase and cytokine levels. The pancreas was rapidly removed for morphologic examination and scoring. A portion of pancreas was stored at -70℃ and prepared for the measurement of tissue myeloperoxidase (MPO) activity, an indicator of neutrophil sequestration, and for reverse-transcriptase PCR (RT-PCR) and real-time PCR measurements.
RESULTS: Treatment with GJ decreased significantly the severity of pancreatitis and pancreatitis-associated lung injury. Treatment with GJ attenuated the severity of AP compared with saline-treated mice, as shown by reduction in pancreatic edema, neutrophil infiltration, serum amylase and lipase levels, serum cytokine levels, and mRNA expression of multiple inflammatory mediators.
CONCLUSION: These results suggest that GJ attenuated the severity of AP as well as pancreatitis-associated lung injury.

Key words: Gardenia jasminoides; Acute pancreatitis; Cerulein

Peer reviewer: James H Grendell, Professor of Medicine, Chief-Division of Gastroenterology, Hepatology, & Nutrition, Winthrop University Hospital, 222 Station Plaza N. #429, Mineola, New York 11501, United States

Jung WS, Chae YS, Kim DY, Seo SW, Park HJ, Bae GS, Kim TH, Oh HJ, Yun KJ, Park RK, Kim JS, Kim EC, Hwang SY, Park SJ, Song HJ. *Gardenia jasminoides* protects against cerulein-induced acute pancreatitis. *World J Gastroenterol* 2008; 14(40): 6188-6194 Available from: URL: http://www.wjgnet.com/1007-9327/14/6188.asp DOI: http://dx.doi.org/10.3748/wjg.14.6188

INTRODUCTION

Acute pancreatitis (AP) is an acute inflammatory process of the pancreas that frequently involves peri-pancreatic

tissues and remote organ systems. The severity of the disease varies widely, the clinical course is unpredictable, and specific therapy is limited[1-4]. It is generally believed that the severity of pancreatitis is determined by events that occur after acinar cell injury. Pancreatic acinar cells synthesize and release cytokines and chemokines, resulting in the recruitment of inflammatory cells such as neutrophils and macrophages. This leads to further acinar cell injury, resulting in the elevation of various pro-inflammatory mediators such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α[5,6]. The release of inflammatory mediators such as TNF-α and IL-1β during AP propagates a complex cascade of events between tissue vasculature and inflammatory cells. These inflammatory cells and mediators play a role in the systemic manifestations besides modulating pancreatic acinar cell injury; blocking the cytokine cascade in its early stage, and ameliorating the disease and its systemic complications.

Gardenia jasminoides (GJ) is widely employed in several Asian countries as a natural colorant, and has been used in Chinese traditional medicine for its homeostatic, antiphlogistic, analgesic and antipyretic effects. Its main components include geniposide and crocin[7]. These components exhibit antioxidant, cytotoxic, antitumor and neuroprotective effects[8-10]. However, the impact of GJ and its components on cerulein-induced AP have not been examined.

The present study was designed to confirm the preventive effects of GJ in a mouse model of cerulein-induced AP. In order to gain a better insight into the mechanism of action of the observed anti-inflammatory effects of GJ, we investigated the effects of GJ on (1) pancreas weight/body weight (PW/BW) ratio, (2) pancreatic histology, (3) serum amylase and lipase levels, (4) serum level of pro-inflammatory mediators, such as TNF-α, IL-1β and IL-6, and (5) lung histology.

MATERIALS AND METHODS

Materials

Avidin-peroxidase and 2'-AZINO-bis (3-ethylbenzithiazoline-6-sulfonic acid) tablets, cerulein, Tris-HCl, NaCl, Triton X-100, hexadecyltrimethylammonium bromide and etramethylbenzidine were purchased from Sigma (St. Louis, MO, USA). Anti-mouse TNF-α, IL-6 and IL-1β antibodies, recombinant TNF-α, IL-6 and IL-1β were purchased from R&D Systems (Minneapolis, MN, USA).

Preparation of GJ

GJ was prepared by decocting the dried prescription of herbs with boiling distilled water. The decoction time was about 3 h. Their voucher specimens were deposited at the Herbarium at the College of Oriental Medicine, Won-Kwang University.

Animal models

All experiments were performed according to protocols approved by the Animal Care Committee of the university. Female C57BL/6 mice (6-7-wk old, weighting 18-20 g) were purchased from Orient Bio Co. (Sungnam, KyungKiDo, Republic of Korea). All animals were bred and housed in standard shoebox cages in a climate-controlled room with an ambient temperature of 23 ± 2℃ and a 12-h light-dark cycle for 7 d. Animals were fed standard laboratory chow, given water and randomly assigned to control or experimental groups. The mice were fasted for 18 h before induction of AP. Six mice were included in each experimental group.

Experimental design

AP was induced by supramaximal concentration of cerulein (50 μg/kg), a stable CCK analogue, by administering it intraperitoneally every hour for a total of 6 h as described previously[11]. The mice were fed orally with GJ (1 g/kg, 0.1 g/kg, $n = 6$ each) or normal saline (control group, $n = 6$), followed by intraperitoneal injection of cerulein (50 μg/kg) or saline every hour for a total of 5 h. The mice were sacrificed at 12 h after the completion of cerulein injections. Blood samples were obtained to determine serum amylase, lipase and cytokine levels. The pancreas was rapidly removed for morphologic examination and scoring. A portion of pancreas was stored at -70℃ and prepared for the measurement of tissue myeloperoxidase (MPO) activity, an indicator of neutrophil sequestration, and for reverse-transcriptase PCR (RT-PCR) and real-time PCR measurements.

Histological analyses

The entire pancreas of at least six mice from each treatment group was examined and semi-quantitated based on the degree of necrosis, vacuolization, inflammation, and edema. Using the previously described method of Ethridge *et al*[12], entire sections (a minimum of 100 fields) of pancreas were examined from each sample and scored on a scale of 0-3 (0 being normal and 3 being severe), based on the number of necrotic acinar cells, and the presence of vacuolization, interstitial edema, and interstitial inflammation. The characteristics included were: presence of acinar-cell ghosts, vacuolization and swelling of the acinar cells, and/or the destruction of the histo-architecture of the whole or parts of the acini.

Enzyme-linked immunosorbent assay (ELISA)

ELISA for TNF-α, IL-1β and IL-6 (R&D Systems) was carried out in duplicate in 96-well plates (Nunc, Denmark), coated with each of the following: 100 μL aliquots of anti-mouse IL-6, IL-1β and TNF-α monoclonal antibodies at 1.0 μg/mL in PBS at pH 7.4, and incubated overnight at 4℃. The plates were washed in PBS containing 0.05% Tween-20 (Sigma) and blocked with PBS containing 1% BSA, 5% sucrose and 0.05% NaN_3 for 1 h. After additional washes, the standards were added and incubated at 37℃ for 2 h. After incubation for 2 h at 37℃, the wells were washed, and the following were added: 0.2 μg/mL of biotinylated anti-mouse TNF-α, IL-1β and IL-6, and again incubated at 37℃ for 2 h. After the wells were washed, avidin-peroxidase

was added and the plates were incubated for 20 min at 37℃. The wells were again washed and ABTS substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate using recombinant TNF-α, IL-1β and IL-6 in serial dilutions.

Measurement of serum amylase and lipase

Arterial blood samples for determination of serum amylase and lipase were obtained 12 h after induction of pancreatitis. The mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (4 mg/kg). After anesthetization, blood samples were withdrawn from the heart. Serum amylase was measured using ADIVA 1650 (Bayer, USA). Serum lipase was measured using a Cobas-mira (Roche, USA).

mRNA expression

mRNA transcripts were analyzed by RT-PCR in mouse pancreatic tissues. Total RNA was isolated from the mouse pancreas using Qiagen RNeasy kit and subjected to reverse transcription using SuperScript Ⅱ RT (Invitrogen). Taqman quantitative RT-PCR with a 7700 Sequence Detection System was done according to the instructions of the manufacturer (Applied Biosystems). For each sample, triplicate test reactions and a control reaction lacking reverse transcriptase were analyzed for expression of the gene of interest, and results were normalized to those of the 'housekeeping' HPRT mRNA. Arbitrary expression units were calculated by division of expression of the gene of interest by ribosomal protein HPRT mRNA expression. The forward, reverse and probe oligonucleotide primers for multiplex real-time TaqMan PCR were as follows: for mouse TNF-α (forward, 5'-TCTCTTCAAGGGACAAGGCTG-3'; reverse, 5'-ATAGCAAATCGGCTGACGGT-3'; probe, 5'-CCCGACTACGTGCTCCTCACCCA-3'), for mouse IL-1β (forward, 5'-TTGACGGACCCCAAAAGAT-3'; reverse, 5'-GAAGCTGGATGCTCTCATCTG-3'; universal probe, M15131.1-Roche Applied Science), for mouse IL-6 (forward, 5'-TTCATTCTCTTTGCTCTTGAATTAGA-3'; reverse, 5'-GTCTGACCTTTAGCTTCAAATCCT-3'; universal probe, M20572.1-Roche Applied Science), for mouse HPRT (forward, 5'-GACCGGTCCCGTCATGC-3'; reverse, 5'-CATAACCTGGTTCATCATCGCTAA-3'; probe, 5'-ACCCGCAGTCCCAGCGTCGT-3').

MPO estimation

Neutrophil sequestration in the pancreas was quantified by measuring the tissue MPO activity[11,12]. Tissue samples were thawed, homogenized in 20 mmol/L phosphate buffer (pH 7.4), and centrifuged (10000 × *g*, 10 min, 4℃), and the resulting pellet was resuspended in 50 mmol/L phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma). The suspension was subjected to four cycles of freezing and thawing and was further disrupted by sonication (40 s). The sample was then centrifuged (10000 × *g*, 5 min, 4℃), and the supernatant used for the MPO assay. The reaction mixture consisted of the supernatant, 1.6 mmol/L tetramethylbenzidine (Sigma), 80 mmol/L sodium phosphate buffer (pH 5.4), and 0.3 mmol/L hydrogen peroxide. The mixture was incubated at 37℃ for 110 s, the reaction was terminated with 2 mol/L of H_2SO_4, and the absorbance was measured at 450 nm. This absorbance was then corrected for the DNA content of the tissue sample (fold increase over control).

Statistical analysis

The results were expressed as means ± SE. The significance of change was evaluated using Student's *t* test. Differences between the experimental groups were evaluated by using analysis of variance. Values of $P < 0.05$ were accepted as statistically significant.

RESULTS

Effect of GJ on PW/BW ratio, serum amylase and lipase activity in cerulein-induced AP

To assess the effect of GJ on the PW/BW ratio, the pancreatic weight was divided by the body weight of the mice. GJ reduced significantly the PW/BW ratio, compared with the normal saline-treated group, in a dose-dependent manner ($P < 0.05$) (Figure 1A). The serum levels of amylase and lipase are commonly used as markers of AP[13,14]. GJ reduced significantly the serum amylase and lipase levels in cerulein-induced AP (Figure 1B and C).

Effect of GJ on serum levels of TNF-α, IL-1β and IL- in cerulein-induced AP

The serum levels of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6 were increased in cerulein-induced AP[15-17]. GJ decreased significantly the levels of TNF-α and IL-1β in the cerulein-induced AP. Moreover, GJ was associated with a trend towards suppression of IL-6, although the difference was not statistically significant (Figure 2).

Effect of GJ on mRNA expression of TNF-α, IL-1β and IL-6 in cerulein-induced AP

The GJ-pretreated group showed significant reduction of pancreatic tissue mRNA expression of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, in a dose-dependent manner, compared with the saline pretreated group in cerulean-induced AP (Figure 3).

Effect of GJ on pancreatic histology in cerulein-induced AP

In normal mice, the histological features of the pancreas were typical of a normal architecture. Mice treated with i.p. injections of cerulein developed acute necrotizing pancreatitis. Histological examination of the pancreas (at 12 h after the injection of cerulein) revealed tissue damage characterized by inflammatory cell infiltrate and acinar cell necrosis. GJ pretreatment resulted in significant reduction in pancreatic injury (Figure 4A). The presence

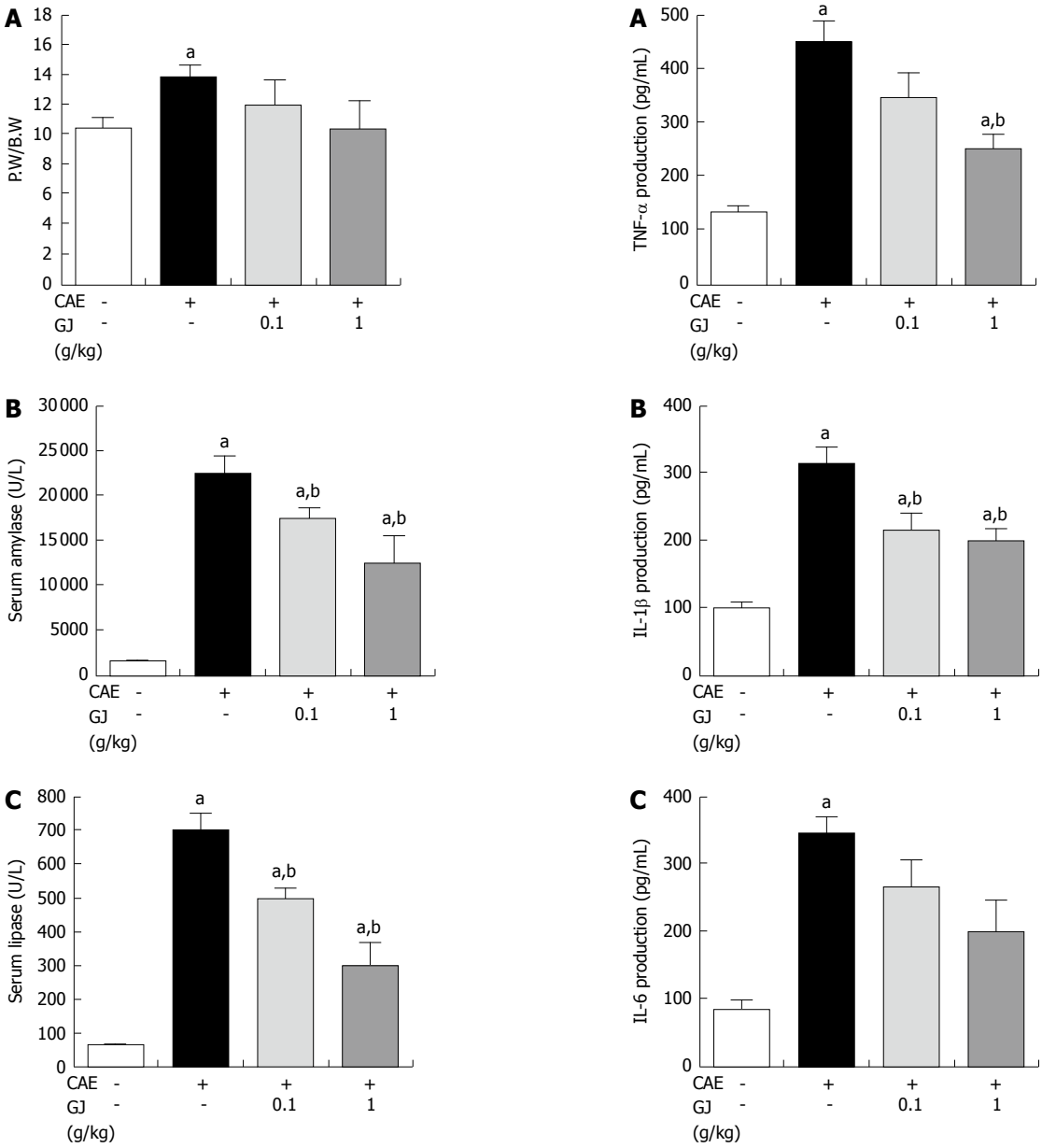


Figure 1 Effects of GJ pretreatment on the (A) PW/BW, (B) serum amylase activity, and (C) serum-lipase activity in cerulein induced AP. The study groups were treated as indicated in the experimental protocol. The mean ± SE of the six animals are shown. [a]*P* < 0.05 *vs* saline treatment; [b]*P* < 0.05 *vs* cerulein treatment alone.

Figure 2 Effect of GJ on (A) TNF-α, (B) IL-1β and (C) IL-6 secretion in cerulein-induced AP. Mice were treated as indicated in the experimental protocol. The mean ± SE of six animals are shown. The study groups were treated as indicated in the experimental protocol. [a]*P* < 0.05 *vs* saline treatment; [b]*P* < 0.05 *vs* cerulein treatment alone.

of edema, inflammation, vacuolization, and necrosis were reduced significantly in the GJ-pretreated group compared with the normal saline-pretreated group, in a dose-dependent manner (Figure 4B).

Effect of GJ on lung histology in cerulein induced AP

Lung injury commonly develops early in AP. AP-associated lung injury is characterized by edema and inflammation[18]. In addition to the pancreas, we assessed the lungs after cerulein administration. Histological examination of lung sections (at 12 h after the injection of cerulein) revealed tissue damage characterized by edema and inflammatory cell infiltrate. GJ pretreatment resulted in significant reduction in lung injury. The histological sections were scored for edema and inflammation. The lungs of the GJ-pretreated mice had significantly less edema and inflammation compared with lungs from saline-injected control animals (Figure 5).

Effect of GJ on MPO activity of lung and pancreas in cerulein induced AP

As an additional quantitative assessment of the severity of the inflammatory response, we measured MPO activity, an indicator of neutrophil sequestration, in the pancreas and lung following induction of AP in the GJ-pretreated mice and saline-injected control animals. MPO activity in the pancreas and lung in the GJ-pretreated mice was significantly less compared with the saline-injected control animals (Figure 6).

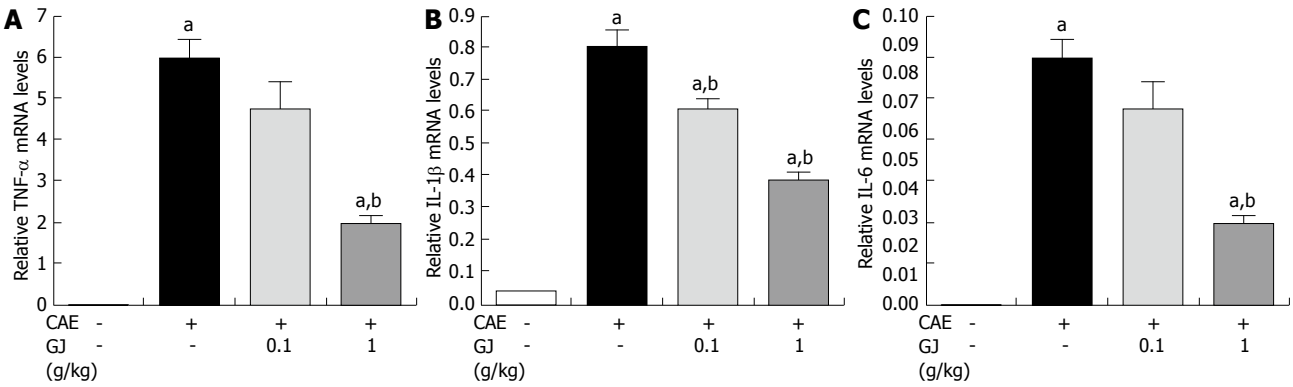


Figure 3 Effect of GJ on TNF-α, IL-6 and IL-1 mRNA levels in cerulein-induced AP. The mice were sacrificed at 1, 3 and 6 h after six injections. Levels of pancreatic mRNA were quantified by real-time PCR for (A) TNF-α, (B) IL-1β, and (C) IL-6. The mean ± SE of six animals are shown. [a]*P* < 0.05 *vs* saline treatment; [b]*P* < 0.05 *vs* cerulein treatment alone.

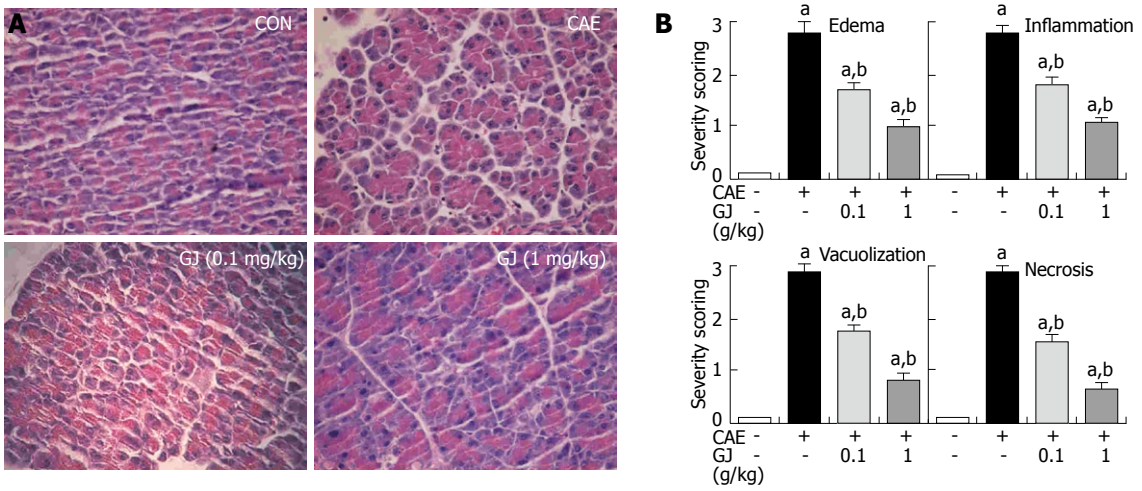


Figure 4 Effects of the GJ on pancreatic inflammatory changes following pancreatitis. A: Representative H&E-stained sections of pancreas in control mice (CON) who were not given cerulein, in mice given cerulein (CAE), and in mice given GJ (1 mg/kg) at the same time as the first cerulein injection; B: Histological sections of pancreas harvested 12 h after injection of saline (CON), cerulein alone, or GJ (1 or 0.1 mg/kg) given at the same time as the first injection of cerulein. The results were scored from 0 (normal) to 3 (severe) for edema, inflammation, vacuolization, and necrosis. [a]*P* < 0.05 *vs* saline treatment; [b]*P* < 0.05 *vs* cerulein treatment alone. The figure shows the results of one experiment in which 4-5 mice were tested per group. The results obtained were similar to those in three additional experiments (x 200).

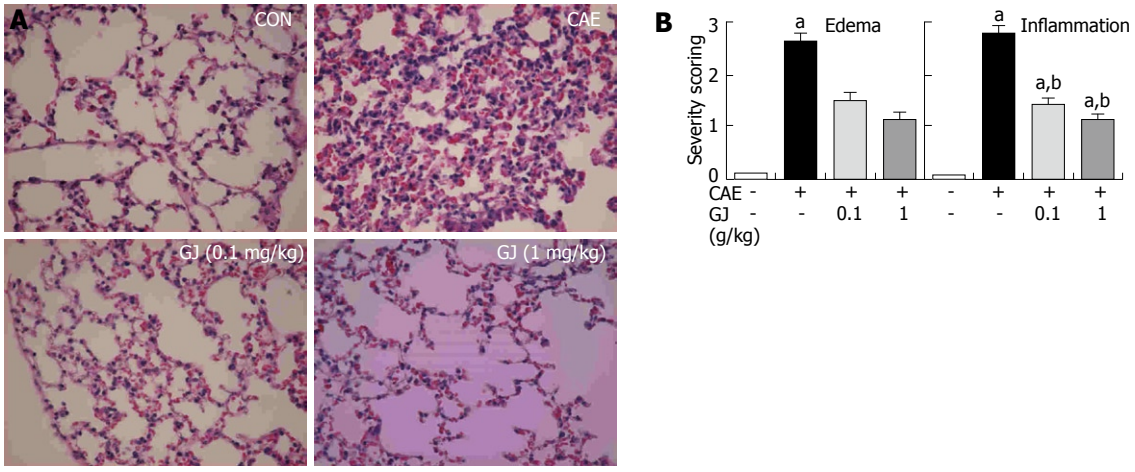


Figure 5 GJ reduced the severity of AP-associated lung injury. A: Representative H&E-stained sections of the pancreas in control mice (CON) not given cerulein, in mice given cerulein (CAE), and in mice given GJ (1 mg/kg) at the same time as the first cerulein injection; B: Histology sections of the lung harvested 12 h after administration of saline (CON), cerulein alone, or GJ (1 or 0.1 mg/kg) given at the same time as the first injection of cerulein. The results were scored from 0 (normal) to 3 (severe) for edema, inflammation, vacuolization, and necrosis. [a]*P* < 0.05 *vs* saline treatment; [b]*P* < 0.05 *vs* cerulein treatment alone. The figure shows one experiment in which 4-5 mice were tested per group. The results obtained were similar to those in three additional experiments (x 200).

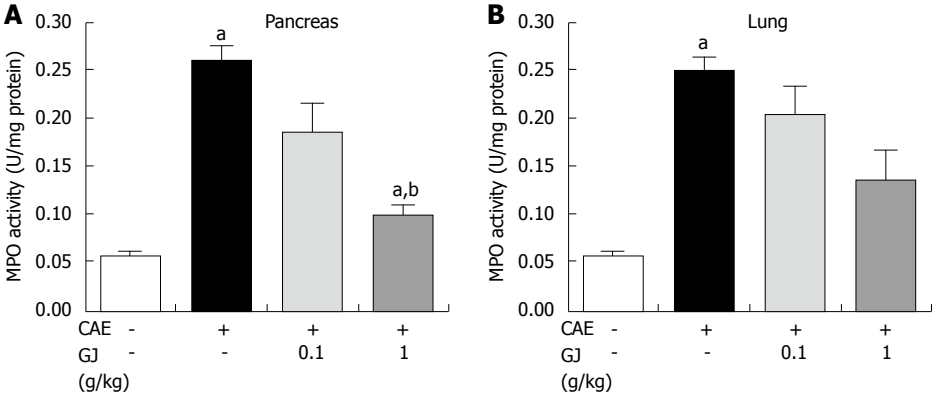


Figure 6 MPO activity was measured in the pancreas 6 h after completion of the cerulein injections and in saline-injected control mice (CON). The data are expressed as MPO activity (U/mg protein). [a]*P* < 0.05 *vs* saline treatment; [b]*P* < 0.05 *vs* cerulein treatment alone. The figure shows the results of one experiment in which 5-6 mice were tested per group.

DISCUSSION

AP is associated with a high rate of morbidity and mortality. The mortality rate in patients with severe AP is as high as 20% to 30%[1,2]. AP, characterized by interstitial edema, vacuolization, inflammation and acinar cell necrosis, is commonly caused by excessive ethanol consumption, biliary tract disease, certain medications, and invasive procedures of the biliary and pancreatic ducts[19-22]. The pathophysiology of AP is poorly understood, and the clinical course is unpredictable[7].

The fruit of GJ has been used as an oriental herbal medicine in traditional formulations. It has been employed in the treatment of inflammation, jaundice, headache, edema, fever, hepatic disorders, and hypertension. In addition, the pigments obtained from the fruit are used as food colorants. The pharmacologic actions of GJ, such as protective effect against oxidative damage, cytotoxic activity, anti-inflammatory actions, and fibrolytic activity have been described in detail[23,24]. The present study was carried out to determine whether GJ could inhibit the severity of cerulein-induced AP.

Several markers of AP such as PW/BW, amylase and lipase activity were reduced (Figure 1). There is much evidence to implicate the involvement of inflammatory mediators, such as cytokines, TNF-α, IL-1β, and IL-6 in the development of pancreatitis[25]. In experimental pancreatitis, the serum levels of TNF-α and IL-1β are elevated and their blockade attenuates the disease process[18,25]. IL-6 is one of the principal cytokine mediators of the acute-phase response, and has been suggested as a marker for predicting the severity of AP[25]. As shown in Figures 2 and 3, GJ may inhibit these cytokines in AP.

The results of the present study indicate that cerulein caused significant morphological abnormalities in the pancreas, as demonstrated by the appearance of vacuolization, inflammatory infiltration and changes in histo-architecture of the pancreatic acini. Pretreatment with GJ inhibited acinar cell death as well as infiltration by inflammatory cells in cerulein-induced AP. To rule out interference by the binding of cerulein to CCK receptors on pancreatic acinar cells, we examined the effect of GJ in pancreatic acinar cells. GJ itself did not have any cytoxicity at 6 h. However, GJ inhibited cerulein-induced aciniar cell death in a dose-dependent manner (data not shown).

Lung injury commonly develops early in AP. AP-associated lung injury is characterized by edema and inflammation[12]. Therefore, we also assessed the lungs after cerulein administration. Histological examination of lung sections (at 12 h after the injection of cerulein) revealed tissue damage characterized by edema and inflammatory cell infiltrate. GJ pretreatment resulted in a significant reduction in lung injury. The histology sections were scored for edema and inflammation and, as shown in Figure 5, the lungs of the GJ pretreated mice had significantly less edema and inflammation compared with lungs from the saline treated control animals.

In conclusion, the present study shows that GJ pretreatment ameliorated the severity of cerulein-induced AP in rats. Additionally GJ pretreatment ameliorated many of the laboratory and biochemical parameters of the disease. Our findings suggest that GJ may be beneficial in the treatment of acute pancreatitis.

REFERENCES

1 **Banks PA**. Medical management of acute pancreatitis and complications. In: Go VLW, DiMagno EP, Gardner JD, Lebenthal E, Reber HA, Scheele GA, eds. The pancreas: biology, pathobiology, and disease. New York: Raven, 1993: 593-611
2 **Rau B**, Uhl W, Buchler MW, Beger HG. Surgical treatment of infected necrosis. *World J Surg* 1997; **21**: 155-161
3 **Baron TH**, Morgan DE. Acute necrotizing pancreatitis. *N Engl J Med* 1999; **340**: 1412-1417
4 **Ho HS**, Frey CF. Gastrointestinal and pancreatic complications associated with severe pancreatitis. *Arch Surg* 1995; **130**: 817-822; discussion 822-823
5 **Norman J**. The role of cytokines in the pathogenesis of acute pancreatitis. *Am J Surg* 1998; **175**: 76-83
6 **Lane JS**, Todd KE, Gloor B, Chandler CF, Kau AW, Ashley SW, Reber HA, McFadden DW. Platelet activating factor antagonism reduces the systemic inflammatory response in a murine model of acute pancreatitis. *J Surg Res* 2001; **99**: 365-370
7 **Hu Z**, Wang Y, Luo G, He L. [Estimation of measurement uncertainty of analytical results for the determination of three active components from Gardenia jasminoides Ellis. by HPLC] *Zhong Yao Cai* 2005; **28**: 991-994
8 **Sheng L**, Qian Z, Zheng S, Xi L. Mechanism of hypolipidemic effect of crocin in rats: crocin inhibits pancreatic lipase. *Eur J Pharmacol* 2006; **543**: 116-122
9 **Pham TQ**, Cormier F, Farnworth E, Tong VH, Van Calsteren

MR. Antioxidant properties of crocin from Gardenia jasminoides Ellis and study of the reactions of crocin with linoleic acid and crocin with oxygen. *J Agric Food Chem* 2000; **48**: 1455-1461

10 **Oh PS**, Lim KT. Plant originated glycoprotein has anti-oxidative and anti-inflammatory effects on dextran sulfate sodium-induced colitis in mouse. *J Biomed Sci* 2006; **13**: 549-560

11 **Van Acker GJ**, Saluja AK, Bhagat L, Singh VP, Song AM, Steer ML. Cathepsin B inhibition prevents trypsinogen activation and reduces pancreatitis severity. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G794-G800

12 **Ethridge RT**, Chung DH, Slogoff M, Ehlers RA, Hellmich MR, Rajaraman S, Saito H, Uchida T, Evers BM. Cyclooxygenase-2 gene disruption attenuates the severity of acute pancreatitis and pancreatitis-associated lung injury. *Gastroenterology* 2002; **123**: 1311-1322

13 **Strowski MZ**, Sparmann G, Weber H, Fiedler F, Printz H, Jonas L, Goke B, Wagner AC. Caerulein pancreatitis increases mRNA but reduces protein levels of rat pancreatic heat shock proteins. *Am J Physiol* 1997; **273**: G937-G945

14 **Tietz AB**, Malo A, Diebold J, Kotlyarov A, Herbst A, Kolligs FT, Brandt-Nedelev B, Halangk W, Gaestel M, Goke B, Schafer C. Gene deletion of MK2 inhibits TNF-alpha and IL-6 and protects against cerulein-induced pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G1298-G1306

15 **Exley AR**, Leese T, Holliday MP, Swann RA, Cohen J. Endotoxaemia and serum tumour necrosis factor as prognostic markers in severe acute pancreatitis. *Gut* 1992; **33**: 1126-1128

16 **Lampel M**, Kern HF. Acute interstitial pancreatitis in the rat induced by excessive doses of a pancreatic secretagogue. *Virchows Arch A Pathol Anat Histol* 1977; **373**: 97-117

17 **Ranson JH**, Turner JW, Roses DF, Rifkind KM, Spencer FC. Respiratory complications in acute pancreatitis. *Ann Surg* 1974; **179**: 557-566

18 **Gross V**, Leser HG, Heinisch A, Scholmerich J. Inflammatory mediators and cytokines--new aspects of the pathophysiology and assessment of severity of acute pancreatitis? *Hepatogastroenterology* 1993; **40**: 522-530

19 **Beger HG**, Rau B, Mayer J, Pralle U. Natural course of acute pancreatitis. *World J Surg* 1997; **21**: 130-135

20 **Kaiser AM**, Saluja AK, Steer ML. Repetitive short-term obstructions of the common bile-pancreatic duct induce severe acute pancreatitis in the opossum. *Dig Dis Sci* 1999; **44**: 1653-1661

21 **Steinberg W**, Tenner S. Acute pancreatitis. *N Engl J Med* 1994; **330**: 1198-1210

22 **Heath DI**, Cruickshank A, Gudgeon M, Jehanli A, Shenkin A, Imrie CW. Role of interleukin-6 in mediating the acute phase protein response and potential as an early means of severity assessment in acute pancreatitis. *Gut* 1993; **34**: 41-45

23 **Tseng TH**, Chu CY, Huang JM, Shiow SJ, Wang CJ. Crocetin protects against oxidative damage in rat primary hepatocytes. *Cancer Lett* 1995; **97**: 61-67

24 **Jagadeeswaran R**, Thirunavukkarasu C, Gunasekaran P, Ramamurty N, Sakthisekaran D. In vitro studies on the selective cytotoxic effect of crocetin and quercetin. *Fitoterapia* 2000; **71**: 395-399

25 **Bhatia M**, Brady M, Shokuhi S, Christmas S, Neoptolemos JP, Slavin J. Inflammatory mediators in acute pancreatitis. *J Pathol* 2000; **190**: 117-125

S- Editor Tian L **L- Editor** Anand BS **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6195

World J Gastroenterol 2008 October 28; 14(40): 6195-6203
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Patient education improves adherence to peg-interferon and ribavirin in chronic genotype 2 or 3 hepatitis C virus infection: A prospective, real-life, observational study

Patrice Cacoub, Denis Ouzan, Pascal Melin, Jean-Philippe Lang, Michel Rotily, Thierry Fontanges, Marina Varastet, Michel Chousterman, Patrick Marcellin

Patrice Cacoub, Department of Internal Medicine, Pitié-Salpêtrière Hospital, AP HP, Paris 75651, France; Pierre and Marie Curie (Paris 6) University, CNRS UMR 7087, Paris 75651, France
Denis Ouzan, Department of Hepato-Gastroenterology, Arnault Tzanck Institute, Saint Laurent du Var 06721, France
Pascal Melin, Department of Polyvalent Medicine, Hospital of Saint Dizier, Saint Dizier 52115, France
Jean-Philippe Lang, Department of Psychiatry and Addiction, Hospital of Erstein, Erstein 67152, France
Michel Rotily, Marina Varastet, ClinSearch, Bagneux 92220, France
Thierry Fontanges, Department of Hepato-Gastroenterology, General Hospital of Bourgoin, Bourgoin Jailleu 38317, France
Michel Chousterman, Department of Hepato-Gastroentrology, Hospital of Créteil (CHIC), Créteil 94010, France
Patrick Marcellin, Department of Hepatology, Beaujon Hospital, Pôle des Maladies de l'Appareil Digestif, Clichy 92118, France; and INSERM U 773, CRB3, University of Paris VII, Beaujon Hospital, Clichy 92118, France
Author contributions: Cacoub P, Ouzan D, Melin P, Lang JP, Rotily M, Fontanges T, Chousterman M, and Marcellin P (The Scientific Committee) designed the research, reviewed the data, supervised the analysis and interpreted the results; Varastet M helped analysing the data, interpreting the results, and wrote the paper; All authors helped writing, critically reviewed and approved the paper.
Supported by Schering-Plough France
Correspondence to: Patrice Cacoub, Professor, Department of Internal Medicine, Pitié-Salpêtrière Hospital, 47-83 boulevard de l'Hôpital, Paris cedex 13 F-75651, France. patrice.cacoub@psl.aphp.fr
Telephone: +33-142-178027 Fax: +33-142-178033
Received: June 19, 2008 Revised: September 16, 2008
Accepted: September 23, 2008
Published online: October 28, 2008

Abstract

AIM: To evaluate the impact of therapeutic education on adherence to antiviral treatment and sustained virological response (SVR) in a real-life setting in genotype 2/3 hepatitis C, as there are few adherence data in genotype 2/3 infection, even from randomized trials.

METHODS: This prospective survey included genotype 2/3 patients who received peg-interferon alfa-2b and ribavirin. There was no intervention. Adherence was self-reported over the past 4 wk (peg-interferon) or 7 d (ribavirin). Adherence to bitherapy was defined as adherence to the two drugs for ⩾ 20 wk. SVR was defined as undetectable RNA ⩾ 12 wk after the end of treatment.

RESULTS: 370/674 patients received education during the first 3 mo of treatment. After 6 mo, adherence to bitherapy was higher in educated patients (61% *vs* 47%, $P = 0.01$). Adherence to peg-interferon was 78% *vs* 69% ($P = 0.06$). Adherence to ribavirin was 70% *vs* 56% ($P = 0.006$). The SVR (77% *vs* 70%, $P = 0.05$) and relapse (10% *vs* 16%, $P = 0.09$) rates tended to be improved. After adjustment for baseline differences, education improved adherence [Odds ratio (OR) 1.58, $P = 0.04$] but not the SVR (OR 1.54, $P = 0.06$).

CONCLUSION: In genotype 2/3 patients, therapeutic education helped maintain real-life adherence to bitherapy.

Key words: Viral hepatitis; Adherence; Therapeutic education; Real life; Peg-interferon; Ribavirin

Peer reviewer: Raymund Rabe Razonable, PhD, Division of Infectious Diseases, Mayo Clinic Institution, 200 First Street SW, Rochester 55905, United States

Cacoub P, Ouzan D, Melin P, Lang JP, Rotily M, Fontanges T, Varastet M, Chousterman M, Marcellin P. Patient education improves adherence to peg-interferon and ribavirin in chronic genotype 2 or 3 hepatitis C virus infection: A prospective, real-life, observational study. *World J Gastroenterol* 2008; 14(40): 6195-6203 Available from: URL: http://www.wjgnet.com/1007-9327/14/6195.asp DOI: http://dx.doi.org/10.3748/wjg.14.6195

INTRODUCTION

As pointed out by the WHO[1], poor adherence to treatment is a worldwide issue in all chronic conditions, which results in poor health outcomes and increased health care costs[2,3]. Even in clinical trials, mean adherence rates are low (43%-78%) in chronic conditions[4-6]. In clinical practice, adherence rates of

about 50% are usually reported[7].

Adherence to therapy is critical in the treatment of chronic hepatitis C virus (HCV) infection. The current gold standard therapy is a combination of peg-interferon alfa and ribavirin[8,9]. Patients with genotype 1 infection have a 42%-51% likelihood of achieving a sustained virological response (SVR) after 48 wk of therapy; 78%-82% of patients with genotype 2 or 3 infection respond to 24 wk of treatment, whereas patients with genotype 3 infection and high viral load are difficult to treat (< 70% responders)[10,11]. Non-responders to prior standard bitherapy respond to retreatment in 13% of the cases (29% in non-1 genotype), and relapsers in 58.5% of the cases[12]. Therapy requires weekly subcutaneous injections, twice-daily oral dosing and frequent visits, with blood tests. Side effects occur in nearly all patients. As a result, 15%-20% of patients in clinical trials and > 25% in clinical practice discontinue therapy.

In clinical trials, SVR was significantly improved in those patients with HCV genotype 1 infection who received > 80% of their total peg-interferon dose and > 80% of their ribavirin dose for > 80% of the scheduled treatment duration, in comparison with those who failed these adherence criteria[13]. A review of the 2002-2007 literature confirmed that treatment response is influenced not only by HCV genotype and viral load, but also by patient-related factors including adherence[14]. Moreover, optimal HCV healthcare requires further efforts from providers in communicating with patients, as advocated in France by hepatitis C experts and the Health Ministry[15,16], and shown in studies using patient questionnaires in North America[17,18].

We carried out a large survey named CheObs to evaluate adherence to chronic hepatitis C treatment in the real-life setting in France. We observed that some patients received therapeutic education by a third party (other than the investigator), at the discretion of the investigators, during the study period. According to the consensus that efforts to boost treatment adherence improve SVR rates, we performed the present analysis to evaluate the impact of patient education on real-life adherence and response to treatment with peg-interferon alfa-2b and ribavirin. This analysis was carried out in patients with genotype 2/3 HCV infection (one third of the CheObs cohort), as there are few adherence data for these patients even from randomized trials, and because their data were available before those of patients with other genotypes, due to shorter treatment duration.

MATERIALS AND METHODS

The prospective, multicenter, CheObs survey was carried out in teaching hospitals, non-teaching hospitals, and private practice offices highly involved in the management of hepatitis C in France, and supervised by a Scientific Committee. Consecutive patients aged ⩾ 18 years with chronic hepatitis C were enrolled if initiation of bitherapy with peg-interferon alfa-2b and ribavirin was scheduled. They could be naive for any chronic hepatitis C therapy or non-responders/relapsers to previous therapy. In accordance with French law, the Ethics Committee's approval was not required as the protocol was strictly observational and usual practice was unchanged. However, all patients gave informed consent to participate.

Included patients saw their physician at a frequency corresponding to the usual practice in the center. The investigator and the patient completed a questionnaire each at inclusion, at the visits occurring approximately every 3 mo during treatment, and at the visit occurring approximately 6 mo after the end of treatment. Patients filled in their questionnaires in the waiting room and either gave it back to the investigator in a sealed envelope or returned it using a prepaid envelope.

The investigators recorded socio-demographic data, history of HCV infection (including previous treatments), risk factors, comorbidities, patient therapeutic education (provided or not), planned/prescribed hepatitis C treatment, modification of treatment during follow-up, concomitant medications, and adverse events. The virological status, documented by qualitative PCR (Amplicor™, Roche) and test date, was recorded at the last visit.

The patient questionnaires concerned adherence to peg-interferon and ribavirin, and the persons involved in the management of their disease (e.g. health professionals of any discipline, patient associations). The following parameters relating to the past 4 wk were recorded to evaluate adherence to peg-interferon: date of injections (or reason for not having an injection), frequency of and reason for taking peg-interferon at a higher/lower dose than prescribed. The following parameters relating to the past 7 d were recorded to evaluate adherence to ribavirin: number of (200 mg) capsules prescribed morning and evening, date of dosing and number of capsules taken in the morning and evening, reasons for missing doses, reasons for and frequency of taking more/less capsules than prescribed.

As for any survey, there was no protocol-specific intervention. Therapeutic education was defined by intervention of a third party (healthcare professionals other than the prescribing physician) and distribution of support documents and educational material during individual sessions. It was provided at the discretion of the physician. No instruction was given related to which patient should be considered or how education should be provided.

Adherence to bitherapy was defined as adherence to both peg-interferon and ribavirin over a sufficient time period, as self-reported by the patients. According to the "80/80/80" criteria previously defined[13], patients were considered to have adhered to peg-interferon if they had received three or four injections during the past 4 wk, and to have adhered to ribavirin if they had taken at least 22 (200 mg) capsules over the past week. Patients were considered to have adhered to bitherapy if they had adhered to the two drugs for at least 20 wk. These rules were defined according to the recommended number of peg-interferon injections (one per week), the recommended ribavirin daily dose (at least 800 mg/d),

and the recommended duration of bitherapy in genotype 2/3 HCV infection (24 wk).

SVR was defined as undetectable HCV RNA in the serum 12 wk after the end of treatment or later. This time interval was considered to be sufficient for this evaluation, since relapse after 12 wk of follow-up is rarely (2%) observed whatever the HCV genotype[19], and the dates of the visits and laboratory tests could not be forced in this observational protocol. Non-response was defined as detectable RNA at the end of treatment, and relapse as undetectable RNA at the end of treatment but detectable at a later time point.

Statistical analysis

Due to the lack of data in the literature, the CheObs sample size calculation was based on real-life adherence observed in chronic conditions other than chronic HCV infection, such as HIV infection[20], diabetes[21], or hypertension[22]. A total of 1537 patients were required to estimate a 50% adherence rate, with a precision of 2.5% and a type I error of 0.05. Assuming 25%-30% of patients were lost to follow-up or discontinued treatment early, approximately 2000 patients were included overall.

The present analysis was carried out in the subset of patients with genotype 2/3 HCV infection from the CheObs cohort. Statistical analysis was conducted using SAS 8.2 (SAS Institute Inc, Cary, NC, USA). Tests were two-sided and type I error was set at 0.05. Descriptive statistics were performed using all available data. Group comparisons were carried out using Kruskal-Wallis or Fisher's exact tests. The relationships between adherence or virological response and a set of potential explanatory variables were analysed by forward stepwise logistic regressions. These variables included not only those for which groups differed significantly at baseline ($P < 0.05$), but also those expected to have a significant impact according to the literature.

RESULTS

Patients' characteristics

Between 2002 and 2006, 184 investigators enrolled 2001 HCV patients in the CheObs survey, including 705 patients infected with the genotype 2/3 HCV (Figure 1). Of these, 674 patients were analyzed. We observed that 370/674 (55%) patients received therapeutic education during the first 3 mo of treatment and 304 (45%) did not. Among the 82 centers which included the analyzed population, 24 (29%) did not educate any patient, 18 (22%) educated > 0 to 50% of their patients, 19 (23%) educated > 50% to < 100% of their patients, and 21 (26%) educated 100% of their patients. The 31 patients excluded from analysis were similar to the analyzed population for the therapeutic education rate (58%) and all other baseline variables, except for the Metavir activity score, which was more frequently A2/A3 (78% *vs* 50%, $P = 0.009$).

In the analyzed population, educated patients had a higher body weight (70.5 ± 14.0 kg *vs* 67.9 ± 14.3 kg, $P = 0.02$) than patients without therapeutic education,

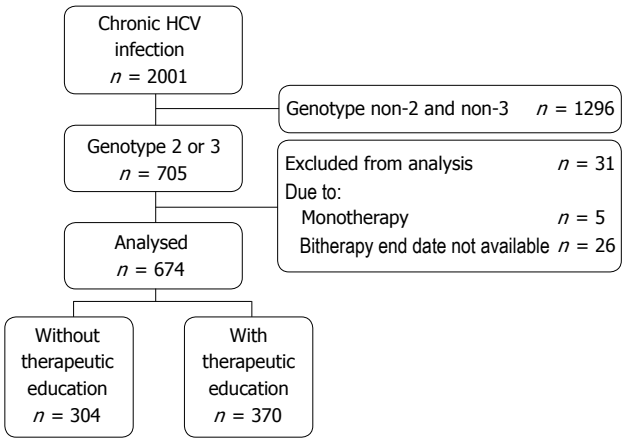


Figure 1 Patient flow.

but a similar body mass index (BMI) (Table 1). They more frequently had a past history of depression ($P = 0.01$) and current psychiatric disorders ($P = 0.04$, mainly depression and anxiety), though there was no difference for the nature or proportion of each disorder. Educated patients were also more frequently psychoactive drug users ($P = 0.02$, mainly cocaine and heroin), but the profile of consumption (injecting behavior and frequency of abuse) was similar. Regarding HCV infection, although educated patients had more frequently significant liver fibrosis than those without therapeutic education ($P = 0.04$), the proportion of cirrhotic patients was similar (13% and 15%).

Treatment

A 24-wk bitherapy was scheduled in most patients (571/667, 86%), with no marked difference between groups (Table 2). Treatment for 48 wk was more frequently planned in non-responders/relapsers to previous therapy (43/125, 34%) than in naive patients (49/542, 9%, $P < 0.001$). The planned weekly dose of peg-interferon was higher in educated patients ($P = 0.01$), at 1.5 μg/kg per week in most cases (82% *vs* 75%) whereas patients without therapeutic education were more frequently prescribed lower doses. However there was no difference between the groups for the proportion of retreated patients (Table 1) or that of relapsers/non-responders to previous treatment ($P = 0.95$). The ribavirin dose prescribed was similar in both groups. It was 800 mg/d in half of the patients (344/669, 51%) and < 800 mg/d in 18 (3%) patients.

The actual duration of bitherapy was shorter than 20 wk in 85/674 (13%) patients ($P = 0.20$ between groups, Table 2), with an average of 11.1 ± 4.9 wk (median 12) in these patients. Reasons for premature discontinuation were: lost to follow-up ($n = 28$), safety ($n = 27$), patient's request ($n = 14$), virological (HCV RNA detectable or decrease < 2 log, $n = 7$), unknown ($n = 3$), investigator's request ($n = 2$), and other ($n = 4$). They were similar in nature and occurrence ($P = 0.79$) in both groups. These patients, who were not included in the analysis of virological response, did not differ significantly from those who received a longer bitherapy

Table 1 Baseline characteristics of the patients (n = 674): Univariate analysis

	Therapeutic education		
	No (n = 304)	Yes (n = 370)	P
Socio-demography			
Men	170/303 (56)	229/370 (62)	0.14
Age (yr)	44.1 ± 11.4	44.9 ± 11.4	0.16
Body mass index (kg/m^2)	23.5 ± 4.1	24.1 ± 4.2	0.06
Employment status			
Professional activity	190/303 (63)	220/370 (59)	0.59
Unemployed	48/303 (16)	69/370 (19)	
Other	65/303 (22)	81/370 (22)	
Educational level			
Low	182/300 (61)	223/365 (61)	0.94
High	118/300 (39)	142/365 (39)	
Origin of incomes			
Paid employment	166/298 (56)	183/368 (50)	0.24
Unemployment incomes	48/298 (16)	60/368 (16)	
Other	84/298 (28)	125/368 (34)	
Debts			
Difficult to manage	8/232 (4)	27/315 (9)	0.02
None or easily managed	224/232 (97)	288/315 (91)	
Comorbidities			
Past psychiatric history			
Depression	67/303 (22)	113/370 (31)	0.01
Suicide attempt	23/302 (8)	29/368 (8)	0.81
Hospitalisation for mental disease	25/301 (8)	36/369 (10)	0.59
Psychiatric disorder	60/300 (20)	98/367 (27)	0.04
Chronic disease	64/298 (22)	89/366 (24)	0.41
Risk factors			
Alcohol consumption > 20 g/d	10/70 (14)	24/100(24)	0.17
Tobacco consumption	167/299 (56)	187/366 (51)	0.24
Drug abuse			
None	139/303 (46)	172/368 (47)	0.02
Former	158/303 (52)	174/368 (47)	
Current	6/303 (2)	22/368 (6)	
HCV infection			
Source of HCV infection[1]			
Transfusion	62/304 (20)	76/370 (21)	1.00
Injection or intranasal drug abuse	157/304 (52)	193/370 (52)	0.94
Other	86/304 (29)	100/370 (27)	0.86
Duration of HCV infection (year)	20.4 ± 8.4	20.0 ± 8.9	0.85
Serum HCV-RNA			
⩽ 800 000 IU/mL or equivalent	121/193 (63)	162/265 (61)	0.77
> 800 000 IU/mL or equivalent	72/193 (37)	103/265 (39)	
HCV genotype			
2	85/304 (28)	117/370 (32)	0.31
3	219/304 (72)	253/370 (68)	0.31
Coinfection			
Human immunodeficiency virus	12/303 (4)	14/369 (4)	1.00
Hepatitis B virus[2]	3/301 (1)	6/369 (2)	0.63
Metavir activity grade or equivalent			
A0 or A1	119/226 (53)	131/272 (48)	0.32
A2 or A3	107/226 (47)	141/272 (52)	
Metavir fibrosis stage or equivalent			
F0 or F1	110/227 (49)	109/272 (40)	0.04
F2 or F3	82/227 (36)	129/272 (47)	
F4	35/227 (15)	34/272 (13)	
Knodell score	7.9 ± 3.0	8.4 ± 3.5	0.19
Previous anti-HCV treatment course			
None	242/303 (80)	303/370 (82)	0.74
One or more	61/303 (20)	67/370 (18)	

Data are expressed as mean ± SD, or proportions of patients. [1]One patient could have more than one presumed source of infection. [2]Hepatitis B antigen positivity.

Table 2 Treatment planned and actually received (n = 674): Univariate analysis

	Therapeutic education		
	No (n = 304)	Yes (n = 370)	P
Duration of bitherapy (wk)[1]			
Planned	27.7 ± 8.9	27.1 ± 8.2	0.49
Actual	28.4 ± 12.7	30.3 ± 14.2	0.25
Premature discontinuation (< 20 wk)	44/304 (15)	41/370 (11)	0.20
Peginterferon weekly dose (μg/kg)			
Planned	1.35 ± 0.29	1.41 ± 0.22	0.01
Actual at 3 mo[2]	1.31 ± 0.30	1.38 ± 0.25	0.006
Actual at 6 mo[2]	1.18 ± 0.35	1.25 ± 0.31	0.02
Ribavirin daily dose (mg)			
Planned	897 ± 147	906 ± 154	0.67
Actual at 3 mo[2]	871 ± 166	885 ± 175	0.25
Actual at 6 mo[2]	771 ± 201	803 ± 209	0.06

Data are expressed as mean ± SD, or proportions of patients. [1]Date of end of bitherapy minus date of inclusion; [2]Cumulated over the past 3 mo (AUC, investigator report).

with regards to the main variables.

Physicians modified the peg-interferon dose less frequently in educated patients (16% *vs* 22% without therapeutic education, P = 0.046), whereas the ribavirin dose was changed in similar proportions of patients in both groups (17% *vs* 18%, P = 0.28) nearly always because of adverse effects and weight loss and depression in particular. The difference in the dose between groups remained constant for peg-interferon whereas it increased over time for ribavirin, in particular after the third month of treatment (Table 2). The occurrence of adverse events over the whole study was 82% of patients in each group (P = 0.92).

Adherence to bitherapy

Overall adherence to bitherapy was 64% (301/468) at 3 mo and 55% (209/383) at 6 mo of treatment. Adherence to peg-interferon (80% and 74% at 3 and 6 mo, respectively) was higher than to ribavirin (72% and 64%, respectively). Patients prescribed high doses of ribavirin (⩾ 1000 mg/d) did not differ significantly from those with lower doses with respect to premature treatment discontinuation and adherence to peg-interferon and/or ribavirin.

At 3 mo, the proportion of adherents to both drugs was 66% with therapeutic education and 63% without therapeutic education (non-significant difference) (Figure 2 and Table 3). At 6 mo, this proportion was still 61% in educated patients, whereas it dropped down to 47% without therapeutic education (P = 0.01). Multivariate analysis showed that, after adjustment, therapeutic education increased the probability of adhering to bitherapy at 6 mo by a factor of 1.58 (95% CI: 1.02 to 2.46).

At 6 mo, the adherence rate was still 78% for peg-interferon and 70% for ribavirin in educated patients, whereas it was reduced to 69% (P = 0.06) and 56% (P = 0.006), respectively, in patients without therapeutic

Table 3 Adherence to treatment, virological response (univariate analysis) and their association with therapeutic education (multivariate analysis)

	Therapeutic education					
	Univariate analysis			Multivariate analysis		
	No (n = 304)	Yes (n = 370)	P	OR	95% CI	P
Adherence[1] at 3 mo						
Bitherapy	137/218 (63)	164/250 (66)	0.56	1.04	0.69 to 1.56	0.87
Peginterferon	175/218 (80)	201/250 (80)	1.00	0.94	0.57 to 1.53	0.79
Ribavirin	152/218 (70)	186/250 (74)	0.30	1.13	0.72 to 1.77	0.59
Adherence[1] at 6 mo						
Bitherapy	83/175 (47)	126/208 (61)	0.01	1.58	1.02 to 2.46	0.04
Peginterferon	121/175 (69)	162/208 (78)	0.06	1.78	1.07 to 2.96	0.03
Ribavirin	98/175 (56)	145/208 (70)	0.006	1.67	1.05 to 2.65	0.03
Virological response[2]						
SVR	171/246 (70)	230/298 (77)	0.05	1.54[3]	0.99 to 2.40	0.06
Nonresponse	37/246 (15)	37/298 (12)	0.38			
Relapse	38/246 (16)	31/298 (10)	0.09			

Data are expressed as proportions of patients. Multivariate analyses were adjusted for sex, weight, BMI, educational level (adherence only), history of depression, psychiatric disorders, alcohol consumption, drug abuse, duration of HCV infection, previous anti-HCV treatment, HCV genotype (SVR only), and peginterferon dose prescribed at inclusion. [1]≥ 3 peginterferon injections during the last 4 wk and/or ≥ 22 ribavirin capsules of 200 mg each during the last 7 d (self-report); [2]≥ 12 wk after the end of treatment; [3]The rate of SVR was used as dependent variable.

education. After adjustment, therapeutic education increased the probability of adherence by a factor of 1.78 for peg-interferon and 1.67 for ribavirin after 6 mo of bitherapy.

Educated patients more frequently reported contacting persons in the hospital for management of their disease, with a median of three persons *vs* two for patients without therapeutic education during the first 3 mo of treatment (P=0.016), and three persons *vs* one during the next 3 mo (P = 0.003). Conversely, patients without therapeutic education reported more frequent contacts with office-based persons, with a median of two persons *vs* one in educated patients during the first 3 mo (P = 0.012), and two persons *vs* none during the next 3 mo (P < 0.001).

Virological response

The overall SVR rate was 74% (401/544). There were 13.6% of non-responders and 12.7% of relapsers. The virological response was better, though not significantly, in educated patients (Figure 2 and Table 3). The SVR rate was higher (77% *vs* 70%, P = 0.05) and the rate of relapse was lower (10% *vs* 16%, P = 0.09) in these patients than in patients without therapeutic education. Multivariate analysis confirmed that, after adjustment, the relationship between SVR and therapeutic education was borderline significant (P = 0.06). Response was better in patients whose ribavirin dose was not reduced during the first 3 mo of treatment (P < 0.001), mostly due to an increased SVR rate (362/473, 77% *vs* 37/67, 55%, P = 0.001) and a decreased rate of non-response (51/473, 11% *vs* 21/67, 31%, P < 0.001).

The virological response is shown taking into account genotype and baseline viral load (> or ≤ 800 000 IU/mL) in Figure 3. Though this effect was not significant, therapeutic education was found to have a beneficial impact in all patients. In those with genotype 2 and low viral load, an impact on the SVR (P = 0.038) and relapse (P = 0.047) rates was observed, but the sample size was small (n = 80).

Moreover, although favorable trends were observed, therapeutic education had no significant impact on the virological response in patients treated for the first time (P = 0.27) or in non-responders/relapsers to previous therapy (P = 0.22).

DISCUSSION

We evaluated the impact of patient therapeutic education by a third party on adherence and virological response to peg-interferon alfa-2b and ribavirin in 674 patients with chronic genotype 2/3 HCV infection. This is the first time that adherence has been evaluated in patients carrying these genotypes, and also the first time that adherence has been assessed in a real-life setting.

Therapeutic education given during the first 3 mo of treatment significantly improved the proportion of patients adhering to bitherapy at 6 mo (OR 1.58, P = 0.01 after adjustment for sex, weight, BMI, educational level, history of depression, psychiatric disorders, alcohol consumption, drug abuse, duration of HCV infection, previous anti-HCV treatment, and peg-interferon dose prescribed at inclusion). The proportion of adherents was stable above 60% until the sixth month of treatment in educated patients, whereas it dropped by more than 10% between the third and sixth month of treatment when there was no therapeutic education. The virological response was also improved in educated patients, with an increased SVR rate (77% *vs* 70%) and a lower relapse rate (10% *vs* 16%), though this effect was statistically not significant (P = 0.06 after adjustment for sex, weight, BMI, history of depression, psychiatric disorders, alcohol consumption, drug abuse, duration of HCV infection, previous anti-HCV treatment, HCV genotype, and peg-interferon dose prescribed at inclusion). Since a 12-wk treatment may be very effective

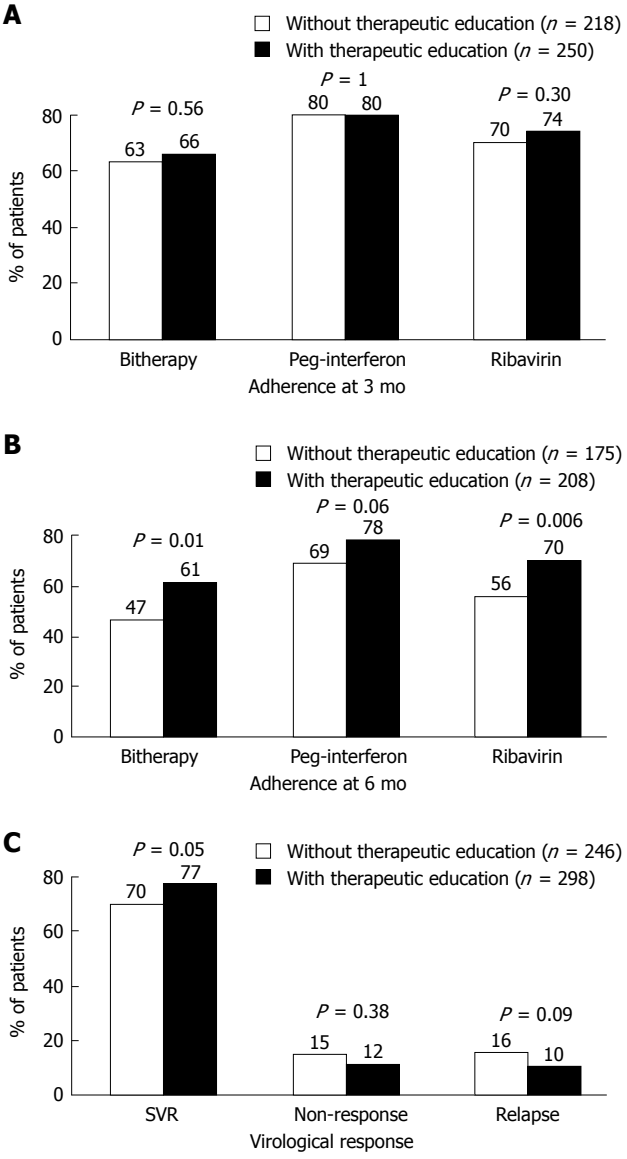


Figure 2 Impact of patient therapeutic education on adherence to treatment (A and B) and virological response (C). At 3 mo of treatment, the proportion of patients adhering to treatment (patient self-report) was similar in the two groups. At 6 mo, the proportion of adherents dropped in patients without therapeutic education only. The virological response was better in educated patients, with an increased rate of SVR and a lower relapse rate.

in naive genotype 2/3 patients, the fact that adherence was the same over 12 wk may explain why adherence in the later part did not translate to significantly higher SVR rates. As suggested by the borderline *P* value, this might also be due to the fact that inclusion of one-third of the CheObs cohort in this analysis limited the power to detect statistical significance.

The overall adherence rate to ribavirin (64%-72%) was consistently lower than that to peg-interferon (74%-80%), as expected from their regimen. The ribavirin regimen is somewhat complex (twice-daily oral dosing) whereas peg-interferon alfa-2b adherence is facilitated by the low frequency of administration (weekly injections) and availability of an injecting pen device. Therapeutic education significantly enhanced adherence to ribavirin, which is particularly important for obtaining an SVR[23]. A positive impact was also observed on adherence to peg-interferon, showing that an improvement can still be obtained.

Our results are consistent with the general consensus that patient therapeutic education in clinical practice effectively improves adherence to treatment in chronic disease[2,15,16] and that adherence, even to placebo, is essential to achieve health outcome goals[3]. It should be noted however, that education required the cooperation of specialists and nursing staff, and therefore probably affected both patient-related and health care team-related factors, which are recognized to improve adherence.

The adherence rate to bitherapy (64%) was low compared to the rates of > 70% reported in clinical trials[13]. The SVR rate observed here (72%) was however, in line with that reported in clinical trials (80%) in genotype 2/3 HCV patients treated with similar therapy[24]. Apart from the fact that results are usually more marked in randomized trials, our population included a high proportion of patients with psychiatric disorders and/or drug addicts. This suggests that in real-life situations such comorbidities and high-risk behavior do not have an impact on adherence or response to treatment, so that they may not be as difficult to treat as usually believed.

Unlike during clinical trials, patients in this observational study were not selected or obliged to follow specific procedures and physicians were not instructed on which patient should be considered for therapeutic education. This may be considered to be a weakness of the study, as comparisons were performed on non-randomized groups. The CheObs study was not designed to perform comparisons, but aimed to provide a picture of the real-life setting. The use of multivariate analysis in the present evaluation reduced such bias by taking into account the differences between groups observed at baseline.

The method used to assess adherence may also be criticized. When the study was designed, we chose to use data reported by patients rather than by investigators, to best reflect the real-life situation. However, asking patients to fill a diary each day would have influenced their behavior and led to an unquantifiable overestimation of our primary endpoint. Adherence data were therefore collected over limited time periods. We also chose not to take into account body weight when calculating adherence to ribavirin. Theoretically, we overestimated adherence to ribavirin in patients over 65 kg body weight, i.e. in approximately half the study population at baseline. This bias was however reduced, as weight is known to decrease markedly over time in most treated patients and, as expected, dose reductions occurred for safety reasons in a large proportion of patients (17%-18%) in both groups.

Our results demonstrate that, although patients with HCV genotype 2/3 are those who usually show the best response rates, further efforts may be made to improve outcomes. There is more than one barrier preventing patients from optimal compliance to their treatment regimen[1]. Five interacting dimensions affect adherence: social and economic factors, and factors related to the

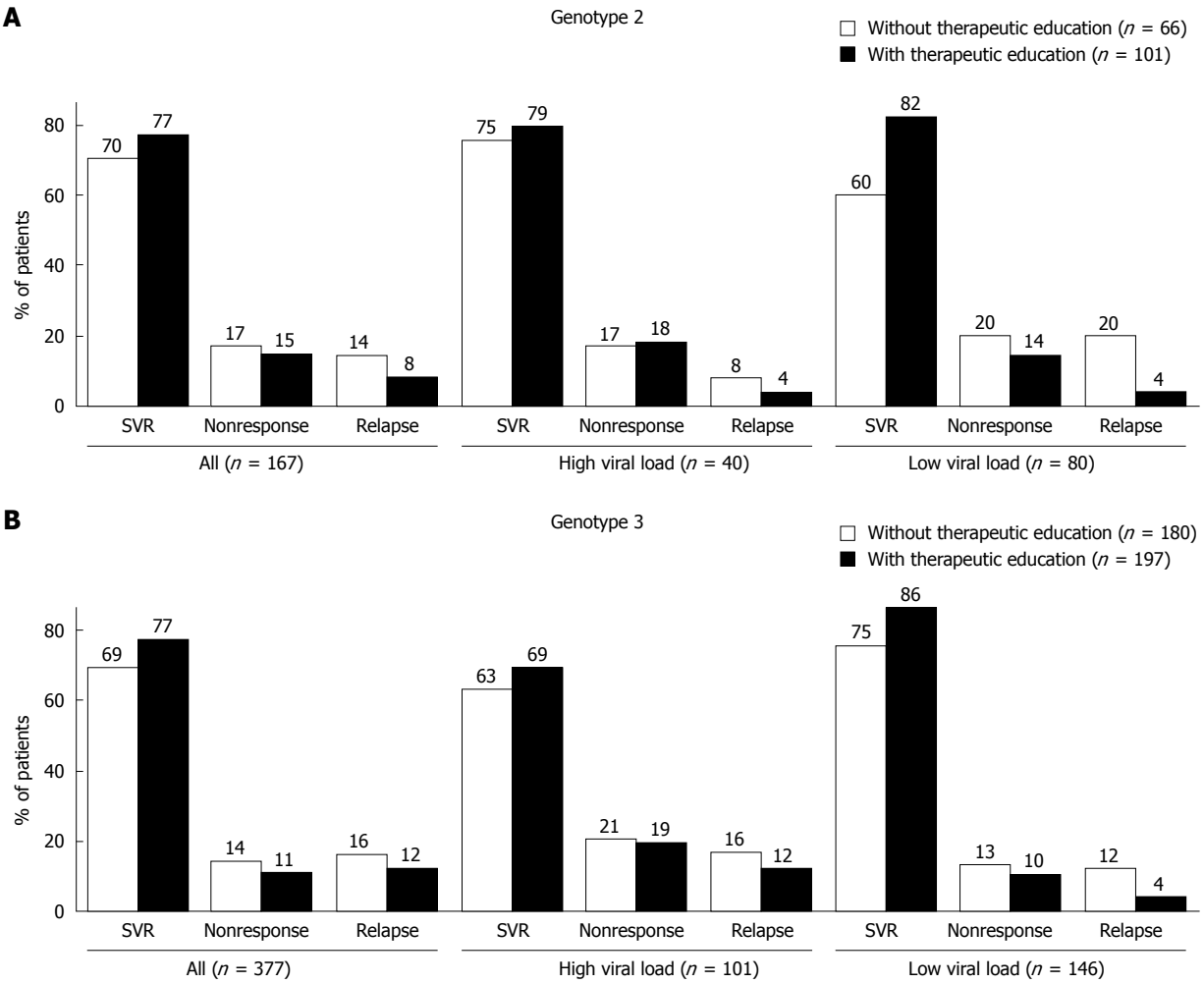


Figure 3 Virological response in patients infected with genotype 2 (A) or genotype 3 (B), and according to viral load (> or ⩽ 800 000 IU/mL). Although not statistically significant, patient therapeutic education was beneficial in all patient subgroups, especially in those with genotype 2 and low viral load, with a marked impact on the SVR (SVR, $P = 0.038$) and relapse ($P = 0.047$) rates.

health care team and system, the condition, treatment, and the patient[2,15,16]. Increasing the impact of interventions aimed at patient-related factors and/or health care team-related factors is essential. Methods that have been shown to be effective in improving therapy include: educational interventions involving patients[25,26]; strategies to improve dosing schedules[6]; interventions that enlist ancillary health care providers such as pharmacists, behavioral specialists, and nursing staff[27,28]; and enhancing communication between physicians and patients[29-31].

To conclude, in the real-life setting, therapeutic education helped maintain adherence to bitherapy in patients with genotype 2/3 infection. There was a trend for a benefit on virological response. This analysis of the real-life impact of patient education by a third party on health outcomes may help to further improve patient quality of life and outcome. The importance of adherence and the role of education should now be studied in a randomized controlled trial in genotype 1/4 infection.

ACKNOWLEDGMENTS

The authors are grateful to Ceri Medical (Garches, France), who helped in field monitoring, Florence Colin-Mercier (Stat Process, Pont Mort, France), who analysed the data, as well as all investigators for participating in this study (the CheObs Study Group).

The CheObs Study Group (France): Laurent Alric (Toulouse), Tony Andreani (Paris), Michel Antoni (Orange), Jean-Pierre Arpurt (Avignon), Tarik Asselah (Clichy), Yannick Bacq (Tours), François Bailly (Lyon), Axel Balian (Clamart), Xavier Ballorain (Châtellerault), Christelle Bapin (Montpellier), Jean-Claude Barbare (Compiègne), Didier Barbereau (Tours), Marc Bardou (Dijon), Jean-Luc Baroni (Martigues), Eric Barriere (Limoges), Marc Bary (Paris), Guy Bellaiche (Aulnay-sous-Bois), Serge Bellon (Avignon), Souaad Benali (Marseille), Serge Benhaim (Marseille), Sylvain Beorchia (Lyon), Pierre Bernard (Pessac), Brigitte Bernard-Chabert (Reims), Isabelle Besson (La Rochelle), Louis Bettan (Villeneuve-St-Georges), Isabelle Beurton (Besançon), Daniel Bianchi (Nice), Pierre Blanc (Montpellier), Alain Blanchi (Le Mans), Gilles Bommelaer (Clermont-Ferrand), Cyril Bonaventure (Lyon), Corinne Bonny (Clermont-Ferrand), Daniele Botta-Fridlund (Marseille), James Boulant (Grasse), Guy Boulay (Arles), Marc Bourliere (Marseille), Nathalie Boyer (Clichy), Slim Bramli (Avignon), Jean-Pierre Bronowicki (Vandoeuvre-les-Nancy), Maud

Brung-Lefebvre (Elbeuf), Yvon Calmus (Paris), Jean-Yves Canva (Roubaix), Valèrie Canva-Delcambre (Lille), Jean Cassigneul (Toulouse), Laurent Castera (Pessac), Xavier Causse (La Source), Jean-Michel Cayla (Saint Brice), Catherine Chevalier (Limoges), Philippe Chevassus (Gap), Renè Chiari (Perpignan), Ariane Chryssostalis (Crèteil), Jean-Marc Combis (Toulouse), Filomena Conti (Paris), Denis Corcos (Narbonne), Olivier Croizet (Toulouse), Emmanuel Cuillerier (Dreux), Bella Czernichow (Sarreguemines), Louis D'Alteroche (Tours), Hélène Danielou (Rennes), Odile Danne (Pontoise), Manh-Thuong Dao (Caen), Cécilia D'Arondel de Haye (Paris), Patrick Delasalle (Grasse), Pierre Deltenre (Lille), Jacques Denis (Corbeil-Essonnes), Salem Dernaika (Paris), Hervé Desmorat (Toulouse), Jacques Doll (Le Chesnay), Sylva Doumet (Villeneuve-St-Georges), Jean-Charles Duclos-Vallee (Villejuif), Michel Duffaut (Toulouse), Samouh Elfadel (Caen), Gilbert Eschmann (Grasse), Claude Eugene (Poissy), Roger Faroux (La Roche-sur-Yon), Laetitia Fartoux Heymann (Paris), Abdelaziz Fatisse (Belfort), Bernard Filoche (Lomme), Antoine Fleury (Pontoise), Isabelle Fouchard-Hubert (Angers), Juliette Foucher (Pessac), Serge Fratte (Belfort),Valérie Garcia (Montpellier), Dany Gargot (Blois), Jean-Louis Gaudin (Le Puy-en-Velay), Nathalie Giuily (Clichy), Odile Goria (Rouen), Alain Gourlaouen (Morlaix), Stéphane Grimaldi (Carcassonne), Catherine Guillemard (Caen), Anne Guillygomarc'H (Rennes), Dominique Guyader (Rennes), François Habersetzer (Strasbourg), Sadia Hachemane (Montpellier), Nabil Haddad (Villejuif), Hervé Hagege (Créteil), Najia Haioun (Cavaillon), Bertrand Hanslik (Montpellier), Cyril Hatem (Dijon), Marie-Laure Hemery (Montpellier), Pascale Hervio (Pontoise), Christophe Hezode (Créteil), Noëlle Hilleret (La Tronche), Patrick Hillon (Dijon), Valérie Hyrailles (Béziers), Jean-Paul Jacques (Toulouse), Pascal Kitmacher (La Rochelle), Alain Landau (Dreux), Emmanuel Lapoile (Villeneuve-St-Georges), Dominique Larrey (Montpellier), Olivier Larroque (Hyères), Sophie Le Calvez (Paris), Pascal Lebray (Paris), Olivier Lejeune (St-Priest-en-Jarez), Alain Lemoine (Nevers), Stéphane Levy (Reims), Fabrice Longo (Fréjus), Véronique Loustaud Ratti (Limoges), Hervé Louvet (Reims), Damien Lucidarme (Lomme), Catherine Mathieu-Chandelier (Lille), Philippe Mathurin (Lille), Marianne Maynard (Lyon), Sophie Metivier (Toulouse), Jean-Philippe Miguet (Besançon), Anne Minello (Dijon), François Mion (Lyon), Joseph Moussalli (Paris), Bernard Nalet (Montélimar), Jean-Baptiste Nousbaum (Brest), Frédéric Oberti (Angers), Valérie Oules (Marseille), Georges Philippe Pageaux (Montpellier), Jean-Louis Payen (Montauban), Christophe Pilette (Le Mans), Didier Pillon (Bourg-en-Bresse), Isabelle Portal (Marseille), Philippe Pouderoux (Nîmes), Claude Raffanel (Nîmes), Baboojaswan Ramgoolam (Apt), Karine Randl (Clermont-Ferrand), Vlad Ratziu (Paris), Corinne Regimbeau (Paris), André-Jean Remy (Perpignan), Christophe Renou (Hyères), Ghassian Riachi (Rouen), Didier Ribard (Nîmes), Olivier Ribiere (Rambouillet), Jean-Philippe Richardet (Créteil), Marie-Pierre Ripault (Clichy), Bruno Roche (Villejuif), Christine Roche (Clermont-Ferrand), Olivier Rogeaux (Chambéry), Rolland Rollux (Thonon-les-Bains), Isabelle Rosa (Créteil), Françoise Roudot-Thoraval (Créteil), Faouzi Saliba (Villejuif), Thierry Sapey (Châteauroux), Olivier Savary (Brest), Olivia Scalone (Lyon), Mathieu Schnee (La-Roche-sur-Yon), Maud Schoeny (Saint-Dizier), Lawrence Serfaty (Paris), Jeanne Serpaggi (Paris), Eric Sgro (Carcassonne), Si Nafa Si Ahmed (La Source), Claude Souvignet (La Tronche), Nathalie Szostak-Talbodec (Tourcoing), Jean-Claude Tellaroli (Thionville), Dominique Thabut (Paris), Gilles Tordjman (Aulnay-sous-Bois), Christian Trepo (Lyon), Régine Truchi (Nice), Claire Vanlemmens (Besançon), René-Louis Vitte (Poissy), Jean-Pierre Zarski (La Tronche).

COMMENTS

Background

Adherence to therapy is critical in the treatment of chronic hepatitis C virus (HCV) infection. The current gold standard therapy is a combination of peg-interferon alfa and ribavirin. Patients with genotype 2 or 3 infection have a 78%-82% likelihood of achieving a sustained virological response (SVR) after 24 wk of therapy, whereas patients with genotype 3 infection and high viral load are difficult to treat (< 70% responders). Therapy requires weekly subcutaneous injections, twice-daily oral dosing and frequent visits, with blood tests. Side effects occur in nearly all patients. As a result, 15%-20% of patients in clinical trials and over 25% in clinical practice discontinue therapy.

Research frontiers

In clinical trials, the SVR rate was significantly improved in those patients with HCV genotype 1 infection who received > 80% of their total peg-interferon dose and > 80% of their ribavirin dose for > 80% of the scheduled treatment duration. A review of the 2002-2007 literature confirmed that treatment response is influenced not only by HCV genotype and viral load, but also by patient-related factors including adherence.

Innovations and breakthroughs

The authors evaluated, perhaps for the first time, the impact of patient therapeutic education by a third party on adherence and virological response to peg-interferon alfa-2b and ribavirin. The analysis was carried out in the 674 patients with chronic genotype 2/3 HCV infection from the CheObs cohort. Therapeutic education given during the first 3 mo of treatment significantly improved the proportion of patients adhering to bitherapy at 6 mo (odds ratio 1.58). Though not significantly, the virological response was also improved in educated patients, with an increased SVR rate (77% *vs* 70%) and a lower relapse rate (10% *vs* 16%). Therapeutic education significantly enhanced adherence to ribavirin, which is particularly important for obtaining a SVR. A positive impact was also observed on adherence to peg-interferon, showing that an improvement can still be obtained.

Applications

This analysis of the real-life impact of patient therapeutic education by a third party on health outcomes may help to further improve patient quality of life and outcome. Five interacting dimensions affect adherence: social and economic factors, and factors related to the health care team and system, the condition, treatment, and the patient. Methods that have been shown to be effective in improving therapy include: educational interventions involving patients; strategies to improve dosing schedules; interventions that enlist ancillary health care providers such as pharmacists, behavioral specialists, and nursing staff; and enhancing communication between physicians and patients.

Peer review

This is a subgroup analysis of a bigger project. Since the a priori power calculation was based on 2000 subjects for meaningful statistical analysis, the inclusion of only 630 or so patients in this study clearly limits the power to detect statistical significance.

REFERENCES

1 **Sabate E**. Adherence to long-term therapies. Evidence for action. Geneva: World Health Organization; 2003. Available from URL: http://whqlibdoc.who.int/publications/2003/9241545992.pdf

2 **Osterberg L**, Blaschke T. Adherence to medication. *N Engl J Med* 2005; **353**: 487-497

3 **Simpson SH**, Eurich DT, Majumdar SR, Padwal RS, Tsuyuki RT, Varney J, Johnson JA. A meta-analysis of the association between adherence to drug therapy and mortality. *BMJ* 2006; **333**: 15

4 **Cramer J**, Rosenheck R, Kirk G, Krol W, Krystal J. Medication compliance feedback and monitoring in a clinical trial: predictors and outcomes. *Value Health* 2003; **6**: 566-573

5 **Waeber B**, Leonetti G, Kolloch R, McInnes GT. Compliance with aspirin or placebo in the Hypertension Optimal Treatment (HOT) study. *J Hypertens* 1999; **17**: 1041-1045

6 **Claxton AJ**, Cramer J, Pierce C. A systematic review of the associations between dose regimens and medication compliance. *Clin Ther* 2001; **23**: 1296-1310

7 **Dunbar-Jacob J**, Mortimer-Stephens MK. Treatment adherence in chronic disease. *J Clin Epidemiol* 2001; **54** Suppl 1: S57-S60

8 **Camma C**, Licata A, Cabibbo G, Latteri F, Craxi A. Treatment of hepatitis C: critical appraisal of the evidence. *Expert Opin Pharmacother* 2005; **6**: 399-408

9 **Manns MP**, Wedemeyer H, Cornberg M. Treating viral hepatitis C: efficacy, side effects, and complications. *Gut* 2006; **55**: 1350-1359

10 **von Wagner M**, Huber M, Berg T, Hinrichsen H, Rasenack J, Heintges T, Bergk A, Bernsmeier C, Haussinger D, Herrmann E, Zeuzem S. Peginterferon-alpha-2a (40KD) and ribavirin for 16 or 24 weeks in patients with genotype 2 or 3 chronic hepatitis C. *Gastroenterology* 2005; **129**: 522-527

11 **Zeuzem S**, Hultcrantz R, Bourliere M, Goeser T, Marcellin P, Sanchez-Tapias J, Sarrazin C, Harvey J, Brass C, Albrecht J. Peginterferon alfa-2b plus ribavirin for treatment of chronic hepatitis C in previously untreated patients infected with HCV genotypes 2 or 3. *J Hepatol* 2004; **40**: 993-999

12 **Moucari R**, Ripault MP, Oules V, Martinot-Peignoux M, Asselah T, Boyer N, El Ray A, Cazals-Hatem D, Vidaud D, Valla D, Bourliere M, Marcellin P. High predictive value of early viral kinetics in retreatment with peginterferon and ribavirin of chronic hepatitis C patients non-responders to standard combination therapy. *J Hepatol* 2007; **46**: 596-604

13 **McHutchison JG**, Manns M, Patel K, Poynard T, Lindsay KL, Trepo C, Dienstag J, Lee WM, Mak C, Garaud JJ, Albrecht JK. Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. *Gastroenterology* 2002; **123**: 1061-1069

14 **Hughes CA**, Shafran SD. Chronic hepatitis C virus management: 2000-2005 update. *Ann Pharmacother* 2006; **40**: 74-82

15 **Consensus conference**. Treatment of hepatitis C. *Gastroenterol Clin Biol* 2002; **26** Spec No 2: B303-B320

16 **Plan National Hépatites Virales C et B** (2002-2005). 2002. Available from URL: http://www.sante.gouv.fr/htm/actu/hepatites/sommaire.htm

17 **Balfour L**, Cooper C, Tasca GA, Kane M, Kowal J, Garber G. Evaluation of health care needs and patient satisfaction among hepatitis C patients treated at a hospital-based, viral hepatitis clinic. *Can J Public Health* 2004; **95**: 272-277

18 **Zickmund S**, Hillis SL, Barnett MJ, Ippolito L, LaBrecque DR. Hepatitis C virus-infected patients report communication problems with physicians. *Hepatology* 2004; **39**: 999-1007

19 **Zeuzem S**, Heathcote EJ, Shiffman ML, Wright TL, Bain VG, Sherman M, Feinman SV, Fried MW, Rasenack J, Sarrazin C, Jensen DM, Lin A, Hoffman JH, Sedarati F. Twelve weeks of follow-up is sufficient for the determination of sustained virologic response in patients treated with interferon alpha for chronic hepatitis C. *J Hepatol* 2003; **39**: 106-111

20 **Moatti JP**, Spire B, Duran S. [A review of socio-behavioural studies on adherence to antiretroviral treatments: beyond biomedical models?] *Rev Epidemiol Sante Publique* 2000; **48**: 182-197

21 **Donnan PT**, MacDonald TM, Morris AD. Adherence to prescribed oral hypoglycaemic medication in a population of patients with Type 2 diabetes: a retrospective cohort study. *Diabet Med* 2002; **19**: 279-284

22 **Rudd P**. Compliance with antihypertensive therapy: raising the bar of expectations. *Am J Manag Care* 1998; **4**: 957-966

23 **Mulhall BP**, Younossi Z. Impact of adherence on the outcome of antiviral therapy for chronic hepatitis C. *J Clin Gastroenterol* 2005; **39**: S23-S27

24 **Manns MP**, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965

25 **Patton K**, Meyers J, Lewis BE. Enhancement of compliance among patients with hypertension. *Am J Manag Care* 1997; **3**: 1693-1698

26 **Ran MS**, Xiang MZ, Chan CL, Leff J, Simpson P, Huang MS, Shan YH, Li SG. Effectiveness of psychoeducational intervention for rural Chinese families experiencing schizophrenia--a randomised controlled trial. *Soc Psychiatry Psychiatr Epidemiol* 2003; **38**: 69-75

27 **Haynes RB**, McDonald H, Garg AX, Montague P. Interventions for helping patients to follow prescriptions for medications. *Cochrane Database Syst Rev* 2002; CD000011

28 **Simoni JM**, Frick PA, Pantalone DW, Turner BJ. Antiretroviral adherence interventions: a review of current literature and ongoing studies. *Top HIV Med* 2003; **11**: 185-198

29 **Burnier M**. Long-term compliance with antihypertensive therapy: another facet of chronotherapeutics in hypertension. *Blood Press Monit* 2000; **5** Suppl 1: S31-S34

30 **Misdrahi D**, Llorca PM, Lancon C, Bayle FJ. [Compliance in schizophrenia: predictive factors, therapeutical considerations and research implications] *Encephale* 2002; **28**: 266-272

31 **Ross FM**. Patient compliance--whose responsibility? *Soc Sci Med* 1991; **32**: 89-94

S- Editor Li DL **L- Editor** Negro F **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6204

World J Gastroenterol 2008 October 28; 14(40): 6204-6207
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Hydrogen breath test for the diagnosis of lactose intolerance, is the routine sugar load the best one?

Fiorenza Argnani, Mauro Di Camillo, Vanessa Marinaro, Tiziana Foglietta, Veronica Avallone, Carlo Cannella, Piero Vernia

Fiorenza Argnani, Mauro Di Camillo, Vanessa Marinaro, Tiziana Foglietta, Veronica Avallone, Carlo Cannella, Piero Vernia, Gastroenterologia A e Scienza dell'Alimentazione, Sapienza Università di Roma, Roma 00161, Italy
Author contributions: Argnani F, Cannella C and Vernia P designed research; Argnani F, Di Camillo M, Foglietta T, Avallone V performed research; Argnani F and Vernia P wrote the paper.
Correspondence to: Piero Vernia, MD, Professor, Gastroenterologia A, Dipartimento di Scienze Cliniche, Policlinico Umberto Ⅰ, Sapienza Università di Roma, Roma 00161, Italy. vernia@uniroma1.it
Telephone: +39-6-49972360 Fax: +39-6-4463737
Received: May 9, 2008 Revised: September 17, 2008
Accepted: September 24, 2008
Published online: October 28, 2008

Abstract

AIM: To evaluate the prevalence of lactose intolerance (LI) following a load of 12.5 g in patients diagnosed as high-grade malabsorbers using the hydrogen breath test (HBT)-25.

METHODS: Ninety patients showing high-grade malabsorption at HBT-25 were submitted to a second HBT with a lactose load of 12.5 g. Peak hydrogen production, area under the curve of hydrogen excretion and occurrence of symptoms were recorded.

RESULTS: Only 16 patients (17.77%) with positive HBT-25 proved positive at HBT-12.5. Hydrogen production was lower as compared to HBT-25 (peak value 21.55 parts per million (ppm) ± 29.54 SD *vs* 99.43 ppm ± 40.01 SD; $P < 0.001$). Symptoms were present in only 13 patients. The absence of symptoms during the high-dose test has a high negative predictive value (0.84) for a negative low-dose test. The presence of symptoms during the first test was not useful for predicting a positive low-dose test (positive predictive value 0.06-0.31).

CONCLUSION: Most patients with a positive HBT-25 normally absorb a lower dose of lactose and a strict lactose restriction on the basis of a "standard" HBT is, in most instances, unnecessary. Thus, the 25 g lactose tolerance test should probably be substituted by the 12.5 g test in the diagnosis of LI, and in providing dietary guidelines to patients with suspected lactose malabsorption/intolerance.

Key words: Lactase deficiency; Lactose malabsorption; Lactose intolerance; Hydrogen breath test

Peer reviewer: Phillip S Oates, PhD, Department of Physiology, School of Biomedical and Chemical Sciences, the University of Western Australia, Western Australia 6009, Australia

Argnani F, Di Camillo M, Marinaro V, Foglietta T, Avallone V, Cannella C, Vernia P. Hydrogen breath test for the diagnosis of lactose intolerance, is the routine sugar load the best one? *World J Gastroenterol* 2008; 14(40): 6204-6207 Available from: URL: http://www.wjgnet.com/1007-9327/14/6204.asp DOI: http://dx.doi.org/10.3748/wjg.14.6204

INTRODUCTION

The enzyme lactase phlorizin hydrolase, located at the intestinal brush border, is necessary for the hydrolysis of lactose, the main sugar in milk. Due to the genetically programmed decrease in intestinal lactase activity that occurs post-weaning (lactase non-persistence), a large proportion of the human population loses, in adult age, the possibility to digest and absorb lactose[1-3]. In Europe, its prevalence increases with a north-south and west-east gradient. Thus, about 50% of adult Italians cannot digest and absorb lactose normally[4,5].

Lactose malabsorption (LM) may be asymptomatic or induce symptoms similar to those of functional bowel disorders and irritable bowel syndrome, consisting of abdominal pain, gaseousness, flatulence and diarrhea. LM is not necessarily a predictor of the occurrence of symptoms, and the term "lactose intolerance (LI)" refers to a condition in which abdominal symptoms are experienced after the ingestion of lactose, in milk or dairy food.

The test for identifying the genotype responsible for lactase deficiency[6-8] is not widely available and its use for the diagnosis of LM is debatable. Thus, the diagnosis of LM is usually based on a positive hydrogen breath test (HBT) with an oral load of 25 g lactose (HBT-25) and is often followed by the institution of a lactose-free diet, also in those patients who do not experience abdominal symptoms. This approach is debatable, as a reduction of calcium intake below the recommended daily allowance (RDA) may ensue. Moreover, also in those cases in which

symptoms are triggered by the 25 g of lactose ingested during the test, such a strict reduction of milk and dairy products is often unnecessary, as the amount of lactose administered during the test considerably exceeds the amount of lactose ingested daily, by most adults.

The present study was aimed at evaluating whether a reduction in the daily intake of milk and lactose-containing food is really necessary in subjects with LM during a standard HBT-25. To this end, we performed the HBT with an oral load of 12.5 g lactose (HBT-12.5) in a group of patients with marked LM documented by means of HBT-25. Positivity of the test, occurrence and type of symptoms during the two tests were compared.

MATERIALS AND METHODS

During the period January, 2001 to May, 2004, 913 outpatients underwent a lactose tolerance test in our laboratory. The HBT was performed after 24 h on a low-fiber diet and a 12-h fasting period with an oral load of lactose at a dose of 0.5 g/kg body weight, up to a maximum of 25 g. End-alveolar air samples were collected in syringes using a modified Haldane-Priestly tube[9], prior to the administration of lactose, and thereafter every 30 min for 4 h. Hydrogen (H_2) and methane (CH_4) concentrations were measured in parts per million (ppm) by means of a Quintron Model DP Microlyzer gas chromatograph (Quintron Instruments, Milwaukee, WI, USA). The test was defined as "positive" when a H_2 peak exceeding 20 ppm over baseline values was observed in two or more samples. Tests not fulfilling the above-mentioned criteria were defined as negative. Those patients with a negative HBT, who did not excrete increased amounts of H_2 after oral administration of 20 g lactulose in a subsequent HBT (24 patients), were defined as hydrogen non-producers. A positive test identified patients with LM, irrespective of the presence or absence of abdominal symptoms. Positivity of LM was arbitrarily defined as "high-grade" when H_2 excretion exceeded 70 ppm in at least two samples, and "low-grade" in all other instances.

Of the 353 patients with positive HBT-25, 147 fulfilled the above-mentioned criteria for high-grade LM. Of these, 50 were excluded from the study due to the presence of small bowel diseases, such as Crohn's disease and celiac disease, in which medical treatment or dietary modifications could result in variations in lactase activity. The remaining 97 patients were considered eligible for entry to the study and were required to undergo a further lactose tolerance test, with a lactose load of 12.5 g. Only seven refused to enter the study (compliance 92.78%) and the test was performed, 4-12 wk after the first test, in 90 patients (12 male, 78 female, mean age 41.81 ± 15 SD years). Of these, 65 had experienced symptoms during HBT-25 and 25 had not. The excretion of gas during HBT-12.5 was quantified as: (1) peak H_2 concentration; (2) area under the curve (AUC) of H_2 concentration from 60 to 240 min, calculated with the triangular rule and expressed in arbitrary units of ppm/h. During the test, occurrence and type of symptoms were recorded.

Statistical analysis

Data were analyzed using the χ^2 test, the inference between proportions and the *t*-test for paired data, when appropriate.

RESULTS

Positivity of the HBT-12.5 g

Only 16 (17.7%) of the 90 patients enrolled still had a positive test during the HBT-12.5 while the remaining 74 (82.3%) were negative. The difference between HBT-12.5 and HBT-25, evaluated by means of the inference between proportions, was highly significant ($P < 0.001$). Of the 65 LI patients, only five experienced symptoms during the HBT-12.5, while another seven had a positive test, but reported no symptoms. Of the 25 patients with LM, only four had a positive test after an oral load of 12.5 g lactose. None reported symptoms.

Peak H_2 excretion

Considering all 90 patients together, the mean value of peak H_2 excretion during HBT-12.5 was 21.55 ppm ± 29.54 SD, whereas in HBT-25, the mean peak H_2 excretion was 99.43 ppm ± 40.01 SD. As expected, the difference from HBT-25 was highly significant ($P < 0.001$). Considering only the data from the 16 patients who proved positive in both tests, the peak H_2 excretion was 97.68 ppm ± 27.37 during HBT-25 and 69 ppm ± 36.53 SD during HBT-12.5. Thus, even in those patients who had LM during HBT-12.5, the amount of hydrogen excretion was significantly lower as compared to the first test ($P < 0.01$). No difference was found between patients who had symptoms during the test (LI) and those who were LM, but did not experience symptoms, as far as concerning the peak H_2 excretion.

Amount of H_2 excreted (AUC)

The amount of H_2 excreted by the entire population of 90 patients was 18 ppm/h ± 27.12 SD in the HBT-12.5 compared to 97.08 ppm/h ± 40.56 SD in the HBT-25 ($P < 0.001$). Again, taking into account only data from the 16 patients who proved positive in both tests, the amount of H_2 excreted during HBT-12.5, the AUC was significantly lower compared to that of the HBT-25 (54.29 ppm/h ± 41.23 SD *vs* 99.21 ppm/h ± 35.58 SD, respectively; $P < 0.01$). Again no difference was observed between LI and LM.

Symptoms

During the test with 25 g lactose in the 65 patients with LI, gaseousness was present in 36 (55.3%), abdominal pain in 34 (52.3%), flatulence in 16 (24.6%) and diarrhea in eight (13.8%), with some patients reporting more than one symptom (Figure 1). Only five patients with a positive HBT-12.5 experienced symptoms, namely gaseousness in three, flatulence in three and abdominal pain in one. None experienced diarrhea. Eight patients with a negative HBT-12.5 reported experiencing minor symptoms, consisting of gaseousness in five and abdominal pain in five. The relationship between

Table 1 Occurrence of symptoms during positive or negative test with a lactose load of 12.5 g, in relation to the outcome of the test with 25 g lactose

	LI (HBT 25) 65 patients		LM (HBT 25) 25 patients	
	Symptoms +	Symptoms -	Symptoms +	Symptoms -
HBT 12.5 +	5	7	0	4
HBT 12.5 -	8	45	0	21

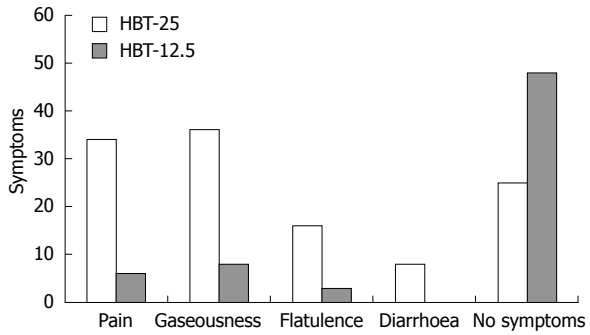


Figure 1 Occurrence of symptoms during the HBT using 25 g lactose (HBT-25) and 12.5 g (HBT-12.5). Some patients reported more than one symptom. It should be noted that, in HBT-12.5, minor symptoms, such as mild abdominal discomfort and abdominal distension, were reported not only by five patients with positive tests, but also by eight patients with a negative one.

positivity/negativity of the low-dose test (HBT-12.5) and occurrence of symptoms during HBT 12.5 is reported in Table 1.

Probability of predicting a positive HBT-12.5

Due to the small number of patients who proved positive during the low-dose test, positivity of HBT-25 does not help predict a positive HBT-12.5 (positive predictive value: 0.17). The occurrence of any symptom during HBT-25 showed only a slightly better positive predictive value for a positive HBT-12.5 (positive predictive value: 0.41). Taking into consideration the occurrence of individual symptoms during HBT-25, the positive predictive value was 0.06 for abdominal pain, 0.25 for gaseousness, 0.31 for flatulence and 0.12 for diarrhea. On the other hand, the absence of abdominal symptoms during HBT-25 had a negative predictive value of 0.84.

DISCUSSION

The diagnosis of LM is usually based upon the positivity of HBT after an oral load of lactose[10-15]. The most commonly used load of lactose is 20-25 g, corresponding to an intake of 400-500 mL of milk, which is rarely ingested in a single dose. Indeed, 400-500 mL of milk exceeds in most instances, the total daily intake of milk and diary products. As HBT has been found to correlate with lactase activity in duodenal biopsies, the HBT-25 is, indeed, useful for population studies[16,17]. HBT, however, is used in the clinical setting with the primary aim of diagnosing LM and LI, the rate of positive tests depending not only upon the degree of hypolactasia, but also the amount in the oral load used for the test. Moreover, the prevalence of symptoms, which is clinically relevant and of great importance for the patient, is dose-related. Thus, the traditional test with 25 g lactose likely overestimates the prevalence of LI. This may lead to unnecessary restrictions in the intake of foods that represent the main source of dietary calcium[18,19]. The present study was aimed at evaluating whether, and to what extent, the use of an oral load of 12.5 g lactose, instead of 25 g, could influence the prevalence of positive tests for diagnosing LI and LM. The present data confirm, in a large series of patients, previous observations showing that high loads of lactose (50 g, corresponding to 1 L of milk) induce abdominal pain and diarrhea in most lactose malabsorbers[20]. Conversely, small amounts of the sugar were usually well tolerated[21-24]. The present data indicates that the absence of abdominal symptoms during an HBT-25 is, in most instances, associated with a negative HBT-12.5. Unexpectedly, the presence of symptoms during HBT-25 was not useful for predicting a positive HBT-12.5. Less than 50% of the patients with abdominal symptoms (LI) display malabsorption of lactose in detectable amounts when the sugar load is reduced and the occurrence of symptoms are relatively rare. Thus, a moderate intake of lactose during a standard HBT-25 may prove harmless in the large majority of patients diagnosed as LI or LM[25].

Interestingly, during the HBT-12.5, eight patients reported symptoms despite a negative test with prevalence similar to that observed in a previous study performed in normal subjects and in patients with irritable bowel syndrome[26-29]. In the present series, symptoms consisted of gaseousness and mild abdominal pain, whereas none of the patients had diarrhea. As patients were asked to report even minor symptoms, a "nocebo", or "inverse placebo", effect may have been elicited by the investigators.

Finally, false-negative results cannot be completely ruled out in these patients, due to a better sensitivity of late (> 240 min) increases in hydrogen excretion, as suggested by Di Stefano *et al*[30]. These data, however, are debatable as these authors, using different hydrogen cutoff levels, considered definitely as lactose intolerant with a false-negative breath test those patients reporting symptoms during the HBT, irrespective of the test results. This is unlikely, as negative expectations often induce non-specific abdominal symptoms not only during lactose HBT, but also after a sham lactose load (personal unpublished data).

In conclusion, these data reaffirm that LI is dose-dependent. Considering the daily mean lactose intake in the general population, 50 or 25 g lactose tolerance breath tests may prove useful for epidemiological studies, looking for lactose deficiency. The widespread availability of genetic testing for lactase polymorphism may render obsolete this technique. Conversely, in the clinical setting, the use of the 12.5 g lactose tolerance test should be probably preferred to the 25 g test, at least in Caucasians and in the populations of the Mediterranean basin, as it may help to identify those patients who would profit from dietary restriction of lactose-containing food, minimizing the risk of inappropriately reducing calcium intake to those who do not need it.

COMMENTS

Background

Hydrogen breath test (HBT), after a lactose load of 25-50 g is widely used in the clinical setting for diagnosing lactose malabsorption (LM) and, when abdominal symptoms are present, of lactose intolerance (LI). The positivity of the test often induces dietary modifications, leading to the reduction of calcium intake.

Research frontiers

The authors confirmed in a large series of patients previous findings suggesting that most patients with LM, documented by HBT, tolerate well small amounts of lactose.

Innovations and breakthroughs

The present data indicates that an oral load of 12.5 of lactose, corresponding to about 250 mL milk, is well tolerated by the majority of patients unable to completely digest and absorb 25 g lactose. Moreover, in the majority of them, an increased excretion of hydrogen was not documented after ingesting an isoosmolar solution of 12.5 g of lactose, indicating that they can normally digest lactose at least up to a dose corresponding to 250 mL milk.

Applications

The authors suggest that 12.5 g lactose HBT should be preferred to the usual oral load of 25-50 g, in order to identify those patients who could really profit from a reduction of lactose-containing food, and minimize the risk of unnecessary reductions of calcium intake.

Terminology

HBTs are indicated by HBT. The positivity of the test defines a subject's LM, irrespective of the occurrence of abdominal symptoms. The coincident occurrence of symptoms is required for defining LI patients.

Peer review

This is an interesting study in which the authors argue that the use of 25 g of lactose to test for LM may be inappropriate, as this is higher than the average dietary intake, and the removal of lactose from the diet may have other deleterious consequences such as reduced calcium intake.

REFERENCES

1 **Gilat T**, Russo S, Gelman-Malachi E, Aldor TA. Lactase in man: a nonadaptable enzyme. *Gastroenterology* 1972; **62**: 1125-1127
2 **Simoons FJ**. The geographic hypothesis and lactose malabsorption. A weighing of the evidence. *Am J Dig Dis* 1978; **23**: 963-980
3 **Friedl J**. Lactase deficiency: distribution, associated problems and implications for nutritional policy. *Ecol Food Nutr* 1981; **11**: 37-48
4 **Burgio GR**, Flatz G, Barbera C, Patane R, Boner A, Cajozzo C, Flatz SD. Prevalence of primary adult lactose malabsorption and awareness of milk intolerance in Italy. *Am J Clin Nutr* 1984; **39**: 100-104
5 **Bozzani A**, Penagini R, Velio P, Camboni G, Corbellini A, Quatrini M, Conte D, Bianchi PA. Lactose malabsorption and intolerance in Italians. Clinical implications. *Dig Dis Sci* 1986; **31**: 1313-1316
6 **Enattah NS**, Sahi T, Savilahti E, Terwilliger JD, Peltonen L, Jarvela I. Identification of a variant associated with adult-type hypolactasia. *Nat Genet* 2002; **30**: 233-237
7 **Bodlaj G**, Stocher M, Hufnagl P, Hubmann R, Biesenbach G, Stekel H, Berg J. Genotyping of the lactase-phlorizin hydrolase -13910 polymorphism by LightCycler PCR and implications for the diagnosis of lactose intolerance. *Clin Chem* 2006; **52**: 148-151
8 **Szilagyi A**, Malolepszy P, Hamard E, Xue X, Hilzenrat N, Ponniah M, MacNamara E, Chong G. Comparison of a real-time polymerase chain reaction assay for lactase genetic polymorphism with standard indirect tests for lactose maldigestion. *Clin Gastroenterol Hepatol* 2007; **5**: 192-196
9 **Metz G**, Gassull MA, Leeds AR, Blendis LM, Jenkins DJ. A simple method of measuring breath hydrogen in carbohydrate malabsorption by end-expiratory sampling. *Clin Sci Mol Med* 1976; **50**: 237-240
10 **Levitt MD**, Donaldson RM. Use of respiratory hydrogen (H2) excretion to detect carbohydrate malabsorption. *J Lab Clin Med* 1970; **75**: 937-945
11 **Solomons NW**, Viteri FE, Hamilton LH. Application of a simple gas chromatographic technique for measuring breath hydrogen. *J Lab Clin Med* 1977; **90**: 856-862
12 **Corazza GR**, Sorge M, Strocchi A, Lattanzi MC, Benati G, Gasbarrini G. Methodology of the H2 breath test. II. Importance of the test duration in the diagnosis of carbohydrate malabsorption. *Ital J Gastroenterol* 1990; **22**: 303-305
13 **Levitt MD**, Bond JH Jr. Volume, composition, and source of intestinal gas. *Gastroenterology* 1970; **59**: 921-929
14 **Corazza GR**, Sorge M, Maurino E, Strocchi A, Lattanzi MC, Gasbarrini G. Methodology of the H2 breath test. I. Collection and storage for gas measurement. *Ital J Gastroenterol* 1990; **22**: 200-204
15 **Romagnuolo J**, Schiller D, Bailey RJ. Using breath tests wisely in a gastroenterology practice: an evidence-based review of indications and pitfalls in interpretation. *Am J Gastroenterol* 2002; **97**: 1113-1126
16 **Newcomer AD**, McGill DB, Thomas PJ, Hofmann AF. Prospective comparison of indirect methods for detecting lactase deficiency. *N Engl J Med* 1975; **293**: 1232-1236
17 **Davidson GP**, Robb TA. Value of breath hydrogen analysis in management of diarrheal illness in childhood: comparison with duodenal biopsy. *J Pediatr Gastroenterol Nutr* 1985; **4**: 381-387
18 **Savaiano D**. Lactose intolerance: a self-fulfilling prophecy leading to osteoporosis? *Nutr Rev* 2003; **61**: 221-223
19 **Prentice A**. Diet, nutrition and the prevention of osteoporosis. *Public Health Nutr* 2004; **7**: 227-243
20 **Shaw AD**, Davies GJ. Lactose intolerance: problems in diagnosis and treatment. *J Clin Gastroenterol* 1999; **28**: 208-216
21 **Vesa TH**, Korpela RA, Sahi T. Tolerance to small amounts of lactose in lactose maldigesters. *Am J Clin Nutr* 1996; **64**: 197-201
22 **Suarez FL**, Savaiano D, Arbisi P, Levitt MD. Tolerance to the daily ingestion of two cups of milk by individuals claiming lactose intolerance. *Am J Clin Nutr* 1997; **65**: 1502-1506
23 **Suarez FL**, Adshead J, Furne JK, Levitt MD. Lactose maldigestion is not an impediment to the intake of 1500 mg calcium daily as dairy products. *Am J Clin Nutr* 1998; **68**: 1118-1122
24 **Suarez FL**, Savaiano DA, Levitt MD. A comparison of symptoms after the consumption of milk or lactose-hydrolyzed milk by people with self-reported severe lactose intolerance. *N Engl J Med* 1995; **333**: 1-4
25 **Hertzler SR**, Huynh BC, Savaiano DA. How much lactose is low lactose? *J Am Diet Assoc* 1996; **96**: 243-246
26 **Savaiano DA**, Boushey CJ, McCabe GP. Lactose intolerance symptoms assessed by meta-analysis: a grain of truth that leads to exaggeration. *J Nutr* 2006; **136**: 1107-1113
27 **Vernia P**, Di Camillo M, Marinaro V. Lactose malabsorption, irritable bowel syndrome and self-reported milk intolerance. *Dig Liver Dis* 2001; **33**: 234-239
28 **Carroccio A**, Montalto G, Cavera G, Notarbatolo A. Lactose intolerance and self-reported milk intolerance: relationship with lactose maldigestion and nutrient intake. Lactase Deficiency Study Group. *J Am Coll Nutr* 1998; **17**: 631-636
29 **Vernia P**, Di Camillo M, Marinaro V. Lactose malabsorption, irritable bowel syndrome and self-reported milk intolerance. *Dig Liver Dis* 2001; **33**: 234-239
30 **Di Stefano M**, Missanelli A, Miceli E, Strocchi A, Corazza GR. Hydrogen breath test in the diagnosis of lactose malabsorption: accuracy of new versus conventional criteria. *J Lab Clin Med* 2004; **144**: 313-318

S- Editor Li DL **L- Editor** Rippe RA **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6208

World J Gastroenterol 2008 October 28; 14(40): 6208-6212
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Sildenafil does not influence hepatic venous pressure gradient in patients with cirrhosis

Jens Otto Clemmesen, Annamaria Giraldi, Peter Ott, Kim Dalhoff, Bent Adel Hansen, Fin Stolze Larsen

Jens Otto Clemmesen, Peter Ott, Bent Adel Hansen, Fin Stolze Larsen, Department of Hepatology, Rigshospitalet, University Hospital of Copenhagen, Copenhagen DK-2100, Denmark
Annamaria Giraldi, Kim Dalhoff, Clinical Pharmacology, Rigshospitalet, University Hospital of Copenhagen, Copenhagen DK-2100, Denmark
Author contributions: Clemmesen JO, Giraldi A, Ott P, and Dalhoff K designed research; Clemmesen JO performed research; Dalhoff K contributed with analytic tools; Clemmesen JO and Ott P analyzed data; Clemmesen JO, Ott P, Hansen BA, and Larsen FS wrote the paper.
Supported by Rigshospitalet, University of Copenhagen, The Laerdal Foundation for Acute Medicine; Savvaerksejer Jeppe Juhl and wife Ovita Juhls Foundation; The Novo Nordisk Foundation; The AP-Møller Foundation; and an unrestricted grant from Pfizer, Denmark
Correspondence to: Jens Otto Clemmesen, MD, DMSc, Department of Hepatology A-2121, Rigshospitalet, Blegdamsvej 9, Copenhagen DK-2100, Denmark. otto.clemmesen@rh.regionh.dk
Telephone: +45-35-450596 Fax: +45-35-452913
Received: December 8, 2007 Revised: July 26, 2008
Accepted: August 3, 2008
Published online: October 28, 2008

Abstract

AIM: To investigate if sildenafil increases splanchnic blood flow and changes the hepatic venous pressure gradient (HVPG) in patients with cirrhosis. Phosphodiesterase type-5 inhibitors are valuable in the treatment of erectile dysfunction and pulmonary hypertension in patients with end-stage liver disease. However, the effect of phosphodiesterase type-5 inhibitors on splanchnic blood flow and portal hypertension remains essentially unknown.

METHODS: Ten patients with biopsy proven cirrhosis (five females/five males, mean age 54 ± 8 years) and an HVPG above 12 mmHg were studied after informed consent. Measurement of splanchnic blood flow and the HVPG during liver vein catheterization were done before and 80 min after oral administration of 50 mg sildenafil. Blood flow was estimated by use of indocyanine green clearance technique and Fick's principle, with correction for non-steady state.

RESULTS: The plasma concentration of sildenafil was 222 ± 136 ng/mL 80 min after administration. Mean arterial blood pressure decreased from 77 ± 7 mmHg to 66 ± 12 mmHg, P = 0.003, while the splanchnic blood flow and oxygen consumption remained unchanged at 1.14 ± 0.71 L/min and 2.3 ± 0.6 mmol/min, respectively. Also the HVPG remained unchanged (18 ± 2 mmHg *vs* 16 ± 2 mmHg) with individual changes ranging from -8 mmHg to +2 mmHg. In seven patients, HVPG decreased and in three it increased.

CONCLUSION: In spite of arterial blood pressure decreases 80 min after administration of the phosphodiesterase type-5 inhibitor sildenafil, the present study could not demonstrate any clinical relevant influence on splanchnic blood flow, oxygen consumption or the HVPG.

Key words: Cirrhosis; Sildenafil; Portal hypertension; Portal hemodynamics; Hepatic blood flow; Erectile dysfunction; Hepatic venous pressure gradient

Peer reviewer: Philip Abraham, PhD, Professor, Consultant Gastroenterologist & Hepatologist, P. D. Hinduja National Hospital & Medical Research Centre, Veer Savarkar Marg, Mahim, Mumbai 400016, India

Clemmesen JO, Giraldi A, Ott P, Dalhoff K, Hansen BA, Larsen FS. Sildenafil does not influence hepatic venous pressure gradient in patients with cirrhosis. *World J Gastroenterol* 2008; 14(40): 6208-6212 Available from: URL: http://www.wjgnet.com/1007-9327/14/6208.asp DOI: http://dx.doi.org/10.3748/wjg.14.6208

INTRODUCTION

Erectile dysfunction is a common problem affecting about half of all patients with end-stage liver disease[1,2]. The systematic use of beta-adrenergic blockade[3,4], initiated in order to reduce the hepatic venous pressure gradient (HVPG), may also per se induce impotence[5]. Selective phosphodiesterase type-5 inhibitors, such as sildenafil, represent an important advance in management of erectile dysfunction[6,7]. Inhibition of phosphodiesterase type-5, the prominent isoform in corpora cavernosa, leads to diminished degradation of cyclic guanosine monophosphate, relaxation of smooth muscle and blood filling of corpora cavernosa[8].

In rats, phosphodiesterase type-5 is also present in the superior mesenteric artery[9]. Administration of sildenafil

results in a dose-dependent increase in mesenteric blood flow and a minor increase in portal venous pressure, in both the bile-duct-ligated rat, as well as in control rats[10]. It has been suggested that phosphodiesterase type-5 is also present in human mesenteric arteries[11]. The use of sildenafil could, therefore, be hazardous in patients with cirrhosis and portal hypertension. Indeed, acute variceal bleeding has been described after intake of 25 mg sildenafil[12,13]. On the other hand, a more recent study in patients with Child A liver cirrhosis has shown a decrease in portal and sinusoidal resistance after inhibition of phosphodiesterase-5[14], which was introduced as a new potential treatment modality of portal hypertension[14].

Since sildenafil seems to be valuable in the treatment erectile dysfunction[14] and portopulmonary hypertension[15] in patients with cirrhosis, the aim of the present study was to determine if splanchnic blood flow and HVPG are influenced by sildenafil in patients with cirrhosis.

MATERIALS AND METHODS

Twelve patients with biopsy-proven cirrhosis were included in the study during diagnostic work-up and/or evaluation for liver transplantation. None of the patients were treated with nitrates or beta-adrenergic drugs at the time of the study. Furthermore, only patients with an HVPG above 12 mmHg were considered for inclusion in this study. Two patients were excluded due to this last criterion. Thus, 10 patients participated (five females/five males, mean age 54 ± 8 years). The reason for referral to our tertiary liver failure unit was chronic hepatic insufficiency in five, recurrent bleeding from esophageal varices in four, and therapy-resistant ascites in one patient. None of the patients had suffered from variceal bleeding within 14 d of the study. Participation in the study included measurement of the HVPG and the estimation of splanchnic blood flow before and 80 min after oral administration of 50 mg of sildenafil. All patients were included after written informed consent, and after the local scientific-ethical committee had approved the study.

Hepatic venous catheterization (Cordis, MP-A1, open end catheter, Miami, USA) was performed through the right or left intermedian cubital vein. During the advance of the catheter, blood pressure in the right atrium was registered. After location of a hepatic vein, the catheter was advanced to the wedge position. Pressure was measured *via* a pressure monitoring set and was continuously recorded (Hewlett Packard, 78354C) and could be printed (Hewlett Packard, M1125A). The pressure transducer was calibrated to zero pressure at the level of the right atrium. The wedge pressure was only accepted if the wedge position afterwards could be confirmed by visualization of a characteristic wedge pattern on the fluoroscope by use of X-ray contrast media (Omnipaque 240 mg/mL, Amersham Health). Free hepatic venous pressure was measured as close as possible to the inferior caval vein. Pressures, free and wedged, were measured in three different hepatic veins and the mean was used. The catheter was then positioned in a hepatic vein half way between wedge position and the caval vein and used for blood sampling.

Splanchnic blood flow was estimated by use of indocyanine green as previously described[16]. In short, indocyanine green was continuously infused and simultaneously, five paired samples of arterial and hepatic venous blood were drawn at intervals of 5 min. Calculation of hepatic blood flow was based on Fick's principle with correction for non-steady state[16]. Splanchnic oxygen consumption was calculated as the arterio-venous oxygen content difference times splanchnic blood flow.

Patients were given 50 mg sildenafil with 100 mL of tap water. From 60 to 80 min thereafter, blood was again sampled for estimation of hepatic blood flow. HVPG was measured again, as described above, in three different veins. Pressure in the right atrium was recorded during withdrawal of the catheter.

Indocyanine green was determined by use of HPLC as earlier described[17]. To ensure absorption of sildenafil, blood was sampled 80 min after administration and plasma sildenafil concentration was later analyzed using automated sequential trace enrichment of dialyzates and HPLC[18].

Statistical analysis

Data are shown as mean ± SD. Paired *t* test was used for comparison, $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics and clinical data from the 10 participating patients showed that the majority had advanced cirrhosis (Table 1). None of the patients had allergy to sildenafil. The concentration of sildenafil in plasma was 222 ± 136 ng/mL, 80 min after administration of the drug.

There was a statistically significant decrease in mean arterial pressure from 77 ± 7 mmHg to 66 ± 12 mmHg, $P = 0.003$, after sildenafil administration (Table 2). No statistically significant changes were observed in other hemodynamic variables or in splanchnic oxygen consumption (Table 2). In particular, the HVPG remained statistically unchanged, i.e. 18 ± 2 mmHg before *vs* 16 ± 2 mmHg, after administration of sildenafil.

The plasma concentration of sildenafil at 80 min was not statistically significantly correlated to the changes in HVPG, splanchnic blood flow, mean arterial pressure, or right atrial pressure.

Four patients had a decrease in HVPG i.e. 3, 3, 3 and, 8 mmHg, respectively. Two of these where Child class A/B and all four had alcoholic cirrhosis.

DISCUSSION

This study reports the effects of sildenafil on the splanchnic hemodynamics in patients with biopsy proven

Table 1 Patient characteristics and clinical data (mean ± SD)

Characteristics	Data
Age (yr)	54 ± 8
Weight (kg)	75 ± 16
Height (cm)	175 ± 7
Female/Male	5/5
Aetiology (ALC/AIH/PBC)	8/1/1
Varices, present	9
Bleeding, earlier	5
Ascites, present	8
Child Pugh (A/B/C)	1/2/7
GEC (μmol/kg per min)	20 ± 4
INR	1.7 ± 0.5
Bilirubin (mmol/L)	64 ± 50
Albumin (g/L)	24 ± 5

ALC: Alcoholic cirrhosis; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis. INR: International normalization ratio; GEC: Galactose elimination capacity, normal > 32 μmol/kg per min.

Table 2 Effect of 50 mg sildenafil (mean ± SD, *n* = 10)

	Baseline	80 min after
MAP (mmHg)	77 ± 7	66 ± 12[1]
HF (beats/min)	83 ± 13	85 ± 11
RA (mmHg)	5 ± 1	6 ± 2
Hepatic blood flow (L/min)	1.14 ± 0.71	1.14 ± 0.94
VO_2 (mmol/min)	2.3 ± 0.6	2.5 ± 1.2
Wedged pressure (mmHg)	29 ± 6	27 ± 6
Free pressure (mmHg)	12 ± 6	11 ± 5
Gradient (mmHg)	18 ± 2	16 ± 2

[1]P = 0.003, *vs* baseline. VO_2: Splanchnic oxygen consumption. MAP: Mean arterial pressure; RA: Right atrial pressure.

cirrhosis and manifest portal hypertension. Despite a small decrease in mean arterial pressure, the main finding in this study was that the splanchnic blood flow, oxygen consumption and the mean HVPG did not change 80 min after administration of sildenafil, i.e. at a time-point when a therapeutic plasma concentration of sildenafil[19] could be documented. These data appear to be in accordance with preliminary results testing the effect of an oral dose of 25 mg of sildenafil with which no effect on portal pressure was reported, in spite a decrease in arterial pressure[20]. However, our results appear to be in contrast to an earlier study reported by Deibert *et al*[14], in which vardenafil (another phosphodiesterase type-5 inhibitor) was found to lower portal pressure in four of five patients with Child A cirrhosis. The reason for this discrepancy is not clear, but three of the five patients in that latter study[14] had HVPG < 12 mmHg before administration of vardenafil.

As illustrated in Figure 1, the individual HVPG changes ranged from -8 to +2 mmHg. In an animal study with bile-duct-ligated rats, a statistically significant increase in the HVPG of 3% ± 1% after intramesenteric (10 mg/kg) administration of sildenafil has been reported[10]. Thus, our finding suggests that the effect of sildenafil on splanchnic hemodynamics in humans with cirrhosis is different from that in rats. This could be due to the uncertainty concerning which phosphodiesterase

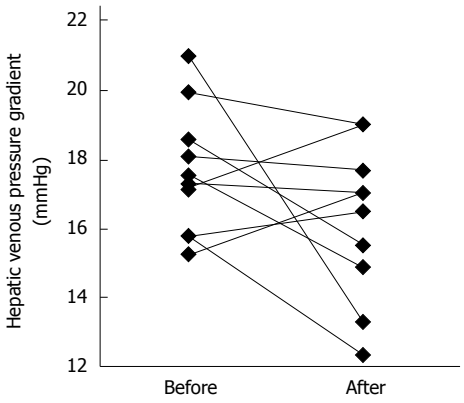


Figure 1 HVPG in ten patients with cirrhosis of the liver before and 80 min after oral intake of 50 mg sildenafil.

isoform, PDE5 and/or PDE1 is present in the human mesenteric artery[11]. It can be argued that even a 10%-15% increase in HVPG, as seen in one out of 10 patients in our study, might be problematic in the individual. However, small changes in the gradient probably take place during everyday life. For example, digestion of food increases hepatic blood flow by almost 100%. In particular, a major increase is seen in the superior mesenteric artery and thus portal blood flow[21]. Moreover, it has been demonstrated in patients with cirrhosis that a meal increases the mean HVPG from 16 to 20 mmHg, corresponding to 25%[22]. This could be even higher in the individual. The risk of experiencing a bleeding episode after intake of sildenafil should, therefore, be interpreted in the light of such everyday events.

The splanchnic hemodynamic data were collected during rest in the present study. However, supposedly the user of sildenafil is rarely in a resting position, but is more likely to increase the level of physical activity, which is known to influence the splanchnic hemodynamics[23]. In fact, physical activity, with an exercise level of 30% of peak workload, appears to increase HVPG from 16.7 to 19.2 mmHg[23]. Working at 50% of peak workload will not increase this HVPG gradient any further. Thus, the physical activity, which often is required to engage in sexual activity, may itself slightly increase the gradient. However, and of importance, the opposite has been observed in patients with cirrhosis during exercise and beta-adrenergic blockade[24]. In these patients, exercise at 30% of peak workload was associated with a decrease in HVPG from 16.3 to 12.9 mmHg[19]. This was explained by a lesser increase in cardiac output and a larger decrease in hepatic blood flow in the propranolol group compared to the non-beta-blocker-treated group of patients.

The possible effect on splanchninc hemodynamics after longer use of sildenafil is elusive and has not been examined. One concern about chronic use may be the possible renal effects. In patients with cirrhosis, administration of a single dose of sildenafil was followed by a decrease in sodium excretion[25]. However, the long-term renal effects may be insignificant as chronic

administration of sildenafil prevented the decrease in sodium excretion seen in bile-duct-ligated rats[26].

The present study shows that sildenafil does not induce any profound clinically relevant changes in splanchnic blood flow, oxygen consumption and HVPG. This indicates that phosphodiesterase type-5 inhibition is of no use as a therapeutic agent for alleviating portal hypertension in patients with chronic end-stage liver disease. On the other hand, the use of sildenafil in such patients, initiated because of erectile dysfunction, appears to be safe per se. However,, clearly more studies on safety are needed, as the use of sildenafil is usually associated with physical activity and often also the use of beta blockers.

COMMENTS

Background

Erectile dysfunction is a common problem affecting about half of all patients with end-stage liver disease. Selective phosphodiesterase type-5 inhibitors, such as sildenafil, represent an important advance in management of erectile dysfunction. In rats, administration of sildenafil results in a dose-dependent increase in mesenteric blood flow and a minor increase in portal venous pressure. The use of sildenafil could, therefore, be hazardous in patients with cirrhosis and portal hypertension and acute variceal bleeding has been described after intake of 25 mg sildenafil.

Research frontiers

Bleeding from esophageal varices is a major contributor to death in patients with end-stage liver disease and it could be hazardous to recommend treatment of erectile dysfunction with sildenafil if this treatment, in turn, leads to an increase in hepatic venous pressure gradient (HVPG).

Innovations and breakthroughs

Another human study addressing the presented problem has been published in abstract form, but the patients investigated all had Child class A cirrhosis whereas, erectile dysfunction as a "quality of life problem" tends to increase with increasing severity of liver disease i.e. Child class B and C, and such patients where investigated in the present study.

Applications

The present study does not support a restrictive use of sildenafil in patients with cirrhosis. Future research should be done with larger patient groups in order to determine the long-term risk of bleeding (if any) during treatment with sildenafil.

Terminology

Liver disease leads to accumulation of fibrous tissue in the liver, and in turn, this leads to increased resistance to the blood flow through the portal vein. The resistance is estimated by the HVPG and the risk of bleeding from esophageal varices in cirrhosis increases with this gradient.

Peer review

This is an interesting and informative study. The authors investigated if sildenafil increases splanchnic blood flow and changes the HVPG in patients with cirrhosis. Phosphodiesterase type-5 inhibitors are valuable in the treatment of erectile dysfunction and pulmonary hypertension in patients with end-stage liver disease.

REFERENCES

1 **Cornely CM**, Schade RR, Van Thiel DH, Gavaler JS. Chronic advanced liver disease and impotence: cause and effect? *Hepatology* 1984; **4**: 1227-1230

2 **Jensen SB**, Gluud C. Sexual dysfunction in men with alcoholic liver cirrhosis. A comparative study. *Liver* 1985; **5**: 94-100

3 **Lebrec D**, Nouel O, Corbic M, Benhamou JP. Propranolol--a medical treatment for portal hypertension? *Lancet* 1980; **2**: 180-182

4 **Groszmann RJ**, Bosch J, Grace ND, Conn HO, Garcia-Tsao G, Navasa M, Alberts J, Rodes J, Fischer R, Bermann M. Hemodynamic events in a prospective randomized trial of propranolol versus placebo in the prevention of a first variceal hemorrhage. *Gastroenterology* 1990; **99**: 1401-1407

5 **Burris JF**, Goldstein J, Zager PG, Sutton JM, Sirgo MA, Plachetka JR. Comparative tolerability of labetalol versus propranolol, atenolol, pindolol, metoprolol, and nadolol. *J Clin Hypertens* 1986; **2**: 285-293

6 **Boolell M**, Gepi-Attee S, Gingell JC, Allen MJ. Sildenafil, a novel effective oral therapy for male erectile dysfunction. *Br J Urol* 1996; **78**: 257-261

7 **Goldstein I**, Lue TF, Padma-Nathan H, Rosen RC, Steers WD, Wicker PA. Oral sildenafil in the treatment of erectile dysfunction. Sildenafil Study Group. *N Engl J Med* 1998; **338**: 1397-1404

8 **Corbin JD**, Francis SH. Cyclic GMP phosphodiesterase-5: target of sildenafil. *J Biol Chem* 1999; **274**: 13729-13732

9 **Sampson LJ**, Hinton JM, Garland CJ. Evidence for expression and function of phosphodiesterase type 5 (PDE-V) in rat resistance arteries. *Br J Pharmacol* 2001; **132**: 13-17

10 **Colle I**, De Vriese AS, Van Vlierberghe H, Lameire NH, DeVos M. Systemic and splanchnic haemodynamic effects of sildenafil in an in vivo animal model of cirrhosis support for a risk in cirrhotic patients. *Liver Int* 2004; **24**: 63-68

11 **Wallis RM**, Corbin JD, Francis SH, Ellis P. Tissue distribution of phosphodiesterase families and the effects of sildenafil on tissue cyclic nucleotides, platelet function, and the contractile responses of trabeculae carneae and aortic rings in vitro. *Am J Cardiol* 1999; **83**: 3C-12C

12 **Tzathas C**, Christidou A, Ladas SD. Sildenafil (viagra) is a risk factor for acute variceal bleeding. *Am J Gastroenterol* 2002; **97**: 1856

13 **Finley DS**, Lugo B, Ridgway J, Teng W, Imagawa DK. Fatal variceal rupture after sildenafil use: report of a case. *Curr Surg* 2005; **62**: 55-56

14 **Deibert P**, Schumacher YO, Ruecker G, Opitz OG, Blum HE, Rossle M, Kreisel W. Effect of vardenafil, an inhibitor of phosphodiesterase-5, on portal haemodynamics in normal and cirrhotic liver -- results of a pilot study. *Aliment Pharmacol Ther* 2006; **23**: 121-128

15 **Callejas Rubio JL**, Salmerón Escobar J, González-Calvín J, Ortego Centeno N. Successful treatment of severe portopulmonary hypertension in a patient with Child C cirrhosis by sildenafil. *Liver Transpl* 2006; **12**: 690-691

16 **Skak C**, Keiding S. Methodological problems in the use of indocyanine green to estimate hepatic blood flow and ICG clearance in man. *Liver* 1987; **7**: 155-162

17 **Ott P**, Keiding S, Bass L. Plasma elimination of indocyanine green in the intact pig after bolus injection and during constant infusion: comparison of spectrophotometry and high-pressure liquid chromatography for concentration analysis. *Hepatology* 1993; **18**: 1504-1515

18 **Cooper JD**, Muirhead DC, Taylor JE, Baker PR. Development of an assay for the simultaneous determination of sildenafil (Viagra) and its metabolite (UK-103,320) using automated sequential trace enrichment of dialysates and high-performance liquid chromatography. *J Chromatogr B Biomed Sci Appl* 1997; **701**: 87-95

19 **Muirhead GJ**, Wilner K, Colburn W, Haug-Pihale G, Rouviex B. The effects of age and renal and hepatic impairment on the pharmacokinetics of sildenafil. *Br J Clin Pharmacol* 2002; **53** Suppl 1: 21S-30S

20 **Inayat I**, Tandon P, Tal MG, Spector M, Bari K, Groszmann RJ, Garcia-Tsao G. Sildenafil has no effect on portal pressure but lowers arterial pressure. *Hepatology* 2007; **46**; 569A

21 **Perko MJ**, Nielsen HB, Skak C, Clemmesen JO, Schroeder TV, Secher NH. Mesenteric, coeliac and splanchnic blood flow in humans during exercise. *J Physiol* 1998; **513** (Pt 3): 907-913

22 **Bellis L**, Berzigotti A, Abraldes JG, Moitinho E, Garcia-Pagan JC, Bosch J, Rodes J. Low doses of isosorbide mononitrate attenuate the postprandial increase in portal

pressure in patients with cirrhosis. *Hepatology* 2003; **37**: 378-384

23 **Garcia-Pagan JC**, Santos C, Barbera JA, Luca A, Roca J, Rodriguez-Roisin R, Bosch J, Rodes J. Physical exercise increases portal pressure in patients with cirrhosis and portal hypertension. *Gastroenterology* 1996; **111**: 1300-1306

24 **Bandi JC**, Garcia-Pagan JC, Escorsell A, Francois E, Moitinho E, Rodes J, Bosch J. Effects of propranolol on the hepatic hemodynamic response to physical exercise in patients with cirrhosis. *Hepatology* 1998; **28**: 677-682

25 **Thiesson HC**, Jensen BL, Jespersen B, Schaffalitzky de Muckadell OB, Bistrup C, Walter S, Ottosen PD, Veje A, Skott O. Inhibition of cGMP-specific phosphodiesterase type 5 reduces sodium excretion and arterial blood pressure in patients with NaCl retention and ascites. *Am J Physiol Renal Physiol* 2005; **288**: F1044-F1052

26 **Ghali-Ghoul R**, Tahseldar-Roumieh R, Sabra R. Effect of chronic administration of sildenafil on sodium retention and on the hemodynamic complications associated with liver cirrhosis in the rat. *Eur J Pharmacol* 2007; **572**: 49-56

S- Editor Li DL **L- Editor** Rippe RA **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6213

World J Gastroenterol 2008 October 28; 14(40): 6213-6217
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Cholangiocarcinoma: A 7-year experience at a single center in Greece

Alexandra Alexopoulou, Aspasia Soultati, Spyros P Dourakis, Larissa Vasilieva, Athanasios J Archimandritis

Alexandra Alexopoulou, Aspasia Soultati, Spyros P Dourakis, Larissa Vasilieva, Athanasios J Archimandritis, 2nd Department of Medicine, University of Athens Medical School, Hippokration General Hospital, Athens, Greece
Author contributions: Alexopoulou A and Soultati A analysed the data and wrote the paper; Dourakis SP and Archimandritis AJ designed research and supervised the project; Vasilieva L performed research and collected the data.
Correspondence to: Alexandra Alexopoulou, 2nd Department of Medicine, University of Athens Medical School, Hippokration General Hospital, 40 Konstantinoupoleos St, Hilioupolis, Athens 16342, Greece. alexopou@ath.forthnet.gr
Telephone: +30-210-7774742 Fax: +30-210-7706871
Received: March 13, 2008 Revised: October 13, 2008
Accepted: October 20, 2008
Published online: October 28, 2008

Abstract

AIM: To evaluate survival rate and clinical outcome of cholangiocarcinoma.

METHODS: The medical records of 34 patients with cholangiocarcinoma, seen at a single hospital between the years 1999-2006, were retrospectively reviewed.

RESULTS: Thirty-four patients with a median age of 75 years were included. Seventeen (50%) had painless jaundice at presentation. Sixteen (47.1%) were perihilar, 15 (44.1%) extrahepatic and three (8.8%) intrahepatic. Endoscopic retrograde cholangiography (ERCP) and/or magnetic resonance cholangiography (MRCP) were used for the diagnosis. Pathologic confirmation was obtained in seven and positive cytological examination in three. Thirteen patients had co-morbidities (38.2%). Four cases were managed with complete surgical resection. All the rest of the cases (30) were characterized as non-resectable due to advanced stage of the disease. Palliative biliary drainage was performed in 26/30 (86.6%). The mean follow-up was 32 mo (95% CI, 20-43 mo). Overall median survival was 8.7 mo (95% CI, 2-16 mo). The probability of 1-year, 2-year and 3-year survival was 46%, 20% and 7%, respectively. The survival was slightly longer in patients who underwent resection compared to those who did not, but this difference failed to reach statistical significance. Patients who underwent biliary drainage had an advantage in survival compared to those who did not (probability of survival 53% *vs* 0% at 1 year, respectively, $P = 0.038$).

CONCLUSION: Patients with cholangiocarcinoma were usually elderly with co-morbidities and/or advanced disease at presentation. Even though a slight amelioration in survival with palliative biliary drainage was observed, patients had dismal outcome without resection of the tumor.

Key words: Cholangiocarcinoma; Surgical resection; Palliative biliary drainage; Survival

Peer reviewer: Jose JG Marin, Professor, Department of Physiology and Pharmacology, University of Salamanca, CIBERehd, Campus Miguel de Unamuno, ED-S09, Salamanca 37007, Spain

Alexopoulou A, Soultati A, Dourakis SP, Vasilieva L, Archimandritis AJ. Cholangiocarcinoma: A 7-year experience at a single center in Greece. *World J Gastroenterol* 2008; 14(40): 6213-6217 Available from: URL: http://www.wjgnet.com/1007-9327/14/6213.asp DOI: http://dx.doi.org/10.3748/wjg.14.6213

INTRODUCTION

Cholangiocarcinoma is the second commonest primary hepatic malignant disease, after hepatocellular carcinoma[1]. Several studies have shown that the incidence and mortality of the disease are rising worldwide[1,2]. The high fatality rate has been attributed to the poor knowledge of the tumor pathogenesis and the paucity of effective methods of diagnosis and management. First, the diagnosis of perihilar and extrahepatic cholangiocarcinoma still remains a clinical challenge, particularly in the presence of primary sclerosing cholangitis. Second, surgical resection is the only curative option for cholangiocarcinoma, but only a minority of patients are suitable for resection[3,4]. Factors that have been considered as contraindications for resectability include, among others, metastatic disease, multiple comorbidities, invasion of the hepatic artery or portal vein and extension of cholangiocarcinoma to involve segmental bile ducts on both liver lobes[3,4,5]. Sometimes the lack of available surgical expertise renders the surgical approach difficult to apply.

We aimed at evaluating the clinical features, diagnostic modalities, therapeutic options and survival rates in a series of 34 patients with cholangiocarcinoma hospitalized

at the Hippokration University Hospital, Athens, Greece. We also attempted to investigate whether there were differences in survival, compared to data published in the literature, and to identify factors associated with survival.

MATERIALS AND METHODS

The medical records of 34 patients diagnosed with cholangiocarcinoma at the Hippokration University Hospital between January 1999 and December 2006 were retrospectively reviewed. The study was approved by the local Ethics Committee. Our hospital has a liver unit and several patients with cholangiocarcinoma are referred from other hospitals. Diagnosis of cholangiocarcinoma was based upon clinical, imaging, cytologic and histopathologic findings. Medical records were scrutinized for epidemiologic characteristics, predisposing factors, initial manifestations of the disease, method of diagnosis, laboratory findings, surgical or palliative therapy, and overall morbidity and mortality.

Cholangiocarcinoma was classified as intrahepatic, perihilar and distal extrahepatic type[3,6]. The staging of the tumor was based on the tumor-node-metastasis system[7]. The perihilar tumors were classified according to the Bismuth classification[8] and the resectability was evaluated according to T-stage criteria[4]. All patients had ultrasound of the liver and gallbladder as the first diagnostic imaging procedure. Metastatic disease was evaluated by imaging of the chest, abdomen and pelvis by helical computed tomography (CT) or magnetic resonance imaging (MRI). No laparoscopic staging was performed.

Statistical data were analyzed using SPSS 13.0 for Windows. Descriptive statistics including mean, ranges and standard deviation values were calculated for all the continuous baseline demographic and laboratory characteristics. We used the Kruskal-Wallis non-parametric test to compare continuous data. The results are presented as means ± 95% confidence intervals (CI). The χ^2 test was used to compare categorical data; the results are presented as counts with percentages. All reported *P* values are based on two-tailed tests of significance. Comparisons were considered significantly different if $P < 0.05$. Overall survival was estimated from the admission of the patient to the hospital until death or last follow-up visit. Survival probabilities were estimated using the Kaplan-Meier method and compared by the log-rank test. Univariate analysis using the Cox regression test was used to determine factors associated with survival.

RESULTS

The study group included 18 men and 16 women, all of Greek origin. Sixteen of 34 (47.1%) were perihilar, 15 (44.1%) extrahepatic and three (8.8%) intrahepatic tumors. Demographic characteristics and laboratory data on presentation are shown in Table 1. Initial manifestations were painless jaundice in 17 patients (50%), whereas 12 (35.3%) presented with abdominal pain and weight loss.

Table 1 Demographic and biochemical characteristics of all patients at baseline (mean ± SD)

Patient characteristics	
Gender (M %)	18/34 (53%)
Age (years)	71.7 ± 13.3
Bilirubin (mg/dL)	13.3 ± 9.1
AST (U/mL)	117 ± 80
ALP (U/mL)	472 ± 237
Albumin (g/dL)	3.6 ± 0.5
Predisposing factor (%)	4/34 (11.7%)
Tobacco use (%)	10/34 (29.4%)

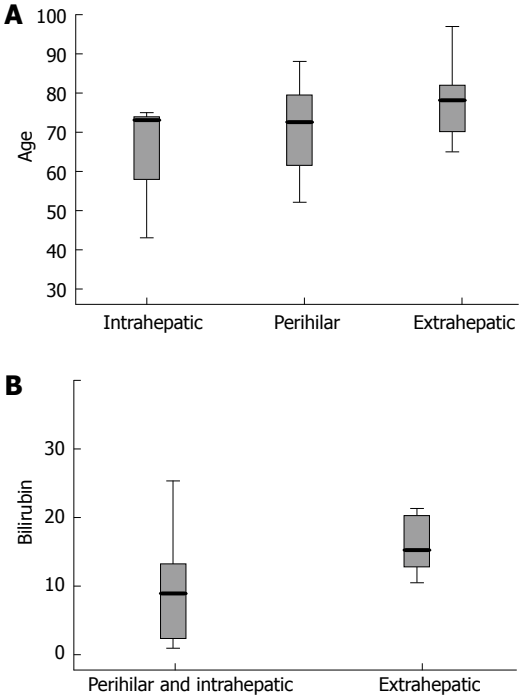


Figure 1 Comparison of baseline characteristics of the patients with respect to site. A: Age distribution with respect to site (intrahepatic, perihilar and extrahepatic) is significantly not different; B: Bilirubin values at presentation with respect to site (intrahepatic, and perihilar *vs* extrahepatic; $P = 0.027$).

Patients with extrahepatic type of cholangiocarcinoma were older than those with perihilar and intrahepatic type, but the difference was not statistically significant (79 ± 8.4 years *vs* 72.5 ± 11 years and 63.6 ± 17.9 years respectively, $P = 0.181$, Figure 1A). Mean values of total bilirubin were higher in the extrahepatic than in perihilar and intrahepatic type (17 ± 9 mg/dL *vs* 8.9 ± 7.5 mg/dL, $P = 0.027$, Figure 1B).

A history of a predisposing factor was recognized in four patients, two with primary sclerosing cholangitis and two with chronic hepatitis B and cirrhosis. No case of Caroli's syndrome, congenital hepatic fibrosis, choledochal cyst or occupation in the chemical industry was found. Moderate consumption of alcohol and use of tobacco were present in four (11.7%) and 10 (29.4%) patients, respectively. Thirteen (38.2%) patients presented with co-morbidities, mostly diabetes mellitus with complications and/or coronary disease.

Intrahepatic tumors were histologically proven using

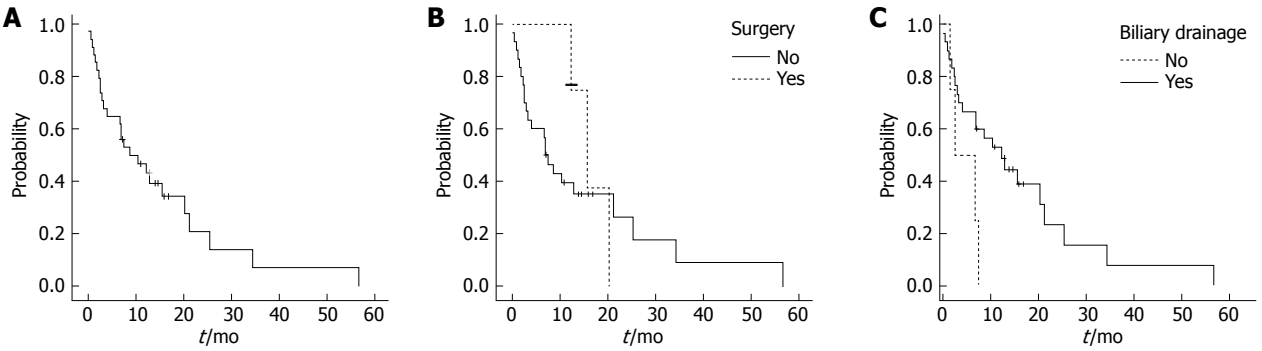


Figure 2 Survival rates in different groups of patients according to treatment. A: Survival of all the 34 patients with cholangiocarcinoma; B: Survival rates of patients with cholangiocarcinoma, stratified by surgical (n = 4) or not surgical resection (n = 30) were not significantly different; C: Survival of patients with unresected cholangiocarcinoma among those who had any kind of biliary decompression (n = 26) and those who had no interventional therapy (n = 4, P = 0.038).

a CT-guided biopsy. Eight of 16 (50%) perihilar tumors were diagnosed by magnetic resonance cholangiography (MRCP) and magnetic resonance imaging (MRI), 4/16 (25%) by endoscopic retrograde cholangiography (ERCP) and 4/16 (25%) by histology (biopsies were obtained at laparoscopy in three cases and at endoscopic ultrasound in one case). Five of 15 (33%) extrahepatic tumors were diagnosed by MRCP/MRI, and 10/15 (66%) by ERCP. Overall, tissue for pathologic confirmation was obtained from seven patients, three with intrahepatic and four with perihilar tumors. A positive cytologic examination was obtained in three further cases.

A local surgical team evaluated the cases as resectable or non-resectable. Four cases were managed with complete surgical resection aiming at histologically negative resection margins (two extrahepatic, one perihilar and one intrahepatic type). All of the remaining cases were characterized as non-resectable. Among the 15 unresectable perihilar tumors, three (20%) were classified as T2-T3 stage, and extension of the hepatectomy was considered too risky compared to the poor patients' performance status; nine others (60%) had evidence of either metastases or extensive local lymphadenopathy or portal vein involvement; and the three remaining patients (20%) had multiple co-morbidities. Two of three intrahepatic tumors were multifocal and were assessed as non-resectable. Three of 13 (23%) cases with unresectable extrahepatic tumors had evidence of either metastases or extensive local lymphadenopathy or portal vein involvement, and 10 (77%) had multiple co-morbidities, including cardiopulmonary disease or diabetes with complications, poor performance status and advanced age (> 80 years) and thus were considered as unsuitable for curative resection.

Palliative biliary drainage to relieve symptoms was performed in the vast majority of cases who did not undergo surgical curative therapy (12/15 perihilar, 13/13 extrahepatic, 1/2 intrahepatic). More specifically, among the perihilar-type cholangiocarcinoma cases who were not resected, eight were managed by stent placement (seven biliary stents were inserted by endoscopic and one by percutaneous routes), three by palliative percutaneous biliary drainage, and one by palliative surgical biliary drainage. No biliary decompression was decided in three cases. Among the two intrahepatic cases not resected, one was managed with palliative percutaneous biliary drainage and no biliary drainage was decided in one case. Among the 13 extrahepatic cases not resected, 12 were managed by stent placement endoscopically and one by palliative percutaneous biliary drainage.

A high number of patients who underwent a palliative biliary drainage (11/26, 42%) were managed with two or more endoscopic or percutaneous sessions for biliary decompression because of stent occlusion (median number of procedures for each patient was two, range 1-7). Metal stents were placed in the vast majority of patients.

Four cases (one intrahepatic, one extrahepatic and two perihilar type) were managed with chemotherapy without surgical resection (two in combination with biliary drainage and two without any other intervention). The causes of death in two-thirds of the patients were infective complications (acute cholangitis) following an occluded biliary stent or acute pancreatitis. Other causes of death were hepatic failure and acute myocardial infarction.

The mean follow-up for all patients was 32 mo, 95% CI 20-43 mo. Overall median survival was 8.7 mo (events, 26/34; 95% CI, 2-16 mo). The probability of 1-year, 2-year and 3-year survival was 46%, 20% and 7%, respectively (Figure 2A). The survival was longer in patients who underwent surgical resection (n = 4) compared to those who did not (n = 30), but the difference failed to reach statistical significance. The probability of survival for the former was significantly higher than for the latter (75% *vs* 39% and 37% *vs* 26% at 1 and 2 years respectively, P = 0.6, Figure 2B). The median survival was 15.7 mo, (95% CI 11-20.6 mo) in the former and 7 mo, (95% CI, 4.4-9.6 mo) in the latter group.

Patients who received any interventional treatment for biliary drainage (either stent replacement or percutaneous biliary drainage or palliative surgical procedure, n = 26) had an advantage in survival in comparison to those who did not (n = 4). The probability of survival for the former was significantly higher than for the latter (53% *vs* 0% at 1 year respectively; median survival, 12.3 mo; 95% CI, 6-18.7 mo and 2.5 mo, 95% CI, 0-7.6 mo, respectively; P = 0.038; Figure 2C). None of the four patients who did not undergo biliary drainage survived beyond 6 mo. None of the following

factors were associated with a statistically significant difference in survival: age, gender, bilirubin, site of the tumour, albumin, stent placement, and chemotherapy.

DISCUSSION

Cholangiocarcinoma is a relatively rare disease accounting for less than 2% of all human malignancies[9]. The specific features of cholangiocarcinoma depend on the anatomical location of the tumor, which are useful to optimize the appropriate therapy. The most common location of these tumors (60%-70%) is the bifurcation of the hepatic ducts (perihilar or Klatskin tumours), while 20%-30% are extrahepatic, arising from the distal common bile duct, and 5%-10% are peripheral or intrahepatic tumors, originating from the small bile ducts of the liver parenchyma[10]. In our series, the perihilar and extrahepatic tumors had the same incidence (47% and 44%, respectively) while intrahepatic tumors accounted for a small minority (8.8%).

The distribution of age among our patients was different in the three types of tumor, with the extrahepatic cases being older, in agreement with the literature[7]. Co-morbidities were common in the extrahepatic type, as older individuals have more illnesses than younger ones. The clinical presentation and risk factors were rather similar with respect to the site of the malignancy. Painless jaundice, abdominal pain, weight loss, use of tobacco and consumption of alcohol did not show predilection for any site of the tumor. It appeared however that jaundice at presentation was more profound in the extrahepatic type of disease than in the perihilar cases. Only a few cases of cholangiocarcinoma were associated with a predisposing factor such as primary sclerosing cholangitis and chronic hepatitis B, a rather low rate compared with other series[11]. Other chronic inflammatory diseases of biliary epithelium such as parasitic infections or intrahepatic biliary stones are not endemic in the Greek population.

At present, only surgical excision of all detectable tumors is associated with improvement in survival. The median survival time for patients with distal bile duct cancers who undergo resection has been reported to be about 38 mo[12,13]. The median survival time for patients with perihilar cholangiocarcinoma varies from 12 to 46 mo[4,14,15,16]. However, factors associated with both the patient and the tumor may preclude surgical resection. It is generally accepted that many patients are not considered surgical candidates because of co-morbidities and advanced age, despite evidence of resectable disease. On the other hand, more than half of cholangiocarcinoma cases usually present with advanced unresectable malignancy[4,17]. In our series, 50% of the patients were considered to have advanced disease and the remaining had multiple co-morbidities and/or advanced age. In the literature, neither a T2 or T3 stage nor portal involvement are considered absolute contraindications for resection for perihilar tumours[4,5]. Similarly, local lymphadenopathy is not a contraindication to resection of extrahepatic tumors[17]. Co-morbidities and advanced age along with the available local surgical expertise determined the low rate of resectability in our series. In an old series from Mayo Clinic, only 22 of 125 patients (18%) underwent curative resection, whereas 82% were candidates for palliative intervention[18]. In another more recent series of 225 patients with hilar cholangiocarcinoma, 80 (35%) underwent resection[4] while resectability rates were higher for intrahepatic cholangiocarcinoma ranging from 45% to 90%[19,20].

No significant survival advantage was found in patients who underwent surgical resection in our series. Curative surgical resection was attempted in only four patients and the disease recurred after resection in three of the four. It is noteworthy that no free-of-cancer surgical margins were found at histology among those three surgical specimens. The natural history of resected cholangiocarcinoma with no disease-free surgical margins is comparable to unresected cholangiocarcinoma receiving palliative therapy[4,21,22]. The overall median survival in our series was 8.7 mo, with a survival rate comparable to that reported for unresected cholangiocarcinoma from previous investigators[18,23,24].

Palliative treatment to relieve symptoms and resolve obstructive jaundice has an important role in the management of cholangiocarcinoma, since the majority of cases are not suitable for resection, as stated above, or they recur after resection[4,25]. Palliation and relief of jaundice can be accomplished by either endoscopic, percutaneous, or operative means. We found that the single factor that may provide a survival benefit in unresected cases is successful biliary decompression. It is noteworthy that of our four patients with cholangiocarcinoma with no interventional procedure for biliary decompression, none survived beyond 6 mo. Similarly, median survival for non-resectable cholangiocarcinoma has been considered as favourable in cases in which biliary drainage is performed, since the median survival has been reported to be 3 mo without and 6 mo with biliary drainage[18,26]. Our attempt to identify other factors associated with survival, e.g. gender or additional palliative therapy (i.e. chemotherapy), failed to show any survival advantage for any of them, even if some were shown to be important in previous studies[18].

Despite the benefit in survival afforded by biliary drainage, the enhancement in quality of life with this kind of intervention seemed to be minimal, since the majority of patients had repeat procedures to ensure the patency of the stents. Patency rates of self-expanding metal stents are higher than those of plastic ones[27]. Even if metal stents remain patent longer, a high percentage of our patients needed the replacement of two or more stents in repeat procedures for resolution of jaundice.

The major limitation of our study was that it involved only a single center and the number of patients was small, and thus the results may not be generalizable.

In conclusion, the present study confirms that cholangiocarcinoma is a tumor with high incidence among the elderly with multiple co-morbidities, which precludes aggressive curative resection. Moreover, survival of patients with unresected cholangiocarcinoma

is short and the benefit in survival, but not in quality of life, from endoscopic or percutaneous biliary drainage, although evident, is rather weak. Therefore, early diagnosis and surgical resection of the tumor are crucial in the management of these patients.

COMMENTS

Background

Cholangiocarcinoma is difficult to diagnose and its prognosis is dismal due to late stage at presentation. The incidence and mortality of the disease are rising worldwide.

Research frontiers

The high fatality rate of cholangiocarcinoma is attributed to the poor knowledge of disease pathogenesis and the paucity of effective methods of diagnosis and management. Surgical resection is the only curative option for cholangiocarcinoma, but only a minority of patients are suitable for resection.

Innovations and breakthroughs

This study confirms that cholangiocarcinoma is diagnosed at an advanced stage, with high incidence among the elderly with multiple co-morbidities, which precludes aggressive curative resection. Survival of patients with unresected cholangiocarcinoma is short and the benefit in survival, but not in quality of life, from endoscopic or percutaneous biliary drainage, although evident, is rather weak.

Applications

The study suggested that early diagnosis and surgical resection of the tumor are crucial in the management of these patients.

Peer review

This is a retrospective study of the outcome of cholangiocarcinoma in a group of 34 patients seen at a single hospital, characterized by a homogeneous ethnic population of Greek origin. The aims are not ambitious and the originality of study is not high. However, the study is still relevant due to the severity of the disease and the scarce information available.

REFERENCES

1 **Khan SA**, Taylor-Robinson SD, Toledano MB, Beck A, Elliott P, Thomas HC. Changing international trends in mortality rates for liver, biliary and pancreatic tumours. *J Hepatol* 2002; **37**: 806-813
2 **Patel T**. Increasing incidence and mortality of primary intrahepatic cholangiocarcinoma in the United States. *Hepatology* 2001; **33**: 1353-1357
3 **de Groen PC**, Gores GJ, LaRusso NF, Gunderson LL, Nagorney DM. Biliary tract cancers. *N Engl J Med* 1999; **341**: 1368-1378
4 **Jarnagin WR**, Fong Y, DeMatteo RP, Gonen M, Burke EC, Bodniewicz BS J, Youssef BA M, Klimstra D, Blumgart LH. Staging, resectability, and outcome in 225 patients with hilar cholangiocarcinoma. *Ann Surg* 2001; **234**: 507-517; discussion 517-519
5 **Ebata T**, Nagino M, Kamiya J, Uesaka K, Nagasaka T, Nimura Y. Hepatectomy with portal vein resection for hilar cholangiocarcinoma: audit of 52 consecutive cases. *Ann Surg* 2003; **238**: 720-727
6 **Singh P**, Patel T. Advances in the diagnosis, evaluation and management of cholangiocarcinoma. *Curr Opin Gastroenterol* 2006; **22**: 294-299
7 **Khan SA**, Thomas HC, Davidson BR, Taylor-Robinson SD. Cholangiocarcinoma. *Lancet* 2005; **366**: 1303-1314
8 **Bismuth H**, Nakache R, Diamond T. Management strategies in resection for hilar cholangiocarcinoma. *Ann Surg* 1992; **215**: 31-38
9 **Parker SL**, Tong T, Bolden S, Wingo PA. Cancer statistics, 1996. *CA Cancer J Clin* 1996; **46**: 5-27
10 **Nakeeb A**, Pitt HA, Sohn TA, Coleman J, Abrams RA, Piantadosi S, Hruban RH, Lillemoe KD, Yeo CJ, Cameron JL. Cholangiocarcinoma. A spectrum of intrahepatic, perihilar, and distal tumors. *Ann Surg* 1996; **224**: 463-473; discussion 473-475
11 **Donato F**, Gelatti U, Tagger A, Favret M, Ribero ML, Callea F, Martelli C, Savio A, Trevisi P, Nardi G. Intrahepatic cholangiocarcinoma and hepatitis C and B virus infection, alcohol intake, and hepatolithiasis: a case-control study in Italy. *Cancer Causes Control* 2001; **12**: 959-964
12 **Nakeeb A**, Tran KQ, Black MJ, Erickson BA, Ritch PS, Quebbeman EJ, Wilson SD, Demeure MJ, Rilling WS, Dua KS, Pitt HA. Improved survival in resected biliary malignancies. *Surgery* 2002; **132**: 555-563; discission 563-564
13 **Nagorney DM**, Donohue JH, Farnell MB, Schleck CD, Ilstrup DM. Outcomes after curative resections of cholangiocarcinoma. *Arch Surg* 1993; **128**: 871-877; discussion 877-879
14 **Iwatsuki S**, Todo S, Marsh JW, Madariaga JR, Lee RG, Dvorchik I, Fung JJ, Starzl TE. Treatment of hilar cholangiocarcinoma (Klatskin tumors) with hepatic resection or transplantation. *J Am Coll Surg* 1998; **187**: 358-364
15 **Klempnauer J**, Ridder GJ, Werner M, Weimann A, Pichlmayr R. What constitutes long-term survival after surgery for hilar cholangiocarcinoma? *Cancer* 1997; **79**: 26-34
16 **Madariaga JR**, Iwatsuki S, Todo S, Lee RG, Irish W, Starzl TE. Liver resection for hilar and peripheral cholangiocarcinomas: a study of 62 cases. *Ann Surg* 1998; **227**: 70-79
17 **Lazaridis KN**, Gores GJ. Cholangiocarcinoma. *Gastroenterology* 2005; **128**: 1655-1667
18 **Farley DR**, Weaver AL, Nagorney DM. "Natural history" of unresected cholangiocarcinoma: patient outcome after noncurative intervention. *Mayo Clin Proc* 1995; **70**: 425-429
19 **Weber SM**, Jarnagin WR, Klimstra D, DeMatteo RP, Fong Y, Blumgart LH. Intrahepatic cholangiocarcinoma: resectability, recurrence pattern, and outcomes. *J Am Coll Surg* 2001; **193**: 384-391
20 **Lieser MJ**, Barry MK, Rowland C, Ilstrup DM, Nagorney DM. Surgical management of intrahepatic cholangiocarcinoma: a 31-year experience. *J Hepatobiliary Pancreat Surg* 1998; **5**: 41-47
21 **Rea DJ**, Munoz-Juarez M, Farnell MB, Donohue JH, Que FG, Crownhart B, Larson D, Nagorney DM. Major hepatic resection for hilar cholangiocarcinoma: analysis of 46 patients. *Arch Surg* 2004; **139**: 514-523; discussion 523-525
22 **Burke EC**, Jarnagin WR, Hochwald SN, Pisters PW, Fong Y, Blumgart LH. Hilar Cholangiocarcinoma: patterns of spread, the importance of hepatic resection for curative operation, and a presurgical clinical staging system. *Ann Surg* 1998; **228**: 385-394
23 **Fong Y**, Blumgart LH, Lin E, Fortner JG, Brennan MF. Outcome of treatment for distal bile duct cancer. *Br J Surg* 1996; **83**: 1712-1715
24 **Nordback IH**, Pitt HA, Coleman J, Venbrux AC, Dooley WC, Yeu NN, Cameron JL. Unresectable hilar cholangiocarcinoma: percutaneous versus operative palliation. *Surgery* 1994; **115**: 597-603
25 **Pichlmayr R**, Weimann A, Klempnauer J, Oldhafer KJ, Maschek H, Tusch G, Ringe B. Surgical treatment in proximal bile duct cancer. A single-center experience. *Ann Surg* 1996; **224**: 628-638
26 **Chang WH**, Kortan P, Haber GB. Outcome in patients with bifurcation tumors who undergo unilateral versus bilateral hepatic duct drainage. *Gastrointest Endosc* 1998; **47**: 354-362
27 **Kaassis M**, Boyer J, Dumas R, Ponchon T, Coumaros D, Delcenserie R, Canard JM, Fritsch J, Rey JF, Burtin P. Plastic or metal stents for malignant stricture of the common bile duct? Results of a randomized prospective study. *Gastrointest Endosc* 2003; **57**: 178-182

S- Editor Tian L **L- Editor** Negro F **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6218

World J Gastroenterol 2008 October 28; 14(40): 6218-6223
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Bcl-2 expression significantly correlates with thymidylate synthase expression in colorectal cancer patients

Riyad Bendardaf, Raija Ristämaki, Kari Syrjänen, Seppo Pyrhönen

Riyad Bendardaf, Raija Ristämaki, Kari Syrjänen, Seppo Pyrhönen, Department of Oncology and Radiotherapy, Turku University Central Hospital, Savitehtaankatu 1, PB 52, Turku FIN 20521, Finland
Correspondence to: Dr. Riyad Bendardaf, Department of Oncology and Radiotherapy, Turku University Hospital, Savitehtaankatu 1, PB 52, Turku FIN-20521, Finland. riyben@utu.fi
Telephone: +358-2-3132800 Fax: +358-2-3132809
Received: November 24, 2007 Revised: July 14, 2008
Accepted: July 21, 2008
Published online: October 28, 2008

Department of Surgery, Colorectal Unit, University Hospital, SE 751 85, Uppsala, Sweden; Damian Casadesus, MD, PhD, Calixto Garcia University Hospital, J and University, Vedado, Havana City, Cuba

Bendardaf R, Ristämaki R, Syrjänen K, Pyrhönen S. Bcl-2 expression significantly correlates with thymidylate synthase expression in colorectal cancer patients. *World J Gastroenterol* 2008; 14(40): 6218-6223 Available from: URL: http://www.wjgnet.com/1007-9327/14/6218.asp DOI: http://dx.doi.org/10.3748/wjg.14.6218

Abstract

AIM: To examine the expression of thymidylate synthase (TS) and oncoprotein Bcl-2 in advanced colorectal cancer (CRC) patients, and to determine their mutual relationship, association to therapeutic response and impact on disease outcome.

METHODS: Tumor samples from 67 patients with CRC, who were treated at advanced stage with either irinotecan alone or in combination with 5-fluorouracil/leucovorin, were analyzed for expression of TS and Bcl-2 using immunohistochemistry.

RESULTS: A significant linear correlation between lower expression levels of Bcl-2 and lower levels of TS expression was found (P = 0.033). Patients with high levels of both TS and Bcl-2 expression had a significantly longer disease-free survival (DFS) (42.6 mo *vs* 5.4 mo, n = 25) than those with low TS/Bcl-2 index (P = 0.001). Tumors with low levels of both TS and Bcl-2 were associated with a longer survival with metastasis (WMS) interval in the whole patients group (n = 67, P = 0.035). TS/Bcl-2 index was not significantly related to disease-specific survival.

CONCLUSION: The present data suggest that CRC patients with low TS/Bcl-2 demonstrate a significantly shorter DFS and longer WMS.

Key words: Thymidylate synthase; Bcl-2; Colorectal cancer; Disease-free survival; Survival with metastases

Peer reviewers: Shu Zheng, Professor, Scientific Director of Cancer Institute, Zhejiang University, Secondary Affiliated Hospital, Zhejiang University, 88# Jiefang Road, Hangzhou 310009, Zhejiang Province, China; Lars A Pahlman, Professor,

INTRODUCTION

Colorectal cancer (CRC) is the second most frequent cancer in Europe in 2004, responsible for 13% (376 400) of all incident cancer cases. It is also the second most frequent cause of cancer mortality in Europe, with an annual mortality of 11.9%, 203 700 annual deaths[1]. In the early stages, CRC is often a curable disease, but the overall prognosis is determined by the extent of local and particularly metastatic tumor spread. However, disease outlook is relatively poor for advanced disease and thus is a significant cause of worldwide cancer-related mortality[1]. For locally advanced and metastatic CRC, fluoropyrimidine, 5-fluorouracil (5-FU) has been the standard cytostatic drug for the last 50 years, in recent years used as modulated by leucovorin and in combination with irinotecan or oxaliplatin.

Fluoropyrimidine metabolites form a covalent complex with thymidylate synthase (TS). Formation of this complex prevents biosynthesis of intracellular thymidylate, which is essential for DNA biosynthesis. TS expression has been shown to be an independent prognostic factor in several other cancers. Higher TS levels in hepatic metastases and resection margin are independent predictors of disease progression and survival in patients with metastatic CRC[2]. Comparable results have been reported in other tumors e.g. gastric[3], cervical[4], ovarian, and head and neck cancers, for which TS+ tumors have demonstrated significantly worse outcome as compared to TS-negative tumors.

Increased expression of the proto-oncogene Bcl-2, a 24-kDa intracellular membrane protein that is able to inhibit programmed cell death without affecting cell proliferation, has been reported in gastrointestinal adenocarcinoma and its precursor lesions[5,6]. Bcl-2

has been shown to prolong cell survival by inhibiting apoptosis in several cell types[7,8]. Abnormal activation of the Bcl-2 gene appears to be an early event in colorectal tumorigenesis[6].

In this study, we examined the expression of TS and the oncoprotein Bcl-2 in locally advanced and metastatic CRC and determined their inter-relationships, as well as their impact on patient survival.

MATERIALS AND METHODS

Patients, treatment and follow-up

A series of 67 patients were diagnosed and treated for Stage Ⅱ, Ⅲ, Ⅳ CRC at the Department of Oncology and Radiotherapy, Turku University Hospital (TUH) and six other hospitals in the same hospital district, between January 1996 and August 2003. The key clinical characteristics of the patients are summarized in Table 1.

At the time of diagnosis, 11 patients had stage Ⅱ, 14 had stage Ⅲ and 42 had stage Ⅳ disease. When patients developed metastases or inoperable local recurrence, they were entered into the chemotherapy protocol. In the protocol, patients received one of two treatment regimens; 18 received irinotecan alone and 49 received a combination of irinotecan, 5-FU and folinic acid (FA) as first line treatment for metastatic disease. Irinotecan (350 mg/m^2) was administered as a 60-90 min intravenous (i.v.) infusion every 3 wk. In the combination regimen, irinotecan (180-210 mg/m^2) was administered as 60-90 min intravenous infusion and 5-FU (500 mg/m^2, i.v. bolus) modulated with folinic acid (FA) (60 mg/m^2, i.v. bolus). The 5-FU/FA administrations were repeated again on the following day. The cycle was repeated every 2 wk[9]. The mean duration of chemotherapy was 6.3 mo (SD, 3.4 mo). Treatment was continued until disease progression, or occurrence of unacceptable toxicity.

The patients were prospectively followed-up until the end of March 2007; mean follow-up time from diagnosis was 34.4 mo (± 26.2 mo). We used three endpoints to calculate the patient survival: (1) disease-free survival (DFS), which was calculated in 26 patients with stage Ⅱ or Ⅲ diseaseat diagnosis; (2) overall disease-specific survival (DSS); and (3) survival with metastases (WMS). DFS is the time from diagnosis to the appearance of metastatic disease and relevant only for those patients with radically operated stage Ⅱ and Ⅲ patients at the time of diagnosis (n = 25). DSS is the time from diagnosis to death or to the time point when last seen alive at the clinic, and was calculable for all patients in the study. WMS was calculated from the date of recording the appearance of disease recurrence/metastases at the clinical visit, until to death or to the time point when last seen alive.

The study was approved by the Ethical Committee and was conducted in accordance with the Declaration of Helsinki. Samples were collected with the endorsement of the National Authority for Medico-legal Affairs.

Immunohistochemical detection of TS and Bcl-2 expression

Sixty-seven formalin-fixed, paraffin-embedded primary tumors were obtained from 67 patients. Sections were cut serially at 5 μm for routine hematoxylin and eosin staining and for immunohistochemical analysis. An experienced pathologist confirmed all histological diagnoses.

TS expression was studied immunohistochemically using monoclonal antibody (Mouse Clone TS 106) from Zymed Laboratory. Bcl-2 protein expression was studied using anti-Bcl-2 monoclonal mouse antibody, which recognizes a peptide comprising amino acids 41-54 of the human Bcl-2 protein (Clone 124, DAKO A/S, Glostrup, Denmark). Signal detection was performed using the streptavidin-biotin method (Vectastain ABC kit). Formalin-fixed, paraffin-embedded sections were deparaffinized in xylene, rehydrated in graded alcohol, immersed in 0.01 mol/L citrate buffer (pH 6.0), heated in a domestic microwave oven at full power for 2 × 5 min, and left in the buffer to cool to room temperature. The sections were incubated in 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. Incubation with the primary antibody diluted in 1%

Table 1 Characteristics of the patients and their tumors at diagnosis

Variable	Total patient group n (%)
Patients	67 (100)
Female	24 (36)
Male	43 (64)
Age (yr)	
Mean (SD)	57.6 (24-80)
Tumor localization	
Rectum	14 (20.9)
Left colon	31 (46.3)
Right colon	15 (22.4)
Transverse colon	7 (10.4)
Primary tumor status[1]	
T1	0 (0)
T2	5 (7.5)
T3	48 (71.6)
T4	14 (20.9)
Primary nodal status[1]	
N0	18 (26.9)
N1	35 (52.2)
No data	14 (20.9)
Histological tumor grade	
Grade Ⅰ	10 (14.9)
Grade Ⅱ	45 (67.2)
Grade Ⅲ	12 (17.9)
Stage[1]	
Ⅱ	11 (16.4)
Ⅲ	14 (20.9)
Ⅳ	42 (62.7)
Localization of metastases	
Liver	28 (41.8)
Lung	1 (1.5)
Multiple	34 (50.7)
Local	4 (6.0)
Disease-specific survival (months)	
Mean (SD)	34.4 (26.2)
Disease-specific outcome	
Alive	3 (4.5)
Died of disease	64 (95.5)

[1]TNM classification.

bovine serum albumin/Tris-buffered saline, TS (1:25) and Bcl-2 (1:50), were carried out overnight in a humid chamber at 4℃. The following day, the slides were washed and incubated first with the biotinylated secondary antibody (30 min, 20℃), then with avidin-biotin-peroxidase complex (30 min, 20℃). Positive staining was visualized with 3,3' diaminobenzidine (DAB) substrate solution and the sections were counterstained with Mayer's hematoxylin. As negative controls, slides were processed with the omission of the primary antibody.

Evaluation of TS and Bcl-2 expression

Expression of TS and Bcl-2 was assessed by an observer blinded to the clinical data. The slides were first screened for an overview of the general staining pattern. Four pictures of each slide, covering most of the tumor area, were taken with a light microscope (4 × magnification) connected to a camera and AnalySIS v 3.00 software (Soft Imaging System GmbH, Munster, Germany). Expression of TS in the four pictures was analyzed using Imaging Research Inc., St. Catharine's, Ontario, Canada), which detected the brown color of the positively stained tumor cells and counted the area of those cells in pixels, and also counted in pixels the total tumor area. The percentage of positively stained tumor cells from the whole tumor area was counted and used in further analysis. This method of evaluating TS expression was able to distinguish between the presence of many cells expressing low amounts and a few cells expressing high amounts of TS, such that the percentage of TS expression reflected total TS expression in the tumor, which may be more relevant biologically.

For statistical purposes, expression profiles of each marker were treated as dichotomous variables, where tumors with negative or weak expression of Bcl-2 was one category (reduced expression), and all those with moderate or strong expression were grouped into the second category. For TS, we used median values as cut-off to build up the dichotomous variable of low- and high TS expression. In addition, combined TS-Bcl-2 indices were created, using the dichotomous Bcl-2 variables and TS variables (median cut-offs), resulting in four possible combinations of TS/Bcl-2: low/low (L/L); low/high (L/H); high/low (H/L); and high/high (H/H). Finally, these were converted to a 3-class index as follows: class 1, TS/Bcl-2, L/L; class 2, TS/Bcl-2, L/H or H/L; and class 3, TS/Bcl-2 H/H.

Statistical analysis

Statistical analyses were performed using the SPSS® (SPSS, Inc., Chicago, IL, USA) and STATA (Stata Corp., TX, USA) software packages (SPSS for Windows, version 14.0.1 and STATA/SE 10.1). Frequency tables were analyzed using the χ^2 test, with likelihood ratio (LR) or Fisher's exact test being used to assess the significance of the correlation between categorical variables. Differences in the means of continuous variables were analyzed using non-parametric tests (Mann-Whitney or Kruskal-Wallis tests) for two and multiple independent samples, respectively. ANOVA was only used for deriving the mean values in each stratum. Univariate survival (life-table) analysis for the outcome measure, DSS, DFS and WMS was based on Kaplan-Meier method, and the groups were compared with the log-rank (Mantel-Cox) test. In all tests, $P < 0.05$ was regarded as statistically significant.

RESULTS

Expression of Bcl-2 and staining of TS are shown in Figure 1. Bcl-2 index and TS index (absolute values) were significantly correlated ($r = 0.286$, Spearman rho, $P = 0.019$). Similarly, using the median-cut off values, there was a significant correlation between Bcl-2 and TS expression profile ($P = 0.039$, Spearman rho or $P = 0.037$, χ^2). In pair-wise comparison, individual samples did not significantly deviate in their Bcl-2 and TS expression profile (median cut-off; $P = 0.841$, Wilcoxon signed ranks test), indicating that in individual tumors, co-detection of H/H and L/L of both markers was a frequent occurrence.

The combined TS-Bcl-2 index was evaluated in relation to all clinical variables recorded, including the response to treatment and survival (DSS, DFS and WMS). Interestingly, for the first time, a significant correlation between TS/Bcl- 2 index and DFS was observed. Among stage Ⅱ and Ⅲ disease ($n = 25$), tumors with H/H profile of TS and Bcl-2 (class 3) were associated with substantially longer DFS (42.6 mo) than those with L/L (5.4 mo) or those with H/L or L/H profile (20.7 mo; $P = 0.031$, Kruskal-Wallis), and this was even more evident in life-table analysis ($P = 0.001$, Mantel-Cox; Figure 2A).

There was a close correlation between TS/Bcl-2 expression and WMS; tumors with L/L profile of TS and Bcl-2 (class 1) were associated with a longer WMS (31.9 mo) than those with H/H (25.7 mo) or H/L and L/H (18.9 mo) in the whole series ($n = 67$, $P = 0.076$, Kruskal-Wallis and $P = 0.092$ Mantel-Cox) (Figure 2B). There was no such difference in DSS among the three TS/Bcl-2 expression profiles, however. TS/Bcl-2 expression was not significantly associated with any other clinicopathological variables, including age, sex, TNM status, grade, stage or carcinoembryonic antigen (CEA) levels.

DISCUSSION

The treatment of CRC has become increasingly complex over recent years. With the emergence of new chemotherapy drugs and targeted agents, there has been a major improvement in the prognosis of patients with metastatic CRC. The identification of prognostic and predictive markers is clinically important, because CRC is a heterogeneous disease with various biological and clinical characteristics.

The present study analyzed the combined TS and Bcl-2 expression in CRC, with particular reference to disease outlook. An increase in TS levels has been

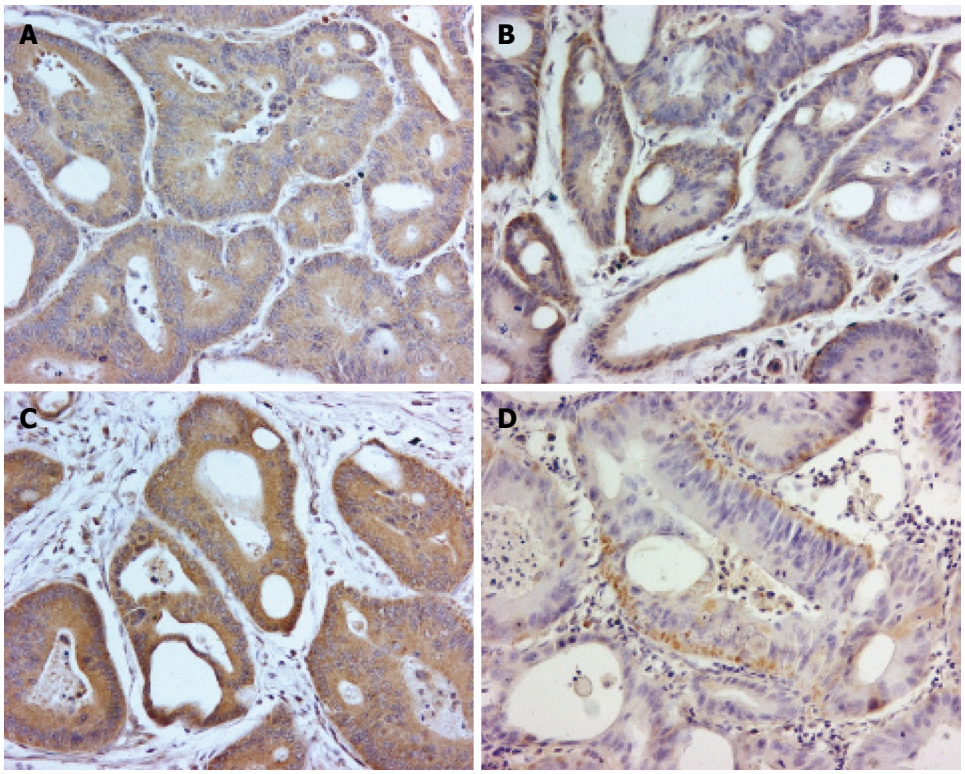


Figure 1 Examples of expression of Bcl-2 and staining of TS. A: High expression of Bcl-2; B: Low expression of Bcl-2; C: High staining of TS expression; D: Low staining of TS expression.

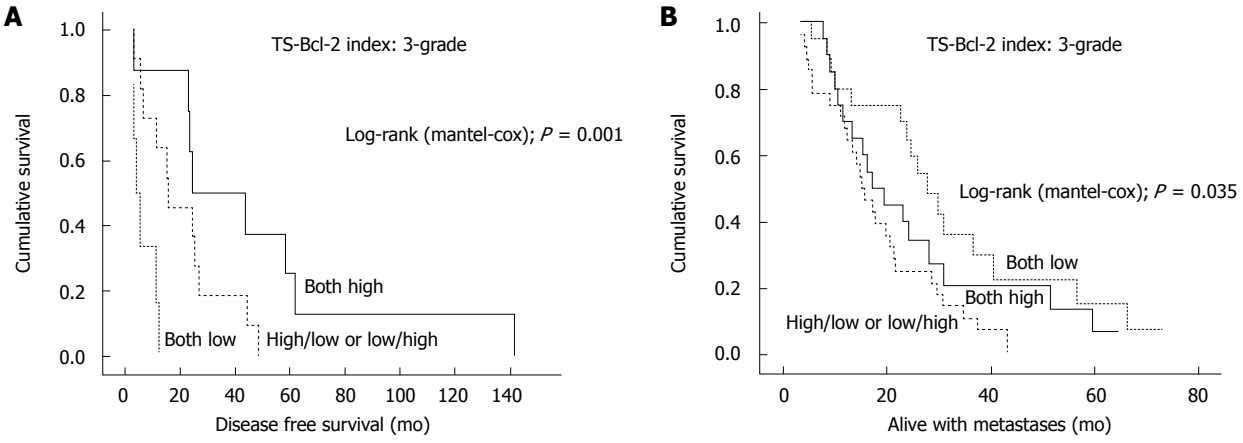


Figure 2 Univariate (Kaplan-Meier) analysis. A:TS/Bcl-2 expression and disease free survival (DFS); B:TS/Bcl-2 expression and survival with metastases (WMS).

suggested to be an important mechanism of resistance to fluoropyrimidine-based chemotherapy[10]. In several studies, TS is reported to be a predictor of both survival in CRC[11-14] and response to 5-FU therapy, with higher levels being associated with poorer prognosis and response to therapy[11,12,15,16]. This suggests that TS levels are not only of importance in predicting the natural course of the disease, but may also predict the sensitivity of cancer cells to 5-FU, which is widely used both in the adjuvant setting and in the treatment of metastases[17,18].

The role of the Bcl-2 family of proteins in chemoresponse has been evaluated extensively in *in vitro* models. Over-expression of Bcl-2 and Bcl-XL has been shown to induce drug resistance[19-21]. In relation to treatment, it has been demonstrated that elevated levels of Bcl-2 protein confers cytotoxic drug resistance to tumor cell lines[7,22]. As Bcl-2 blocks apoptosis *in vitro* and thus contributes to malignant cell accumulation, its over-expression is expected to be associated with more aggressive tumor biology. Indeed, genetic alteration of the Bcl-2 gene located on chromosome 18 is considered to be a key process in the pathogenesis and chemoresistance of human tumors, such as follicular lymphoma[23].

In our previous analysis of Bcl-2 expression in a subset of these tumors (n = 49), we found a weak association of lower levels of Bcl-2 expression with longer overall survival[24]. In another small series (n = 28)[25], we described that lower levels of Bcl-2 expression were significantly associated with lower levels of TS expression. The present study clearly confirmed this observation in a larger series of CRC patients (n = 67), for which a significant linear correlation was established between the two markers (P = 0.039, Spearman). High expression of

TS (above the median) significantly correlated with higher Bcl-2 expression levels. In our previous study, some evidence has suggested that higher levels of TS and Bcl-2 are associated with shorter overall survival[24,25], implicating that elevated levels of TS and Bcl-2 may confer cytotoxic drug resistance among these patients.

To the best of our knowledge, our study shows for the first time the relationship between these two key molecules in a group of patients with locally advanced or metastatic CRC receiving similar treatment. As to the patient survival, there was a significant correlation of TS/Bcl-2 expression with DFS, in that the patients with high TS/Bcl-2 index had a longer DFS (Figure 2A). No such effect was shown for DSS, which was not significantly different among the patients with low- and high TS/Bcl-2 indices.

To conclude, the present data suggest that patients with CRC whose tumors have high TS and Bcl-2 demonstrate a significantly longer DFS and shorter WMS, as compared to patients with low expression of these markers.

COMMENTS

Background

Thymidylate synthase (TS) is reported to be a predictor of both survival in colorectal cancer (CRC) and response to 5-FU therapy, with higher levels being associated with poorer prognosis and response to therapy. Elevated levels of Bcl-2 protein also confer cytotoxic drug resistance to tumor cell lines. We here examined the expression of TS and oncoprotein Bcl-2 in advanced CRC patients, and determined their mutual relationship, association to therapeutic response and impact on disease outcome.

Research frontiers

This study represents a translational study in which a clinical series of CRC samples were analyzed for two important molecular markers: TS and Bcl-2. Accordingly, this study represents a combined molecular analysis and a clinical study, whereby some key molecular pathways were analyzed and their relevance to clinical data was assessed in a series of 67 CRC patients with well-characterized treatment history and long-term follow-up data.

Innovations and breakthroughs

To the best of our knowledge, this is the first study to show the relationship between these two key molecules in a group of patients with locally advanced or metastatic CRC receiving similar treatment. As to the patient survival, there was a significant correlation of TS/Bcl-2 expression with DFS, in that the patients with high TS/Bcl-2 index had a longer DFS.

Applications

The present data suggest that patients with CRC whose tumors have high TS and Bcl-2 demonstrate a significantly longer DFS and shorter WMS, as compared to patients with low expression of these markers. This information should have potential clinical implications in the management of these patients.

Peer review

The importance of this paper to the reader resides in the fact that this study is believed to be the first to suggest that CRC patients with low TS/Bcl-2 demonstrate a significantly shorter DFS, but keep alive longer with a metastatic disease. In relevant cases, one might consider utilizing this type of molecular marker data in more individualized tailoring of the appropriate therapies.

REFERENCES

1 **Boyle P**, Ferlay J. Cancer incidence and mortality in Europe, 2004. *Ann Oncol* 2005; **16**: 481-488

2 **Gonen M**, Hummer A, Zervoudakis A, Sullivan D, Fong Y, Banerjee D, Klimstra D, Cordon-Cardo C, Bertino J, Kemeny N. Thymidylate synthase expression in hepatic tumors is a predictor of survival and progression in patients with resectable metastatic colorectal cancer. *J Clin Oncol* 2003; **21**: 406-412

3 **Suda Y**, Kuwashima Y, Tanaka Y, Uchida K, Akazawa S. Immunohistochemical detection of thymidylate synthase in advanced gastric cancer: a prognostic indicator in patients undergoing gastrectomy followed by adjuvant chemotherapy with 5-fluoropyrimidines. *Anticancer Res* 1999; **19**: 805-810

4 **Suzuki M**, Tsukagoshi S, Saga Y, Ohwada M, Sato I. Enhanced expression of thymidylate synthase may be of prognostic importance in advanced cervical cancer. *Oncology* 1999; **57**: 50-54

5 **Bronner MP**, Culin C, Reed JC, Furth EE. The bcl-2 proto-oncogene and the gastrointestinal epithelial tumor progression model. *Am J Pathol* 1995; **146**: 20-26

6 **Sinicrope FA**, Ruan SB, Cleary KR, Stephens LC, Lee JJ, Levin B. bcl-2 and p53 oncoprotein expression during colorectal tumorigenesis. *Cancer Res* 1995; **55**: 237-241

7 **Hockenbery D**, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 1990; **348**: 334-336

8 **Pidgeon GP**, Barr MP, Harmey JH, Foley DA, Bouchier-Hayes DJ. Vascular endothelial growth factor (VEGF) upregulates BCL-2 and inhibits apoptosis in human and murine mammary adenocarcinoma cells. *Br J Cancer* 2001; **85**: 273-278

9 **Glimelius B**, Ristamaki R, Kjaer M, Pfeiffer P, Skovsgaard T, Tveit KM, Linne T, Frodin JE, Boussard B, Oulid-Aessa D, Pyrhonen S. Irinotecan combined with bolus 5-fluorouracil and folinic acid Nordic schedule as first-line therapy in advanced colorectal cancer. *Ann Oncol* 2002; **13**: 1868-1873

10 **Peters GJ**, van der Wilt CL, van Groeningen CJ, Smid K, Meijer S, Pinedo HM. Thymidylate synthase inhibition after administration of fluorouracil with or without leucovorin in colon cancer patients: implications for treatment with fluorouracil. *J Clin Oncol* 1994; **12**: 2035-2042

11 **Johnston PG**, Fisher ER, Rockette HE, Fisher B, Wolmark N, Drake JC, Chabner BA, Allegra CJ. The role of thymidylate synthase expression in prognosis and outcome of adjuvant chemotherapy in patients with rectal cancer. *J Clin Oncol* 1994; **12**: 2640-2647

12 **Lenz HJ**, Hayashi K, Salonga D, Danenberg KD, Danenberg PV, Metzger R, Banerjee D, Bertino JR, Groshen S, Leichma n LP, Leichman CG. p53 point mutations and thymidylate synthase messenger RNA levels in disseminated colorectal cancer: an analysis of response and survival. *Clin Cancer Res* 1998; **4**: 1243-1250

13 **Yamachika T**, Nakanishi H, Inada K, Tsukamoto T, Kato T, Fukushima M, Inoue M, Tatematsu M. A new prognostic factor for colorectal carcinoma, thymidylate synthase, and its therapeutic significance. *Cancer* 1998; **82**: 70-77

14 **Edler D**, Hallstrom M, Johnston PG, Magnusson I, Ragnhammar P, Blomgren H. Thymidylate synthase expression: an independent prognostic factor for local recurrence, distant metastasis, disease-free and overall survival in rectal cancer. *Clin Cancer Res* 2000; **6**: 1378-1384

15 **Johnston PG**, Lenz HJ, Leichman CG, Danenberg KD, Allegra CJ, Danenberg PV, Leichman L. Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors. *Cancer Res* 1995; **55**: 1407-1412

16 **Leichman L**, Lenz HJ, Leichman CG, Groshen S, Danenberg K, Baranda J, Spears CP, Boswell W, Silberman H, Ortega A. Quantitation of intratumoral thymidylate synthase expression predicts for resistance to protracted infusion of 5-fluorouracil and weekly leucovorin in disseminated colorectal cancers: preliminary report from an ongoing trial. *Eur J Cancer* 1995; **31**A: 1306-1310

17 **Edler D**, Glimelius B, Hallstrom M, Jakobsen A, Johnston PG, Magnusson I, Ragnhammar P, Blomgren H.

Thymidylate synthase expression in colorectal cancer: a prognostic and predictive marker of benefit from adjuvant fluorouracil-based chemotherapy. *J Clin Oncol* 2002; **20**: 1721-1728

18 **Shirota Y**, Stoehlmacher J, Brabender J, Xiong YP, Uetake H, Danenberg KD, Groshen S, Tsao-Wei DD, Danenberg PV, Lenz HJ. ERCC1 and thymidylate synthase mRNA levels predict survival for colorectal cancer patients receiving combination oxaliplatin and fluorouracil chemotherapy. *J Clin Oncol* 2001; **19**: 4298-4304

19 **Miyashita T**, Reed JC. bcl-2 gene transfer increases relative resistance of S49.1 and WEHI7.2 lymphoid cells to cell death and DNA fragmentation induced by glucocorticoids and multiple chemotherapeutic drugs. *Cancer Res* 1992; **52**: 5407-5411

20 **Minn AJ**, Rudin CM, Boise LH, Thompson CB. Expression of bcl-xL can confer a multidrug resistance phenotype. *Blood* 1995; **86**: 1903-1910

21 **Walton MI**, Whysong D, O'Connor PM, Hockenbery D, Korsmeyer SJ, Kohn KW. Constitutive expression of human Bcl-2 modulates nitrogen mustard and camptothecin induced apoptosis. *Cancer Res* 1993; **53**: 1853-1861

22 **Leahy DT**, Mulcahy HE, O'Donoghue DP, Parfrey NA. bcl-2 protein expression is associated with better prognosis in colorectal cancer. *Histopathology* 1999; **35**: 360-367

23 **McDonnell TJ**, Korsmeyer SJ. Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14; 18). *Nature* 1991; **349**: 254-256

24 **Bendardaf R**, Ristamaki R, Kujari H, Laine J, Lamlum H, Collan Y, Pyrhonen S. Apoptotic index and bcl-2 expression as prognostic factors in colorectal carcinoma. *Oncology* 2003; **64**: 435-442

25 **Bendardaf R**, Lamlum H, Elzagheid A, Ristamaki R, Pyrhonen S. Thymidylate synthase expression levels: a prognostic and predictive role in advanced colorectal cancer. *Oncol Rep* 2005; **14**: 657-662

S- Editor Zhong XY **L- Editor** Rippe RA **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6224

World J Gastroenterol 2008 October 28; 14(40): 6224-6227
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Quadruple therapy with furazolidone for retreatment in patients with peptic ulcer disease

Guilherme Eduardo Gonçalves Felga, Fernando Marcuz Silva, Ricardo Correa Barbuti, Tomás Navarro-Rodriguez, Schlioma Zaterka, Jaime Natan Eisig

Guilherme Eduardo Gonçalves Felga, Fernando Marcuz Silva, Ricardo Correa Barbuti, Tomás Navarro-Rodriguez, Schlioma Zaterka, Jaime Natan Eisig, Department of Gastroenterology, University of São Paulo, School of Medicine, São Paulo 054003-000, Brazil
Author contributions: Eisig JN designed this work; Barbuti RC, Navarro-Rodriguez T and Zaterka S made substantial contributions to the conception of the study; Silva FM and Barbuti RC performed the acquisition of data and quality control; Silva FM, Felga GEG and Eisig JN performed the statistical analysis and interpretation of data; Felga GEG and Eisig JN prepared and submitted the manuscript; All authors read and approved the final manuscript.
Correspondence to: Jaime Natan Eisig, MD, PhD, Department of Gastroenterology, University of São Paulo, Rua Dr. Enéas de Carvalho Aguiar, 255-Sala 9159, São Paulo 054003-000, Brazil. eisig@uol.com.br
Telephone: +55-11-30696447 Fax: +55-11-30697830
Received: February 2, 2008 Revised: July 19, 2008
Accepted: July 26, 2008
Published online: October 28, 2008

Abstract

AIM: To establish the efficacy and safety of a 7-d therapeutic regimen using omeprazole, bismuth subcitrate, furazolidone and amoxicillin in patients with peptic ulcer disease who had been previously treated with other therapeutic regimens without success.

METHODS: Open cohort study which included patients with peptic ulcer who had previously been treated unsuccessfully with one or more eradication regimens. The therapeutic regimen consisted of 20 mg omeprazole, 240 mg colloidal bismuth subcitrate, 1000 mg amoxicillin, and 200 mg furazolidone, taken twice a day for 7 d. Patients were considered as eradicated when samples taken from the gastric antrum and corpus 12 wk after the end of treatment were negative for *Helicobacter pylori* (*H pylori*) (rapid urease test and histology). Safety was determined by the presence of adverse effects.

RESULTS: Fifty-one patients were enrolled. The eradication rate was 68.8% (31/45). Adverse effects were reported by 31.4% of the patients, and these were usually considered to be slight or moderate in the majority of the cases. Three patients had to withdraw from the treatment due to the presence of severe adverse effects.

CONCLUSION: The association of bismuth, furazolidone, amoxicillin and a proton-pump inhibitor is a valuable alternative for patients who failed to respond to other eradication regimens. It is an effective, cheap and safe option for salvage therapy of positive patients.

Key words: Gastric ulcer; Duodenal ulcer; *Helicobacter pylori*; Retreatment; Furazolidone

Peer reviewer: Jia-Yu Xu, Professor, Shanghai Second Medical University, Rui Jin Hospital, 197 Rui Jin Er Road, Shanghai 200025, China

Felga GEG, Silva FM, Barbuti RC, Navarro-Rodriguez T, Zaterka S, Eisig JN. Quadruple therapy with furazolidone for retreatment in patients with peptic ulcer disease. *World J Gastroenterol* 2008; 14(40): 6224-6227 Available from: URL: http://www.wjgnet.com/1007-9327/14/6224.asp DOI: http://dx.doi.org/10.3748/wjg.14.6224

INTRODUCTION

Since the discovery of the etiological role of *Helicobacter pylori* (*H pylori*) in peptic ulcer disease[1], its eradication became the main objective of therapy, and several treatment regimens were developed. Currently, triple therapy with omeprazole, amoxicillin, and clarithromycin remains the best therapeutic option[2]. Despite its efficacy, 10% to 20% of the patients present with treatment failure, demanding alternative therapeutic regimes with variable success rates[3,4]. The reasons for this considerable rate of failure are several, including low patient compliance with treatment[5], and bacterial resistance to antimicrobial agents[6,7]. The development of effective salvage treatments is of paramount importance in this situation.

Furazolidone is a synthetic nitrofuran derivative with bactericidal or bacteriostatic activity against Gram-positive and Gram-negative bacteria, and it is well absorbed in the intestine with no tissue accumulation[8]. It has anti-*H pylori* activity and resistant strains appear to be rare or non-existent in many areas[9], characteristics

that make it a potential option for retreatment of peptic ulcer disease caused by *H pylori*. One of the main limitations for its widespread use is the relatively high incidence of significant adverse effects, reported mainly in European studies[10]. In a country such as Brazil, which has large populations with low socioeconomic levels and a high bacterial resistance to metronidazole[11], furazolidone emerges as an interesting option.

This study aimed to establish the efficacy and safety of a 7-d therapeutic regimen for *H pylori* eradication (omeprazole, bismuth subcitrate, furazolidone, and amoxicillin) in patients with peptic ulcer disease who had been previously treated with other therapeutic regimens without success.

MATERIALS AND METHODS

The study was performed in accordance with the declaration of Helsinki, and was approved by the institutional Ethics Review Board for clinical research, and all patients signed an informed written consent form.

Sample size calculation was determined for a descriptive study of a dichotomous variable, considering the prevalence of peptic ulcer with resistant *H pylori* in 2% of the general population.

Patients were selected from the Outpatient Gastroenterology Clinic of Hospital das Clínicas, Faculty of Medicine, University of Sao Paulo. Fifty-one patients with peptic ulcer who had previously been treated unsuccessfully with one or more eradication regimens for *H pylori* were included in the study. Bacterial persistence after treatment was confirmed by positive rapid urease test and histological examination through a modified Giemsa staining method. Gastric mucosa samples were obtained from the antrum and corpus during upper digestive endoscopy.

Patients younger than 18 years of age were excluded, as were those who presented with severe comorbidity, pregnant patients, infants, patients who had previously undergone gastrectomy, patients with a known history of allergy to the therapeutic regimen drugs, and patients who had used non-steroidal anti-inflammatory drugs, antibiotic therapy, or bismuth salts up to 4 wk before study inclusion.

In an open, cohort study, the patients were invited to use a therapeutic regimen for 7 d that consisted of 20 mg omeprazole, 240 mg colloidal bismuth subcitrate, 1000 mg amoxicillin, and 200 mg furazolidone, taken twice a day. Patients were advised not to ingest alcoholic beverages and to avoid foods related to potential side effects determined for drugs similar to monoamine oxidase (MAO) inhibitors. They were also encouraged to take the full medication regularly and were informed about the importance of adequate use of the medication for successful treatment. No other medication was allowed until the end of the treatment, when patients were evaluated regarding compliance by counting the remaining tablets. Adverse effects were recorded in a questionnaire, and each adverse effect was specifically investigated.

Table 1 Clinical data

Clinical data	*n*
Patients	51
Age (yr)	
Mean	49
Median	48
Interval	23-77
Women	32
Duodenal ulcers	39
Gastric ulcers	7
Duodenal and gastric ulcers	5
Tobacco users	16

Treatment efficacy was determined by bacterial negativity at the rapid urease test and histological examination of gastric antrum and corpus mucosa samples taken during digestive endoscopy performed 12 wk after the end of treatment.

A confidence interval of 95% was calculated for the eradication rate percentiles. The χ^2 method with Pearson coefficient was used for the comparison among the variables, eradication rate for previous treatment, gender, and age, with significance value of $P < 0.05$. Statistical analysis was performed with the statistics software, version 10.0 (SPSS Inc., USA).

RESULTS

Among the 51 patients enrolled in the study, there was no predominance regarding gender, and the median age was 48 years. Duodenal ulcer was most commonly observed (Table 1). Five (10%) patients had already undergone three or more previous treatments (32 and 14 had undergone one and two, respectively). Six patients were excluded from the analysis, three for not undergoing follow-up evaluation, and three for early interruption of the treatment due to adverse effects.

The eradication rate was 68.8% (31/45). Eradication rates were similar regardless of the number of previous treatments (one treatment: 21/32, 65.6%; two treatments: 8/14, 57.1%; three or more treatments: 2/5, 40%). Age and gender did not correlate with eradication rates ($P > 0.05$). Adverse effects were reported by 31.4% of the patients, most of which were considered to be slight or moderate. Three patients had to withdraw from the treatment due to the severe adverse effects (one with nausea, one with diarrhea, and one with dizziness).

DISCUSSION

H pylori infection is highly prevalent in Brazil. Among blood donors without gastrointestinal complaints, positive serology for *H pylori* is found in 68%[12]. In a country with more than 200 million inhabitants, we may estimate that 140 millions individuals are currently infected. If we consider a 10% incidence of peptic ulcer disease or gastric cancer, we will find 14 million people in whom eradication of *H pylori* is mandatory. Despite the good eradication rates achieved with the combination

of omeprazole, amoxicillin, and clarithromycin, 10% to 15% of the patients present with treatment failure[2]. In face of this reality, it is interesting to pursue the development of alternative therapeutic regimens with satisfactory eradication rates, low incidence of adverse effects, and low cost.

The efficacy of different regimens varies according to patient compliance and bacterial resistance to the antibiotics[13]. Clarithromycyn, quinolones and metronidazole should not be used more than once, due to *H pylori* intrinsic or induced resistance[14,15]. For patients with primary treatment failure it would be ideal to test the *H pylori* antimicrobial sensitivity, but the high cost and the lack of laboratories capable of adequately performing sensitivity tests limit this strategy. Retreatment must be based on knowledge of the antimicrobial agents previously used.

In our country, *H pylori* strains show an intrinsic resistance to metronidazole that reaches 50%, which decreases the efficacy of schemes containing this drug or other nitroimidazolics[16-19]. Furazolidone is widely available in public health care facilities in Brazil. Despite that no decrease in susceptibility has been observed[20], and bacterial resistance appears to be rare or non-existent in many areas[18,21,22], even among metronidazole-resistant isolates[23]. The main concern regarding its use is the adverse effects observed, especially among individuals who do not adhere to the dietary restrictions recommended, since this drug belongs to the group of the MAO inhibitors. Asian and European studies have reported a relatively high incidence of adverse effects (31.4%-35%)[10,24,25]. Conversely, two South American[26,27] studies have shown fewer undesirable symptoms when compared to the previously mentioned studies. In the present study, despite a comparable incidence of adverse effects during treatment, only three patients had severe symptoms demanding treatment discontinuation. Lower doses of furazolidone could decrease the incidence of undesirable symptoms, but this strategy can also lead to a lower eradication rate[22,28,29].

The prevalence of smokers was similar to that in the Brazilian population and no significant differences in the results were observed when analyzing the number of previous treatments, gender and age. Even though compliance was good, and the length of treatment was short, this regimen ought to be considered only as an alternative for patients with previous treatment failures. Extending the antibiotic course to 10 d or 14 d could improve eradication rates, despite a greater likelihood of adverse effects[30,31].

Furazolidone appears to be an excellent choice for combination therapy for *H pylori* infection, especially as a substitute for metronidazole in quadruple therapy regimens in areas with high prevalence of metronidazole-resistant strains[32]. In our study, the eradication rate was 68.8%, which was superior to that previously reported[33,34], and, considering the efficacy, safety and potential cost-effectiveness, it seems reasonable to introduce furazolidone-based regimens following the failure of initial eradication attempts[32]. The differences regarding the safety profile between South American and American and European studies may be attributed to the limited clinical experience with this drug in the former regions, where it is unavailable and expensive[33].

In conclusion, our study shows that the association of bismuth, furazolidone, amoxicillin and a proton-pump inhibitor is a valuable alternative for patients who failed to respond to an initial therapeutic regime in a country with high prevalence of *H pylori* metronidazole-resistant strains. This scheme is an effective, cheap and safe option for salvage therapy of *H pylori* positive patients.

COMMENTS

Background

Since the discovery of the etiological role of *Helicobacter pylori* (*H pylori*) in peptic ulcer disease, its eradication became essential to allow for adequate healing and prevention of recurrence. It is well established that triple therapy with omeprazole, amoxicillin and clarithromycyn is the first-line treatment, but it fails in 10% to 20% of the patients, demanding alternative therapeutic regimens.

Research frontiers

Furazolidone has anti-*H pylori* activity, low incidence of resistance, is cheap, and is widely available. Despite these characteristics, not many studies have been performed to evaluate its efficacy for retreatment.

Innovations and breakthroughs

This study provides further evidence of the efficacy and tolerability of a short-term furazolidone-based quadruple regimen in South America.

Applications

Furazolidone-based regimens may be an interesting option for retreatment due to their low cost and low resistance rate, especially in developing countries such as Brazil, where metronidazole-resistant strains are common.

Peer review

Quadruple therapy with the medicine used in this study is not a new regimen. It has been used widely in China with good results. Unfortunately, it is not popularly used outside China. The present study was a small series with not very satisfactory results (efficacy rate 68.8%). However, I think it is still worthwhile to be published in our journal, at least indicating that the regime was also used in a South American country with some success.

REFERENCES

1 **Marshall BJ**, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984; **1**: 1311-1315

2 **Malfertheiner P**, Megraud F, O'Morain C, Bazzoli F, El-Omar E, Graham D, Hunt R, Rokkas T, Vakil N, Kuipers EJ. Current concepts in the management of Helicobacter pylori infection: the Maastricht III Consensus Report. *Gut* 2007; **56**: 772-781

3 **Gisbert JP**, Pajares JM. Review article: Helicobacter pylori infection and gastric outlet obstruction - prevalence of the infection and role of antimicrobial treatment. *Aliment Pharmacol Ther* 2002; **16**: 1203-1208

4 **Parente F**, Cucino C, Bianchi Porro G. Treatment options for patients with Helicobacter pylori infection resistant to one or more eradication attempts. *Dig Liver Dis* 2003; **35**: 523-528

5 **Graham DY**, Lew GM, Malaty HM, Evans DG, Evans DJ Jr, Klein PD, Alpert LC, Genta RM. Factors influencing the eradication of Helicobacter pylori with triple therapy. *Gastroenterology* 1992; **102**: 493-496

6 **Houben MH**, van de Beek D, Hensen EF, Craen AJ, Rauws EA, Tytgat GN. A systematic review of Helicobacter pylori eradication therapy--the impact of antimicrobial resistance on eradication rates. *Aliment Pharmacol Ther* 1999; **13**: 1047-1055

7 **Mendonca S**, Ecclissato C, Sartori MS, Godoy AP, Guerzoni RA, Degger M, Pedrazzoli J Jr. Prevalence of Helicobacter

pylori resistance to metronidazole, clarithromycin, amoxicillin, tetracycline, and furazolidone in Brazil. *Helicobacter* 2000; **5**: 79-83
8 **White AH**. Absorption, distribution, metabolism, and excretion of furazolidone. A review of the literature. *Scand J Gastroenterol Suppl* 1989; **169**: 4-10
9 **Fakheri H**, Merat S, Hosseini V, Malekzadeh R. Low-dose furazolidone in triple and quadruple regimens for Helicobacter pylori eradication. *Aliment Pharmacol Ther* 2004; **19**: 89-93
10 **Altamirano A**, Bondani A. Adverse reactions to furazolidone and other drugs. A comparative review. *Scand J Gastroenterol Suppl* 1989; **169**: 70-80
11 **Queiroz DM**, Coimbra RS, Mendes EN, Rocha GA, Alves VM, Oliveira CA, Lima Junior GF. Metronidazole-resistant Helicobacter pylori in a developing country. *Am J Gastroenterol* 1993; **88**: 322-323
12 **Zaterka S**, Eisig JN, Chinzon D, Rothstein W. Factors related to Helicobacter pylori prevalence in an adult population in Brazil. *Helicobacter* 2007; **12**: 82-88
13 **Eisig JN**, Andre SB, Silva FM, Hashimoto C, Moraes-Filho JP, Laudanna AA. The impact of Helicobacter pylori resistance on the efficacy of a short course pantoprazole based triple therapy. *Arq Gastroenterol* 2003; **40**: 55-60
14 **Xia HH**, Talley NJ, Kam EP, Young LJ, Hammer J, Horowitz M. Helicobacter pylori infection is not associated with diabetes mellitus, nor with upper gastrointestinal symptoms in diabetes mellitus. *Am J Gastroenterol* 2001; **96**: 1039-1046
15 **Megraud F**. Basis for the management of drug-resistant Helicobacter pylori infection. *Drugs* 2004; **64**: 1893-1904
16 **Silva FM**, Zaterka S, Eisig JN, Chehter EZ, Chinzon D, Laudanna AA. Factors affecting Helicobacter pylori eradication using a seven-day triple therapy with a proton pump inhibitor, tinidazole and clarithromycin, in Brazilian patients with peptic ulcer. *Rev Hosp Clin Fac Med Sao Paulo* 2001; **56**: 11-16
17 **Queiroz DM**, Dani R, Silva LD, Santos A, Moreira LS, Rocha GA, Correa PR, Reis LF, Nogueira AM, Alvares Cabral MM, Esteves AM, Tanure J. Factors associated with treatment failure of Helicobacter pylori infection in a developing country. *J Clin Gastroenterol* 2002; **35**: 315-320
18 **Godoy AP**, Ribeiro ML, Benvengo YH, Vitiello L, Miranda Mde C, Mendonca S, Pedrazzoli J Jr. Analysis of antimicrobial susceptibility and virulence factors in Helicobacter pylori clinical isolates. *BMC Gastroenterol* 2003; **3**: 20
19 **Zaterka S**, Eisig JN, Chinzon D, Boyd HK, Bastos A, Dias T, Laudanna AA. Five day and ten day triple therapy (amoxicillin, furazolidone and metronidazole) in the treatment of duodenal ulcer. *Rev Hosp Clin Fac Med Sao Paulo* 1996; **51**: 162-165
20 **Kwon DH**, Lee M, Kim JJ, Kim JG, El-Zaatari FA, Osato MS, Graham DY. Furazolidone- and nitrofurantoin-resistant Helicobacter pylori: prevalence and role of genes involved in metronidazole resistance. *Antimicrob Agents Chemother* 2001; **45**: 306-308
21 **Osato MS**, Reddy R, Reddy SG, Penland RL, Malaty HM, Graham DY. Pattern of primary resistance of Helicobacter pylori to metronidazole or clarithromycin in the United States. *Arch Intern Med* 2001; **161**: 1217-1220
22 **Malekzadeh R**, Merat S, Derakhshan MH, Siavoshi F, Yazdanbod A, Mikaeli J, Sotoudemanesh R, Sotoudeh M, Farahvash MJ, Nasseri-Moghaddam S, Pourshams A, Dolatshahi S, Abedi B, Babaei M, Arshi S, Majidpour A. Low Helicobacter pylori eradication rates with 4- and 7-day regimens in an Iranian population. *J Gastroenterol Hepatol* 2003; **18**: 13-17
23 **Vakil N**, Megraud F. Eradication therapy for Helicobacter pylori. *Gastroenterology* 2007; **133**: 985-1001
24 **Fakheri H**, Malekzadeh R, Merat S, Khatibian M, Fazel A, Alizadeh BZ, Massarrat S. Clarithromycin vs. furazolidone in quadruple therapy regimens for the treatment of Helicobacter pylori in a population with a high metronidazole resistance rate. *Aliment Pharmacol Ther* 2001; **15**: 411-416
25 **Isakov V**, Domareva I, Koudryavtseva L, Maev I, Ganskaya Z. Furazolidone-based triple 'rescue therapy' vs. quadruple 'rescue therapy' for the eradication of Helicobacter pylori resistant to metronidazole. *Aliment Pharmacol Ther* 2002; **16**: 1277-1282
26 **Eisig JN**, Silva FM, Rodriguez TN, Hashimoto CL, Barbuti RC. A furazolidone-based quadruple therapy for Helicobacter pylori retreatment in patients with peptic ulcer disease. *Clinics* 2005; **60**: 485-488
27 **Segura AM**, Gutierrez O, Otero W, Angel A, Genta RM, Graham DY. Furazolidone, amoxycillin, bismuth triple therapy for Helicobacter pylori infection. *Aliment Pharmacol Ther* 1997; **11**: 529-532
28 **Roghani HS**, Massarrat S, Shirekhoda M, Butorab Z. Effect of different doses of furazolidone with amoxicillin and omeprazole on eradication of Helicobacter pylori. *J Gastroenterol Hepatol* 2003; **18**: 778-782
29 **Di Mario F**, Cavallaro LG, Scarpignato C. 'Rescue' therapies for the management of Helicobacter pylori infection. *Dig Dis* 2006; **24**: 113-130
30 **Ebrahimi-Dariani N**, Mirmomen S, Mansour-Ghanaei F, Noormohammadpoor P, Sotodehmanesh R, Haghpanah B, Bahrami H. The efficacy of furazolidone-based quadruple therapy for eradication of Helicobacter pylori infection in Iranian patients resistant to metronidazole-based quadruple therapy. *Med Sci Monit* 2003; **9**: PI105-PI108
31 **Malekzadeh R**, Ansari R, Vahedi H, Siavoshi F, Alizadeh BZ, Eshraghian MR, Vakili A, Saghari M, Massarrat S. Furazolidone versus metronidazole in quadruple therapy for eradication of Helicobacter pylori in duodenal ulcer disease. *Aliment Pharmacol Ther* 2000; **14**: 299-303
32 **Graham DY**, Osato MS, Hoffman J, Opekun AR, Anderson SY, El-Zimaity HM. Furazolidone combination therapies for Helicobacter pylori infection in the United States. *Aliment Pharmacol Ther* 2000; **14**: 211-215
33 **Qasim A**, Sebastian S, Thornton O, Dobson M, McLoughlin R, Buckley M, O'Connor H, O'Morain C. Rifabutin- and furazolidone-based Helicobacter pylori eradication therapies after failure of standard first- and second-line eradication attempts in dyspepsia patients. *Aliment Pharmacol Ther* 2005; **21**: 91-96
34 **Wong WM**, Wong BC, Lu H, Gu Q, Yin Y, Wang WH, Fung FM, Lai KC, Xia HH, Xiao SD, Lam SK. One-week omeprazole, furazolidone and amoxicillin rescue therapy after failure of Helicobacter pylori eradication with standard triple therapies. *Aliment Pharmacol Ther* 2002; **16**: 793-798

S- Editor Zhong XY **L- Editor** Anand BS **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6228

World J Gastroenterol 2008 October 28; 14(40): 6228-6236
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Identification and characterization of genotype A and D recombinant hepatitis B virus from Indian chronic HBV isolates

Ranjit Chauhan, Syed Naqui Kazim, Manoj Kumar, Jayashree Bhattacharjee, Narayanasamy Krishnamoorthy, Shiv Kumar Sarin

Ranjit Chauhan, Manoj Kumar, Shiv Kumar Sarin, Department of Gastroenterology and Advanced Center for Liver Diseases, G.B. Pant Hospital, New Delhi 110002, India
Syed Naqui Kazim, Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, New Delhi 110025, India
Ranjit Chauhan, Jayashree Bhattacharjee, Department of Biochemistry, Lady Hardinge Medical College, New Delhi 110002, India
Narayanasamy Krishnamoorthy, The Centre for Genomic Application, New Delhi 110002, India
Author contributions: Chauhan R and Sarin SK designed research; Chauhan R and Kumar M performed research; Krishnamoorthy N contributed reagents/analytic tools; Chauhan R and Kazim SN analyzed data; Chauhan R, Bhattacharjee J and Sarin SK wrote the paper.
Supported by Indian Council of Medical Research-Advanced Center for Liver Diseases Project (ICMR-ACLD)
Correspondence to: Dr. Shiv Kumar Sarin, MD, Director Professor and Head, Department of Gastroenterology, G. B. Pant Hospital, Delhi University Affiliated, Room No. 201, Academic Block, New Delhi 110002, India. sksarin@nda.vsnl.net.in
Telephone: +91-11-23232013 Fax: +91-11-23239710
Received: May 12, 2008 Revised: June 23, 2008
Accepted: June 30, 2008
Published online: October 28, 2008

Abstract

AIM: To confirm the presence of recombination, full-length hepatitis B virus (HBV) from chronic patients was sequenced and analyzed.

METHODS: Full-length HBV genomes from 12 patients were amplified and sequenced in an automated sequencer. Phylogenetic analysis was carried out on full-length, Core and preS2/Surface regions using MEGA software. SimPlot Boot Scanning and amino acid sequence analysis were performed for confirmation of recombination.

RESULTS: Eight patients were infected with genotype D strain; one patient with genotype A and three patients had genotype A and D recombination; two of them had cirrhosis and one had hepatocellular carcinoma. Phylogenetic analysis of core and preS2/surface regions separately showed evidence of genotype A and D recombination. The breakpoints of recombination were found to be at the start of preS2 and at the end of surface coding regions.

CONCLUSION: We identified and characterized recombinant A and D genotype HBV in hepatitis B surface antigen (HBsAg)-positive patients.

Key words: Hepatitis B virus; Genotype; Variation; Evolution; Recombination

Peer reviewer: Vasiliy I Reshetnyak, MD, PhD, Professor, Scientist Secretary of the Scientific Research Institute of General Reanimatology, 25-2, Petrovka str., Moscow 107031, Russia

Chauhan R, Kazim SN, Kumar M, Bhattacharjee J, Krishnamoorthy N, Sarin SK. Identification and characterization of genotype A and D recombinant hepatitis B virus from Indian chronic hepatitis B virus isolates. *World J Gastroenterol* 2008; 14(40): 6228-6236 Available from: URL: http://www.wjgnet.com/1007-9327/14/6228.asp DOI: http://dx.doi.org/10.3748/wjg.14.6228

INTRODUCTION

Hepatitis B virus (HBV) is an organ-specific virus causing inflammation of the liver, leading to complications such as chronic liver disease (CLD) and hepatocellular carcinoma (HCC). As compared to Europe and North America, the prevalence of HBV infection in Asia is quite high, with 40 million people harboring chronic HBV infection in India[1].

Two features make HBV unique. First, its way of replication, by which it uses the pregenomic RNA as an intermediate step for reverse transcription. Second, the efficient utilization of its compact genome for production of seven different proteins from four open reading frames (ORFs). Major proteins that are encoded from these four ORFs are the envelope, core the X protein and the polymerase.

Nucleotide substitution, deletion, insertion and recombination are the main factors that results in variation of the HBV genome. HBV genotypes are classified into eight genotypes, from A to H, based

on the inter-group divergence of 8% or more in the complete genome nucleotide sequence, or a 4% or greater divergence of the Surface gene[2-4]. Recent studies have reported recombination between the HBV genomes of two genotypes. Two kinds of HBV genotype B have emerged[5-7] i.e. recombinant with genotype C and without recombination with C. Mixed genotype refers to an infection that contains more than one genotype in the same patient and is usually the result of multiple exposures and super-infection, the complete genome of each strain belongs to one genotype. According to Robertson *et al*[8], recombination can be detected when different genes or different regions within the same gene are placed by phylogenetic analysis into different sequence subtypes.

We and others from India have reported the presence of mixed genotype A and D[9-12]. However, despite the presence of mixed genotypes, there are no reports from India about the presence of recombination, especially using the full-length HBV genome sequencing approach.

In the present study, we have identified recombinant genotype A and D in patients with CLD and HCC due to chronic HBV infection.

MATERIALS AND METHODS

Patients and serological markers

Twelve treatment-naive chronic HBV infected patients [five with cirrhosis, five with chronic hepatitis B (CHB), and two with HCC] were enrolled. The serum from these patients was tested for the presence of hepatitis B surface antigen (HBsAg) by ELISA (Abbot Laboratories, North Chicago, USA and Organon Tecknika, Boxtel, Netherlands). In addition, the serum was tested for hepatitis e antigen (HBeAg), antibody to hepatitis e antigen (anti-HBe), hepatitis B core Antigen (IgG anti-HBc) by ELISA (Organon Tecknika, Boxtel, Netherlands). Assessment of the severity of liver disease was made by Child-Pugh score[13]. Approval of the institutional ethical committee was obtained to undertake this study.

HBV DNA quantitation

HBV DNA was quantified by a commercially available hybrid capture assay (Ultra sensitive kit, Digene, USA) with the lower limit of detection being 4700 copies/mL.

Full-length HBV DNA amplification

HBV DNA was extracted by using 0.5 to 1.0 mL of patient's plasma using Sera Lysis Buffer (10 mmol/L Tris, 5 mmol/L EDTA, 50 mmol/L NaCl), SDS (1%) and proteinase K (1 mg/mL), followed by extraction with Tris-saturated phenol (pH 7.9) chloroform and then precipitation with ethanol. The obtained pellet was dried and dissolved in 30 μL of 1 × TE buffer (10 mmol/L Tris 1 mmol/L EDTA), a method described previously[12]. Full-length HBV DNA amplification was done by polymerase chain reaction (PCR), as described by Gunther's method[14]. The Taq polymerase with DNA proof reading activity was used. (Expand high fidelity Taq-Polymerase Roche GmBH Basel, Switzerland). Primers were: P1-CCGGAAAGC TTGAGCTCTTC TTTTTCACCTCTGCCTAATCA (1821-1841), P2-CGGAAAGCTTGAGCTCTTCAAAAAGTTGCA TGGTGCTGG (1823-1806). The reaction conditions for PCR were 94℃ for 5 min, 94℃ for 1 min, 60℃ for 1.5 min; 68℃ for 7 min and extension at 72℃ for 10 min, 35 cycles were performed. Purified full-length HBV DNA from recombinant vector pCF 80 (Tetramer of 3.2 kb HBV cloned in pBR322) was used as a positive control. DNA extracted from serum samples of healthy individuals and commercially available molecular biology grade water served as the negative control. Every set of PCR amplifications included HBV-positive and-negative controls. Primers were designed using the software Primer Express.

Table 1 Primers used for sequencing

Name	Sequence	(nt.)
P1_F	5-TTTTTCACCTCTGCCTAATCA-3	(1821-1841)
SEQ_F1	5-AGGCAACTATTGTGGTTTCA-3	(2194-2212)
SEQ_F2	5-TCTTTAACCCTCATTGGAAA-3	(2516-2535)
SEQ_F3	5-TCACCATATTCTTGGGAACAAGA-3	(2823-2845)
SEQ_F4	5-CTTCCTGCTGGTGGCTCCAGTTC-3	(53-75)
SEQ_F5	5-CTCGTGGTGGACTTCTCTC-3	(253-272)
SEQ_F6	5-ATCCTCAACCACCAGCACG-3	(492-510)
SEQ_F7	5-TATTGGGGGCCAAGTCTGTA-3	(749-768)
SEQ_F8	5-TTTACCCCGTTGCYAGGCA-3	(1144-1162)
SEQ_F9	5-CTCATCTGCCGGACCGTG-3	(1562-1581)
P2_R0	5-AAAAAGTTGCATGGTGCTGG-3	(1825-1841)

Sequencing full-length HBV genomes

PCR-amplified products were purified using the Qiagen Gel purification kit according to their recommended protocol. Internal primers used for sequencing given in Table 1 were used for sequencing in an automated DNA sequencer (ABI Prism 3730 Applied Biosystems, Foster City, USA). The nucleotide sequence data reported in this paper appears in the GenBank/EMBL/DDBJ nucleotide sequence databases with accession numbers EF103275-EF103285 and AY945305. The genome length has been measured according to Galibert *et al*[15].

Data analysis

HBV genotyping was done by phylogenetic analysis using full-length sequences, core and preS2 and surface regions. Briefly, sequences were aligned using the CLUSTALW software[16]. Phylogenetic trees were constructed using the Kimura two-parameter matrix and neighbor-joining (NJ) method by MEGA software version 3.1[17]. To confirm the reliability of the phylogenetic tree analysis, bootstrap resampling and reconstruction were carried out 1000 times. Recombination was investigated by SimPlot[18] distributed by the author Ray at (http://www.welch.jhu.edu/). Boot scanning was performed for each of the strains using four sequences at a time[19], i.e. putative recombinant sequence, two consensus sequences of the parental genotype and one consensus sequence as an out-group.

Table 2 Baseline characteristics of patients

Patient No.	1	2	3	4	5	6	7	8	9	10	11	12
Age (yr)	8	24	20	20	30	29	88	38	45	40	62	50
Gender	M	F	M	M	M	M	M	F	M	M	M	M
Diagnosis	CHB	CHB	CHB	CHB	CHB	Cirr.	Cirr.	Cirr.	Cirr.	Cirr.	HCC	HCC
Bilirubin (mg/dL)	1.1	0.68	1.1	0.6	1.1	1	0.8	0.4	2.1	1.4	0.9	1.2
ALT (IU/L)	46	54	42	59	52	107	78	29	101	98	31	43
Albumin (g/dL)	4	4.1	4.3	4.8	4.3	4	2.2	3.9	3.0	3.7	4.1	4
PT prolongation (s)	2	2	1	1	2	4	4	18	8	5	2	2
Ascites	No	No	No	No	No	No	No	No	Yes	No	No	No
Encephalopathy	No	No	No	No	No	No	No	No	No	No	No	No
CTPscore	5	5	5	5	5	6	8	7	9	6	5	5
HBeAg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Neg	Neg	Pos	Pos	Neg
HBV DNA (log copies/mL)	5.4	5.6	6.4	7.2	5.6	6.4	4.9	5.1	5.7	6.1	5.3	6.5
HAI	2	5	6	6	5	-	-	-	-	-	-	-
F Score	1	1	1	3	2	-	-	-	-	-	-	-

Cirr: Cirrhosis; ALT: Alanine aminotransferase; PT: Prothrombin time; CTP: Child-Turcotte-Pugh; HAI; Histological activity index; F: Fibrosis.

RESULTS

Patients and virological characteristics

Baseline characteristics of the study population are given in Table 2. The majority of the patients were male (M: 10, F: 2). Of the 12 patients, five had cirrhosis, all diagnosed radiologically; [one decompensated with a Child-Turcotte-Pugh (CTP) score of 8, and four compensated with a CPT score of 5], and five with CHB (all biopsy proven), and two had HCC. Of the 12, eight were HBeAg-positive and the remaining four were anti-HBe positive. The *Eco*RI restriction enzyme site was present in seven of the full-length sequences, whereas it was absent in five. All sequences had a nucleotide (nt.) length of 3182 except genotype A sequence, which had 3221 nt.

Distribution of genotypes

Phylogenetic analysis using complete HBV genomes of genotypes A to H derived from GenBank revealed the presence of genotype A and D in the study population. Genotype D was predominant, accounting for 92% of the study patients (Figure 1). The nature of genotype D was confirmed by the presence of a 33-bp deletion in the preS1 and a 6-bp deletion in the core terminal regions. Whereas in the genotype A sequence, the 33-bp and 6 bp deletions were absent. Phylogenetic analysis of the core revealed the same results as analysis done with complete HBV genomes as shown in Figure 1A and C.

Presence of A and D genotype recombination

Phylogenetic analysis of preS2/surface region of 12 isolates revealed clustering of three more sequences in addition to isolate 60 in the genotype A branch (Figure 1B). Presence of recombination was confirmed by boot scanning SimPlot analysis; all the sequences were subjected to analysis using the consensus sequence of genotype A, D and H as the out-group as shown in Figure 2. Recombination break points, of three recombinant strains were detected in preS2 and surface ORFs. Isolate 113 had break points at nt 595-618; isolate 105 had break points at nt 639-659 and 723-737. Isolate 103 had break points at nt 319-359 and 1170-1184. PreS2 and surface regions showed similarity with genotype A at 18 amino acid positions in the recombinant sequences, whereas it was absent in the surface, core and X ORFs. Four of them were identified in the preS2, whereas 13 in the surface region, as shown in Figure 3A and B.

Major hydrophilic and the "a" determinant regions

As shown in Figure 3C, when analyzed considering only genotype D, the major hydrophilic region (MHR) showed substitutions at 10 amino acid positions. Of the 10 changes, five spanned the "a" determinant region. When similar analysis was done considering genotype A, we could detect a single mutation in isolate 113 at position 144, changing threonine to methionine in the "a" determinant region of the surface region. All the isolates showed the presence of concomitant threonine to proline change at position 131 of the "a" determinant region, which is homologous to the genotype A sequence.

Sequence characteristics of precore/ core and X ORFs

Among the 12 patients, precore stop codon mutation (W28Stop) was found in two patients, and both belonged to the recombinant genotype. We could document the difference in the core nucleotide sequences in the recombinant sequences; however, they were not exactly similar to the typical genotype A pattern. We detected the presence of T1936C nucleotide mutation in the core gene in one of the HCC patients, isolate number 113.

X ORF: In two patients, we detected mutations in X ORF. Both belonged to the recombinant genotype, i.e. isolate 113 mutations were detected at three positions I127T, K130M, V131I, and isolate 105 was harboring a single mutation at position I127L.

DISCUSSION

Phylogenetic analysis based on two different genomic regions, preS2/surface and core, suggested the existence

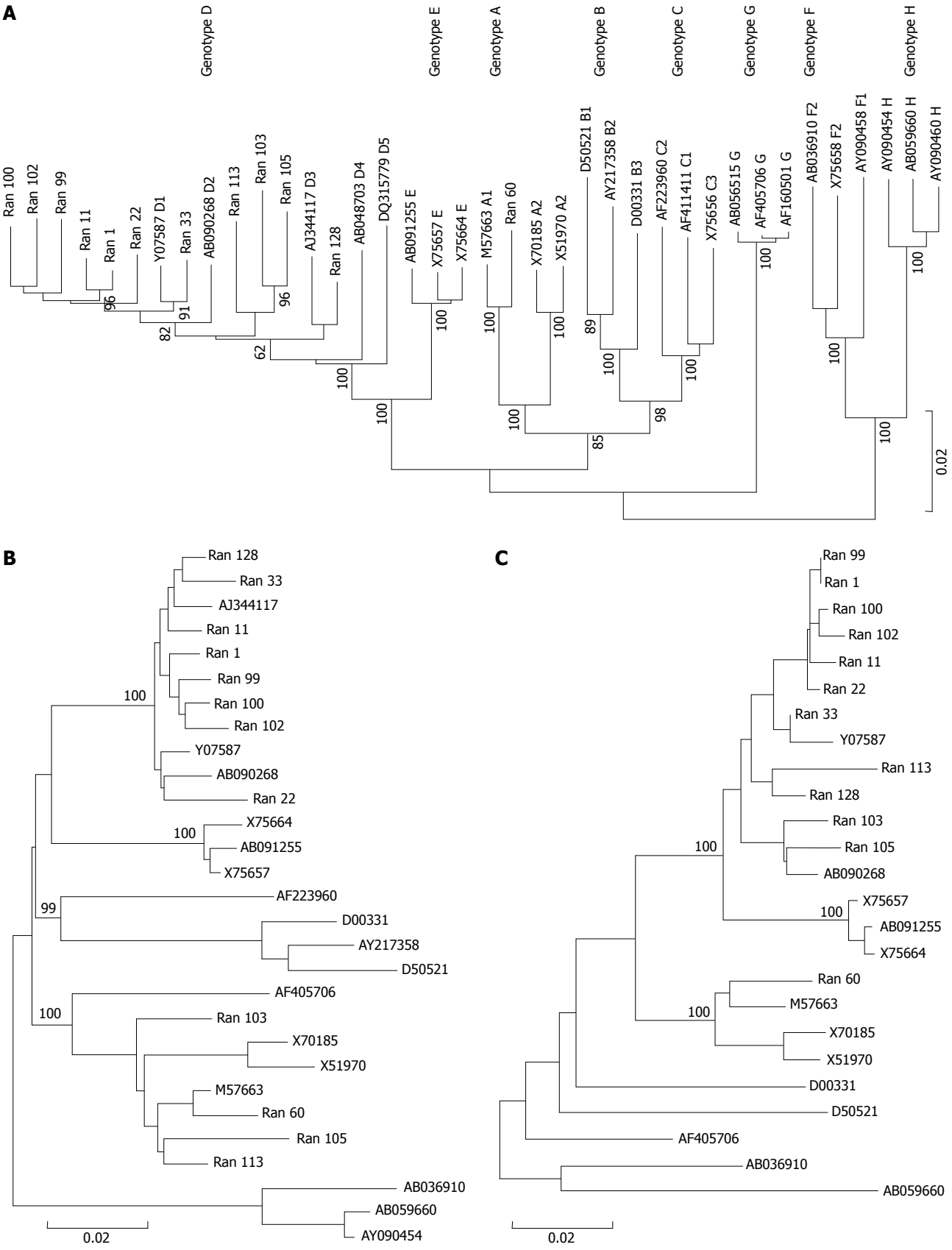


Figure 1 Phylogenetic tree revealed the distribution of genotypes. A: Neighbor joining (NJ) phylogenetic tree was constructed based on the complete genome sequences of 26 HBV reference strains from DDBJ/EMBL/Genbank and 12 Indian HBV isolates in the present study. The bootstrap values obtained from 1000 replicas are shown at the nodes of the main branches. HBV strains from this study are marked as Ran as suffix followed by the isolate number. Reference sequences are denoted by their accession numbers. The capital letter A to H denotes the HBV genotype; B: Phylogenetic analysis done on the PreS2 and surface regions showing three additional (103,105,113) isolates to be grouped in genotype A branch. Phylogenetic trees were constructed by NJ Kimura two-parametric method. Bootstrap values are shown at the nodes of the main branch and at the bottom distance is given; C: Phylogenetic analysis using the Core region. Phylogenetic tree was constructed by NJ Kimura 2-parametric method Bootstrap values are shown at the nodes of the main branch and at the bottom distance given.

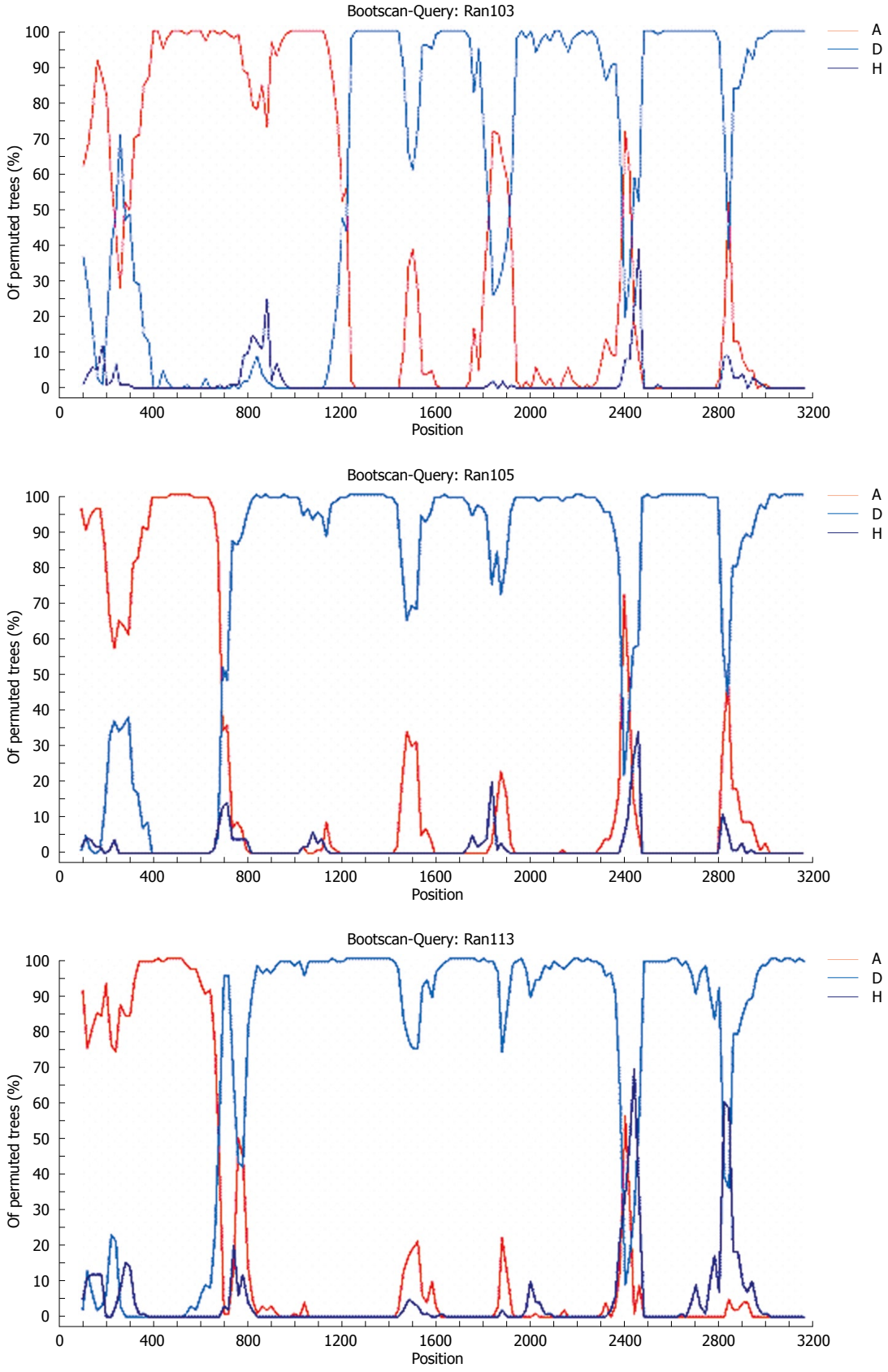


Figure 2 SimPlot analysis demonstrating the recombination in two isolates 103 and105, which were subjected to bootscan analysis over the entire genome using SimPlot program (Lole *et al*[18]).

of recombinant strains in Indian isolates of HBV. On examination of the preS2 and the surface region sequences, a close relation with genotype A sequence was detected in three genotype D sequences respective to core, which was genotype D. Further analysis of corresponding genomes allowed us to map the crossover

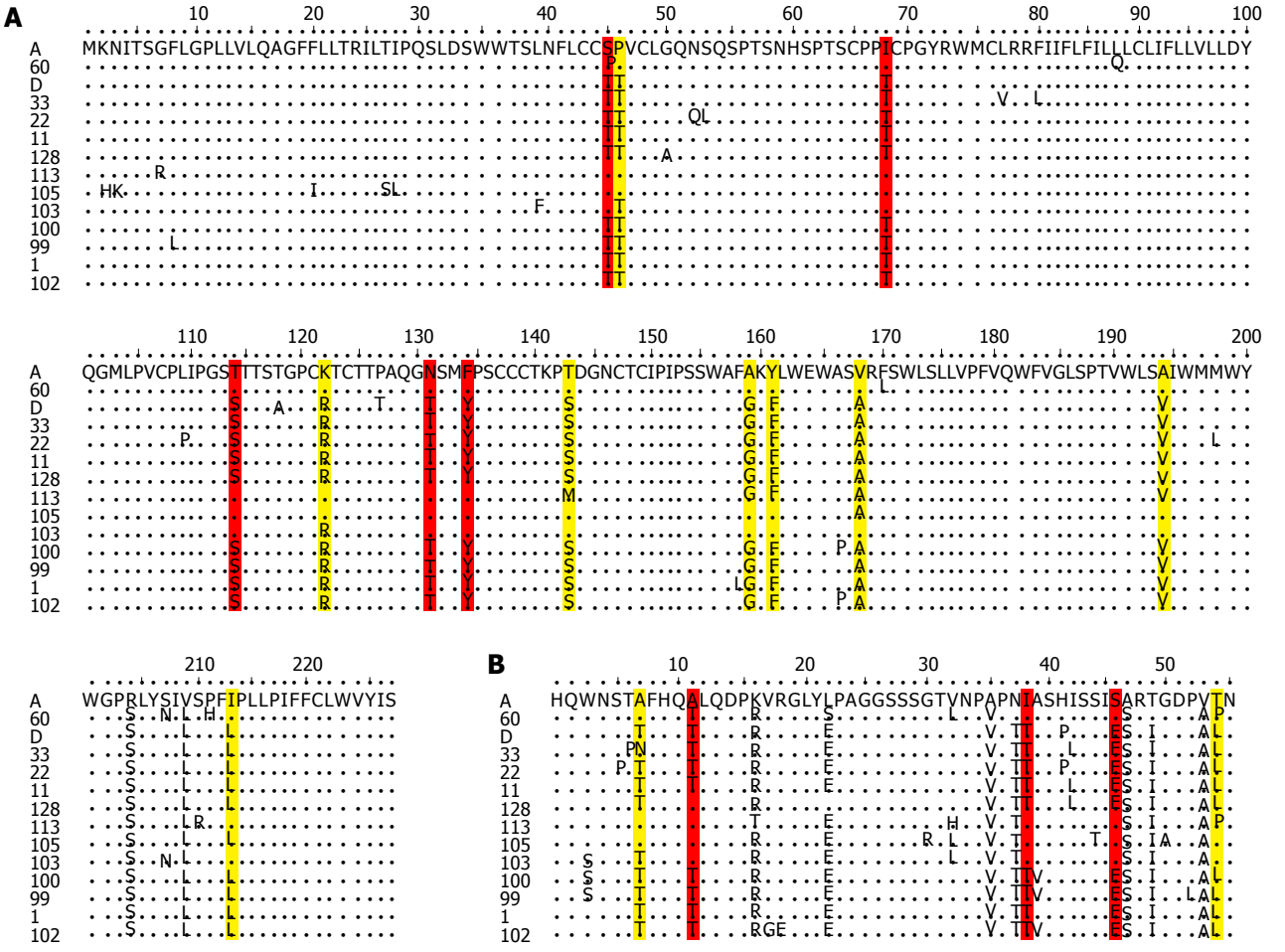


Figure 3 Recombinant sequence similarities with genotype A in PreS2 and Surface region. A: Surface region alignment with genotype A and D amino acids, marked in red shows all three recombinant sequences having similarity with genotype A, whereas yellow marked amino acids shows at least one recombinant sequence similar to genotype A, over all 18 positions; B: Shows same in the preS2 region; C: Shows all the amino acids of MHR of genotype D in black, upward arrow indicates the change of amino acid; D: Shows "a" determinant region and corresponding alignment with 12 isolates.

junctions and to confirm the findings of recombination.

HBV recombination is not a new phenomenon, it is important from an evolutionary as well as epidemiological point of view. As increasing number of full-length HBV genome sequences are reported, and a higher frequency of recombinant hybrid genomes is being recognized. Evidence of HBV recombination from different parts of the world suggests the presence of recombination of HBV genotypes involving A/C, A/D, A/E, B/C, B/D, G/A and G/C strains[20-23]. In Asia, recombination of genotype C/D has been reported from Tibet and China[21,23], whereas recombination of B and C has been detected in Japan[24]. HBV strains from Vietnamese patients also show evidence of

recombination of C and A genotypes[25]. Genotype A and D recombination has only been documented in CHB patients from South Africa[26]. Although the recombination of A and D genotypes has been detected from Italian and Indian HBV strains, such patients were surface-antigen negative[7]. Furthermore, breakpoints of recombination were different from the presently identified recombinant strains[27,28].

A switch in genotype has been documented during change of HBsAg-positive to serologically negative phase[29]. As we detected recombination in the preS2/surface coding region, we focused on the major hydrophilic region, "a" determinant region of surface ORF, speculating that the changes in the MHR and the "a" determinant region could lead to absence of the surface antigen, using standard serological methods. Our analysis revealed changes at 11 amino acid positions when the analysis was done using genotype D (Figure 3C). When the analysis was carried out considering both A and D genotypes, we could detect changes at only a single position substituting (K) lysine with (M) methionine in the "a" determinant region. However, the amino acid substitutions that are supposed to alter the conformation of "a" determinant region were not found.

Compared to genotype D, genotype A is more prevalent in the HBeAg-positive than in the anti-HBe-positive phase[30,31]. It is known that HBV genotype D virus has a selection advantage to form the precore stop codon mutation, as compared to the genotype A virus, the selection being at the pre-genome encapsidation level. However, in the recombinant sequences identified in the present study, we detected the precore stop codon mutation in two of them. The presence of the stop codon in the precore region and co-infection with other genotypes A, C and H are two important features of HBV genotype G[32]. In presently identified recombinant sequences, we detected the presence of Pc G1896A, and this suggests a possible similar situation and a matter for further investigation.

Recent reports suggest the presence of mixed genotypes in Asia. Europe and Africa in CHB patients, including India[9,11,33]. Moreover, higher levels of HBV replication have been shown to be associated with mixed genotype infection[33].

It is quite possible that the PreS, core, X and P proteins are continually expressed but the preS2 region/protein of genotype D is lost for a short interval during recombination with genotype A sequences, mimicking a molecular window period. It is not yet known whether recombination is advantageous for the virus or the host, but it is quite possible that this phenomenon increases the chances of virus survival and doping the host defense system.

One of the reasons for enhanced HCC development in young African adults could be high HBsAg expression in genotype-A-harboring patients[34] HBV genotype A directs the high level of synthesis of HBsAg in proportion to viral DNA, core protein and HBeAg[35].

The frequency of detection of spliced viral genomes is higher in CHB cases compared to acute and resolved HBV infections. The generation of recombinant HBV could be intracellular, as the ratio of full-length and spliced genomes isolated from the intracellular compartment was significantly higher than from extracellular space. This indicates that, compared to those containing spliced genomes, nucleocapsids containing full-length genomes are preferentially enveloped and released from the cell, and could be one of the reasons for severe liver disease[36]. It would be worth while to study the co-infection of two genotypes, and to establish whether the changes accumulate in one cell or together in the newly infected cells. Genetic exchanges between different viral strains within the infected hepatocytes could be one of the possible reasons for recombination.

HBV infection is the predominant factor for the development of HCC in India[37]. Several reports suggest integration of the preS/S region in cancerous liver tissue[38-40]. Binding of the PreS region with fibronectin and transactivation of TGF α could lead to development of cirrhosis and HCC[41-43]. It is quite unique that HBV uses its strongest promoter preS2/S for expression of the host cellular genes, which are advantageous for the virus itself.

HBV recombinant sequences were analyzed with the orangutan and gibbon monkey hepatitis virus. However, we could not find any association of recombinant sequences with them, and the reason for such transmission was clarified (data not shown). Secondly, phylogenetic analysis was done using the Italian and Indian recombinant sequences reported previously[27,28]. However, they were not clustering in the same region as detected in the present recombinant sequences (data not shown).

There are three theories proposed for evolution of HBV: The new world origin theory[5], co-evolution theory[3,44] and co-speciated theory[45,46]. We postulate a competitive selection theory in which the virus and the host cellular machinery compete, and involvement of various unidentified ways by the virus to combat the host defense mechanisms. A few of these could be the splicing, integration, recombination and down-regulation of MHC I . On the other hand, host APOBEC response to edit the viral genome, CTL proteosome complex and various host genetic factors, taking into consideration ethnicity, may play a part as well. Recombinant detection of mixed genotypes, however, may be the tip of the iceberg as a template switch over, splicing and extensive editing by all APOBEC 3 proteins, which have not been well studied.

It can be argued that, in our study, the HBV sequences were analyzed only at one stage of the disease, the process of sequential changes and the time points were not tracked. This was a preliminary study and such studies are quite cumbersome and expensive.

In summary, we identified new A/D recombinants from Indian CLD and HCC patients. To the best of our knowledge, this is the first report which describes recombinant A and D genotype from HBsAg-positive

patients from Asia, and indicates the association of recombinant HBV genotype with HCC. The results of the present study warrants further larger studies to identify populations of recombinant viruses in different clinical categories of HBV patients.

ACKNOWLEDGMENTS

Authors thank Indian Council of Medical Research for carrying out this study and providing funds through Advanced Center for Liver Diseases project.

COMMENTS

Background

Recombination is common in retroviruses, especially human immunodeficiency virus (HIV). As the hepatitis B virus (HBV) uses the reverse transcription step using the pre-genomic RNA, the rate of mutation accumulation is at a much higher rate compared to other DNA viruses. India, being highly populated, harbors the second largest pool of HBV carriers. Recombination is also one of the mechanisms of sequence variability and could account for the non-response to antiviral therapy as well as vaccine. Though recombination from the Indian subcontinent has been detected, authors for the first time report recombination of A and D genotype in HBsAg-positive chronic HBV patients.

Research frontiers

Non-response to antiviral drugs and vaccine is one of the hot research related to the article.

Innovations and breakthroughs

This is believed to be the first report describing the recombinant genotype on the Indian subcontinent.

Applications

Large-scale studies are warranted to determine the prevalence and profile of the recombinant genotype on the Indian subcontinent. The affect of antiviral therapy on the recombinant virus is also warranted.

Terminology

Mixed genotype refers to an infection that contains more than one genotype in the same patient, and is usually the result of multiple exposures and super-infection, the complete genome of each strain belongs to one genotype. Recombinant genotype can be detected when different genes or different regions within the same gene are placed by phylogenetic analysis into different sequence subtypes.

Peer review

This is an interesting paper, which confirms the presence of recombination, and full-length HBV from chronic patients were sequenced and analyzed. Authors identified and characterized recombinant A and D genotype HBV in HBsAg-positive patients.

REFERENCES

1 **Wold Health Organization report.** Available from URL: http://www.who.org
2 **Kramvis A**, Kew M, Francois G. Hepatitis B virus genotypes. *Vaccine* 2005; **23**: 2409-2423
3 **Norder H**, Courouce AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology* 1994; **198**: 489-503
4 **Okamoto H**, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, Mayumi M. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* 1988; **69** (Pt 10): 2575-2583
5 **Bollyky PL**, Rambaut A, Harvey PH, Holmes EC. Recombination between sequences of hepatitis B virus from different genotypes. *J Mol Evol* 1996; **42**: 97-102
6 **Bowyer SM**, Sim JG. Relationships within and between genotypes of hepatitis B virus at points across the genome: footprints of recombination in certain isolates. *J Gen Virol* 2000; **81**: 379-392
7 **Morozov V**, Pisareva M, Groudinin M. Homologous recombination between different genotypes of hepatitis B virus. *Gene* 2000; **260**: 55-65
8 **Robertson DL**, Sharp PM, McCutchan FE, Hahn BH. Recombination in HIV-1. *Nature* 1995; **374**: 124-126
9 **Thakur V**, Guptan RC, Kazim SN, Malhotra V, Sarin SK. Profile, spectrum and significance of HBV genotypes in chronic liver disease patients in the Indian subcontinent. *J Gastroenterol Hepatol* 2002; **17**: 165-170
10 **Vivekanandan P**, Abraham P, Sridharan G, Chandy G, Daniel D, Raghuraman S, Daniel HD, Subramaniam T. Distribution of hepatitis B virus genotypes in blood donors and chronically infected patients in a tertiary care hospital in southern India. *Clin Infect Dis* 2004; **38**: e81-e86
11 **Chattopadhyay S**, Das BC, Kar P. Hepatitis B virus genotypes in chronic liver disease patients from New Delhi, India. *World J Gastroenterol* 2006; **12**: 6702-6706
12 **Chauhan R**, Kazim SN, Bhattacharjee J, Sakhuja P, Sarin SK. Basal core promoter, precore region mutations of HBV and their association with e antigen, genotype, and severity of liver disease in patients with chronic hepatitis B in India. *J Med Virol* 2006; **78**: 1047-1054
13 **Pugh RN**, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. *Br J Surg* 1973; **60**: 646-649
14 **Gunther S**, Li BC, Miska S, Kruger DH, Meisel H, Will H. A novel method for efficient amplification of whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutants in immunosuppressed patients. *J Virol* 1995; **69**: 5437-5444
15 **Galibert F**, Mandart E, Fitoussi F, Tiollais P, Charnay P. Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in E. coli. *Nature* 1979; **281**: 646-650
16 **Thompson JD**, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 1997; **25**: 4876-4882
17 **Kumar S**, Tamura K, Nei M. MEGA: Molecular Evolutionary Genetics Analysis software for microcomputers. *Comput Appl Biosci* 1994; **10**: 189-191
18 **Lole KS**, Bollinger RC, Paranjape RS, Gadkari D, Kulkarni SS, Novak NG, Ingersoll R, Sheppard HW, Ray SC. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. *J Virol* 1999; **73**: 152-160
19 **Salminen MO**, Carr JK, Burke DS, McCutchan FE. Identification of breakpoints in intergenotypic recombinants of HIV type 1 by bootscanning. *AIDS Res Hum Retroviruses* 1995; **11**: 1423-1425
20 **Kurbanov F**, Tanaka Y, Fujiwara K, Sugauchi F, Mbanya D, Zekeng L, Ndembi N, Ngansop C, Kaptue L, Miura T, Ido E, Hayami M, Ichimura H, Mizokami M. A new subtype (subgenotype) Ac (A3) of hepatitis B virus and recombination between genotypes A and E in Cameroon. *J Gen Virol* 2005; **86**: 2047-2056
21 **Cui C**, Shi J, Hui L, Xi H, Zhuoma, Quni, Tsedan, Hu G. The dominant hepatitis B virus genotype identified in Tibet is a C/D hybrid. *J Gen Virol* 2002; **83**: 2773-2777
22 **Suwannakarn K**, Tangkijvanich P, Theamboonlers A, Abe Suwannakarn K, Tangkijvanich P, Theamboonlers A, Abe K, Poovorawan Y. A novel recombinant of Hepatitis B virus genotypes G and C isolated from a Thai patient with hepatocellular carcinoma. *J Gen Virol* 2005; **86**: 3027-3030
23 **Wang Z**, Liu Z, Zeng G, Wen S, Qi Y, Ma S, Naoumov NV, Hou J. A new intertype recombinant between genotypes C and D of hepatitis B virus identified in China. *J Gen Virol* 2005; **86**: 985-990
24 **Sugauchi F**, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, Ishida T, Chutaputti A, Lai CL, Ueda R, Miyakawa Y, Mizokami M. Hepatitis B virus of genotype B with or without recombination with genotype C over the

precore region plus the core gene. *J Virol* 2002; **76**: 5985-5992
25 **Hannoun C**, Norder H, Lindh M. An aberrant genotype revealed in recombinant hepatitis B virus strains from Vietnam. *J Gen Virol* 2000; **81**: 2267-2272
26 **Owiredu WK**, Kramvis A, Kew MC. Hepatitis B virus DNA in serum of healthy black African adults positive for hepatitis B surface antibody alone: possible association with recombination between genotypes A and D. *J Med Virol* 2001; **64**: 441-454
27 **Simmonds P**, Midgley S. Recombination in the genesis and evolution of hepatitis B virus genotypes. *J Virol* 2005; **79**: 15467-15476
28 **Yang J**, Xing K, Deng R, Wang J, Wang X. Identification of Hepatitis B virus putative intergenotype recombinants by using fragment typing. *J Gen Virol* 2006; **87**: 2203-2215
29 **Bahn A**, Gerner P, Martine U, Bortolotti F, Wirth S. Detection of different viral strains of hepatitis B virus in chronically infected children after seroconversion from HBsAg to anti-HBs indicating viral persistence. *J Hepatol* 1997; **27**: 973-978
30 **Li JS**, Tong SP, Wen YM, Vitvitski L, Zhang Q, Trepo C. Hepatitis B virus genotype A rarely circulates as an HBe-minus mutant: possible contribution of a single nucleotide in the precore region. *J Virol* 1993; **67**: 5402-5410
31 **Mangia A**, Chung YH, Hoofnagle JH, Birkenmeyer L, Mushahwar I, Di Bisceglie AM. Pathogenesis of chronic liver disease in patients with chronic hepatitis B virus infection without serum HBeAg. *Dig Dis Sci* 1996; **41**: 2447-2452
32 **Kato H**, Orito E, Gish RG, Bzowej N, Newsom M, Sugauchi F, Suzuki S, Ueda R, Miyakawa Y, Mizokami M. Hepatitis B e antigen in sera from individuals infected with hepatitis B virus of genotype G. *Hepatology* 2002; **35**: 922-929
33 **Toan NL**, Song le H, Kremsner PG, Duy DN, Binh VQ, Koeberlein B, Kaiser S, Kandolf R, Torresi J, Bock CT. Impact of the hepatitis B virus genotype and genotype mixtures on the course of liver disease in Vietnam. *Hepatology* 2006; **43**: 1375-1384
34 **Kew MC**, Kramvis A, Yu MC, Arakawa K, Hodkinson J. Increased hepatocarcinogenic potential of hepatitis B virus genotype A in Bantu-speaking sub-saharan Africans. *J Med Virol* 2005; **75**: 513-521
35 **Sugiyama M**, Tanaka Y, Kato T, Orito E, Ito K, Acharya SK, Gish RG, Kramvis A, Shimada T, Izumi N, Kaito M, Miyakawa Y, Mizokami M. Influence of hepatitis B virus genotypes on the intra- and extracellular expression of viral DNA and antigens. *Hepatology* 2006; **44**: 915-924
36 **Sommer G**, van Bommel F, Will H. Genotype-specific synthesis and secretion of spliced hepatitis B virus genomes in hepatoma cells. *Virology* 2000; **271**: 371-381
37 **Sarin SK**, Thakur V, Guptan RC, Saigal S, Malhotra V, Thyagarajan SP, Das BC. Profile of hepatocellular carcinoma in India: an insight into the possible etiologic associations. *J Gastroenterol Hepatol* 2001; **16**: 666-673
38 **Caselmann WH**, Meyer M, Kekule AS, Lauer U, Hofschneider PH, Koshy R. A trans-activator function is generated by integration of hepatitis B virus preS/S sequences in human hepatocellular carcinoma DNA. *Proc Natl Acad Sci USA* 1990; **87**: 2970-2974
39 **Pineau P**, Marchio A, Mattei MG, Kim WH, Youn JK, Tiollais P, Dejean A. Extensive analysis of duplicated-inverted hepatitis B virus integrations in human hepatocellular carcinoma. *J Gen Virol* 1998; **79** (Pt 3): 591-600
40 **Zhong S**, Chan JY, Yeo W, Tam JS, Johnson PJ. Frequent integration of precore/core mutants of hepatitis B virus in human hepatocellular carcinoma tissues. *J Viral Hepat* 2000; **7**: 115-123
41 **Budkowska A**, Bedossa P, Groh F, Louise A, Pillot J. Fibronectin of human liver sinusoids binds hepatitis B virus: identification by an anti-idiotypic antibody bearing the internal image of the pre-S2 domain. *J Virol* 1995; **69**: 840-848
42 **Ono M**, Morisawa K, Nie J, Ota K, Taniguchi T, Saibara T, Onishi S. Transactivation of transforming growth factor alpha gene by hepatitis B virus preS1. *Cancer Res* 1998; **58**: 1813-1816
43 **Yang J**, Ding X, Zhang Y, Bo X, Zhang M, Wang S. Fibronectin is essential for hepatitis B virus propagation in vitro: may be a potential cellular target? *Biochem Biophys Res Commun* 2006; **344**: 757-764
44 **Magnius LO**, Norder H. Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. *Intervirology* 1995; **38**: 24-34
45 **MacDonald DM**, Holmes EC, Lewis JC, Simmonds P. Detection of hepatitis B virus infection in wild-born chimpanzees (Pan troglodytes verus): phylogenetic relationships with human and other primate genotypes. *J Virol* 2000; **74**: 4253-4257
46 **Simmonds P**. The origin and evolution of hepatitis viruses in humans. *J Gen Virol* 2001; **82**: 693-712

S- Editor Li DL **L- Editor** Alpini GD **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6237

World J Gastroenterol 2008 October 28; 14(40): 6237-6243
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Effect of a fermented milk containing *Bifidobacterium lactis* DN-173010 on Chinese constipated women

Yue-Xin Yang, Mei He, Gang Hu, Jie Wei, Philippe Pages, Xian-Hua Yang, Sophie Bourdu-Naturel

Yue-Xin Yang, Mei He, Institute of Nutrition and Food Safety, China CDC, No. 29, Nanwei Road, Xuanwu District, Beijing 100050, China
Gang Hu, Jie Wei, Beijing Hospital, No. 1 Dahua Avenue, Dongdan, East District, Beijing 100730, China
Philippe Pages, Xian-Hua Yang, Danone Dairy Shanghai R&D Center, RM102, Building 12, 1387 Zhangdong Rd, Shanghai 201203, China
Sophie Bourdu-Naturel, Danone Research, RD128, 91767 Palaiseau, France
Author contributions: Yang YX designed the study; He M analyzed the data and wrote the paper; Wei J and Hu G performed the study; Pages P contributed to the study design; Yang XH coordinated the study and contributed paper writing; Bourdu-Naturel S contributed paper writing and data analysis.
Supported by Danone (Shanghai) Consulting Co. Ltd; 115 national science and technology program
Correspondence to: Sophie Bourdu-Naturel, Danone Research, RD128, 91767 Palaiseau, France. sophie.bourdu-naturel@danone.com
Telephone: +33-169-357249 Fax: +33-169-357696
Received: July 4, 2008 Revised: September 25, 2008
Accepted: October 2, 2008
Published online: October 28, 2008

Abstract

AIM: To investigate the effect of a fermented milk containing *Bifidobacterium lactis* DN-173010 and yogurt strains (BIO®) on adult women with constipation in Beijing.

METHODS: A total of 135 adult females with constipation were randomly allocated to consume for 2 wk either 100 g of the test fermented milk or 100 g of an acidified milk containing non-living bacteria (control). Stool frequency, defecation condition scores, stool consistency and food intake were recorded at baseline and after 1 and 2 wk in an intention-to-treat population of 126 subjects. In parallel, safety evaluation parameters were performed.

RESULTS: At baseline, no differences were found between groups. Following consumption of test product, stool frequency was significantly increased after 1 wk (3.5 ± 1.5 *vs* 2.4 ± 0.6, $P < 0.01$) and 2 wk (4.1 ± 1.7 *vs* 2.4 ± 0.6, $P < 0.01$), *vs* baseline. Similarly, after 1 and 2 wk, of test product consumption, defecation condition (1.1 ± 0.9 *vs* 1.9 ± 1.2, $P < 0.01$ and 0.8 ± 1.0 *vs* 1.9 ± 1.2, $P < 0.01$, respectively) and stool consistency (1.0 ± 0.8 *vs* 1.5 ± 1.1, $P < 0.01$ and 0.6 ± 0.8 *vs* 1.5 ± 1.1, $P < 0.01$, respectively) were significantly improved. Compared with the control group, stool frequency was also significantly increased (3.5 ± 1.5 *vs* 2.5 ± 0.9, $P < 0.01$ and 4.1 ± 1.7 *vs* 2.6 ± 1.0, $P < 0.01$, respectively), and defecation condition (1.1 ± 0.9 *vs* 1.6 ± 1.1, $P < 0.01$ and 0.8 ± 1.0 *vs* 1.6 ± 1.1, $P < 0.01$, respectively) and stool consistency (1.0 ± 0.8 *vs* 1.4 ± 1.0, $P < 0.05$ and 0.6 ± 0.8 *vs* 1.3 ± 1.0, $P < 0.01$, respectively) significantly decreased after 1 and 2 wk of product consumption. During the same period, food intake did not change between the two groups, and safety parameters of the subjects were within normal ranges.

CONCLUSION: This study suggests a beneficial effect of a fermented milk containing *B. lactis* DN-173010 on stool frequency, defecation condition and stool consistency in adult women with constipation constipated women after 1 and 2 wk of consumption.

Key words: Probiotic; *Bifidobacterium lactis* DN-173010; Fermented milk; Constipation; Stool frequency; Stool consistency

Peer reviewer: Beth A McCormick, Associate Professor, Department of Pediatric Gastroenterology and Nutrition, Massachusetts General Hospital, Harvard Medical School, 114, 16th Street (114-3503) Charlestown, MA 02129, United States

Yang YX, He M, Hu G, Wei J, Pages P, Yang XH, Bourdu-Naturel S. Effect of a fermented milk containing *Bifidobacterium lactis* DN-173010 on Chinese constipated women. *World J Gastroenterol* 2008; 14(40): 6237-6243 Available from: URL: http://www.wjgnet.com/1007-9327/14/6237.asp DOI: http://dx.doi.org/10.3748/wjg.14.6237

INTRODUCTION

In the Chinese National Product Standard of GB2746-1999, fermented milk is a product prepared with cow's milk or milk powder as raw material by degreasing, partly degreasing or non-degreasing and fermentation. Bacteria used to ferment milk, typically lactic acid bacteria (LAB), are bacteria that can produce lactic acid during the metabolism process. At present, common LAB used as fermentation agents include: *Streptococcus thermophilus*, *Lactobacillus delbruekii sp bulgaricus*,

Lactobacillus acidophilus, and *Lactobacillus casei*. The health effects of yogurt validated by research include: regulation of intestinal and colon flora, prevention or treatment of diarrhea, regulation of immune function, decreasing symptoms of inflammatory bowel diseases, improvement of lactose intolerance, lowering blood cholesterol level, and prevention of certain types of cancers[1,2]. These effects are a result of the yogurt characteristics due to the fermentation by yogurt symbiosis[3].

Constipation is a common problem, and generally refers to less than 2-3 stools per week, accompanied by small, dry, and/or hard defecation and discharge difficulty. This trouble is commonly reported in many regions of the world including Asia[4] North America[5], and Europe[6], Functional constipation is caused by non-organic or drug factors. Constipation can result in some discomforts such as abdominal distension, abdominal pain, headache, dizziness and poor appetite[7]. The symptoms of constipation can interfere with quality of life. An epidemiological study conducted in Beijing concluded that 6.1% of the adult population was suffering from the symptoms of functional constipation[8]. The disorder is more common in women and elderly people[9]. For example, in Beijing, the incidence of constipation in males and females is 1/4.59. High risk factors include anxiety, work fatigue, bad mood and working at a sedentary job[10].

Probiotics are defined as live micro organisms that, when administered in adequate amounts confer a health benefit on the host[11]. *Bifidobacterium lactis* DN-173010 survives complete transit through the digestive tract and is recovered live in stools in large quantities relative to the quantity initially ingested[12-14]. Three separate clinical studies have demonstrated that daily consumption of fermented milk containing *B. lactis* DN-173010 in association with the yogurt starters *L. bulgaricus* and *S. thermophilus* for 15 d improves gastrointestinal transit. This effect is enhanced with increased daily intake (effect of three pots/d > two pots/d > 1 pot/day) in elderly subjects free of any gastrointestinal pathology[15,16], and in a group of healthy women aged 18-45 years[17]. An exploratory study was designed to examine the effect of this product, compared to heat-treated yogurt, on quality of life and symptoms in irritable bowel syndrome (IBS)[18]. This large scale (267 IBS constipation predominant, Rome II criteria), double-blind, randomized, controlled, parallel group study over 6 wk demonstrated that daily consumption of this product alleviates bloating and improves digestive comfort as assessed by the Functional Digestive Disorders Quality of Life questionnaire. An increase in stool frequency was also observed in subjects with the lowest stool frequency (< 3 or 4 bowel movements/wk) without any change of stool consistency. A fifth study performed on fermented milk containing *B. lactis* DN-173010 alone also demonstrated a reduced transit time in healthy men and women[19]. The results of these five studies support a strong link between improved stool frequency and the strain *B. lactis* DN-173010, and indicate that further research should be carried out to investigate the potential use of this fermented milk product for improving stool parameters in subjects with constipation.

The aim of this study was to investigate the effect of a fermented milk (Bio®) combining *B. lactis* DN-173010 and yogurt strains on functional constipation parameters of adult women in Beijing.

MATERIALS AND METHODS

Study population

A total of 135 women, age 25-65 years old, were recruited in Beijing Hospital. They had a diagnosis of constipation according to the following criteria: less than three stools per week; increased stool hardness; non-organic constipation and habitual constipation.

The following were excluded from the study: Those unable to orally ingest or unable to administer according to instruction; those unable to express complaint clearly; those with constipation symptoms caused by surgical operation within 30 d; those with recent defecation difficulty due to severe organic diseases (colon carcinoma, severe enteritis, intestinal obstruction, inflammatory bowel disease); those with defecation difficulty accompanied by pain; those with acute gastrointestinal tract disease developing within 30 d; those who were pregnant or menstruating; with severe whole body diseases such as cardiovascular, liver, kidney and hematopoietic system; those taking short-term products which may have influenced the results.

Study protocol

One week before product consumption (baseline period), general conditions, safety evaluation parameters, dietary intake and defecation functional parameters (stool frequency, defecation condition scores, stool consistency) were recorded. Thereafter, eligible constipated women were randomly allocated to consume, daily for 2 wk, one pot of either the test product (67 cases) or the control product (68 cases). Subjects were stratified by age, daily eating habits, and constipation causes to the extent possible to ensure inter-group comparability, which could possibly have influenced the results. During the study period, subjects maintained their usual lifestyle and eating habits. The study was approved by the Ethical Review Committee of the Chinese Academy of Preventive Medicine, Institute of Nutrition and Food Hygiene. All subjects provided written informed consent before inclusion in the study.

Study products

The test product was a fermented milk (BIO®, Danone (Shanghai) Consulting Co., Ltd, Shanghai, China), containing *B. lactis* DN-173010 [1.25×10^{10} colony forming unit (cfu) per pot], together with the two classical yogurt ferments, *S. thermophilus* and *L. bulgaricus* (1.2×10^9 cfu/pot).

The control product was an acidified milk without any ferments or probiotics. Both the test and control products were without flavor, and had similar appearance,

color, texture, taste and lactose concentration level. Each serving, corresponding to one pot, contained 100 g. Both products were specially prepared for the study and provided by Danone (Shanghai) Consultation Corp., Ltd.

Assessments and study criteria

The general conditions, such as the mental status, sleep, eating habit, and blood pressure, were evaluated 1 wk before product consumption.

The safety evaluation parameters blood, urine, and stool routine examinations [red blood cell (RBC) count, white blood cells (WBC) count, hemoglobin (Hb) in blood; RBC, WBC, protein in urine; stool properties, RBC and worm ova in feces], liver and kidney function examinations [glutamate pyruvate transaminase (GPT), glutamate oxalate transaminase (GOT), urea nitrogen, creatine, and blood sugar)] were examined once 1 wk before and 1 and 2 wk after product consumption.

Chest X-ray, electrocardiography and abdominal B-ultrasound examinations were done 1 wk before product consumption.

Dietary intake was assessed after 1-2 d, 7-8 d, and 13-14 d of product consumption by food record method for 48 h, to monitor eating habits.

Defecation functional parameters: stool frequency, defecation condition scores and stools were recorded at baseline and 1 and 2 wk after product consumption.

According to extent of defecation difficulty, the defecation condition scores were categorized into four grades[20]: Grade Ⅰ (0 points): Normal defecation; grade Ⅱ (1 point): Only bearing down and discomfortable sensation. grade Ⅲ (2 points): Obvious bearing down and discomfortable sensation, or frequent defecation with difficult and little defecation, seldom abdominal pain or anal burning sensation; grade Ⅳ (3 points): Often abdominal pain or anal burning sensation to influence defecation. According to classification method of Bristol, stool consistency was classified into three grades[20]: Grade Ⅰ (0 points): Like sausage or snake, smooth and soft; like sausage, with fissure on the surface; grade Ⅱ (1 point): Sausage-shaped, with lumps; noncohesive lumps, with coarse edges; grade Ⅲ (2 points): Separating hard lumps, like fruit kernel (difficult discharge).

Statistical analysis

All analysis were conducted on the intention-to-treat (ITT) population, corresponding to subjects having consumed at least one pot of product.

Descriptive statistics were reported as mean ± SD or frequency for all variables, unless otherwise stated. Statistical comparative analysis between two groups was performed by *t* test, χ^2 test and the sum of rank, by SPSS statistical software.

RESULTS

General information of the subjects

Female volunteers (n = 135) were recruited for the study; four cases in test group and five cases in control group were withdrawn from the study. The general conditions of the other 126 subjects in two groups are described in Table 1. A *t* test was used for age and weight, χ^2 test for eating pattern and amount, and non-parametric rank sum tests for the three defecation parameters, to compare difference between the two groups. There were no significant differences ($P > 0.05$).

Table 1 General basic information of subjects before product consumption

Parameters		Control group (n = 63)	Test group (n = 63)	P value
Eating pattern	Regular	59	56	0.344
	Irregular	4	7	
Appetite	Good	17	21	0.437
	Common	46	42	
	Poor	0	0	
Eating amount	Large	3	4	0.927
	Common	52	51	
	Small	8	8	
Age (yr)		46.4 ± 6.7	46.4 ± 9.8	0.992
Body weight (kg)		62.5 ± 10.4	61.2 ± 9.6	0.478
Stool frequency (n/wk)		2.4 ± 0.6	2.4 ± 0.6	0.746
Defecation condition score		1.9 ± 1.2	1.9 ± 1.2	0.914
Stool consistency		1.6 ± 1.1	1.5 ± 1.1	0.408

Table 2 Stool frequency (n/wk, mean ± SD)

	n	Baseline	Week 1	Week 2
Control group	63	2.4 ± 0.6	2.5 ± 0.9	2.6 ± 1.0[a]
Test group	63	2.4 ± 0.6	3.5 ± 1.5[b,c]	4.1 ± 1.7[b,c]

Note: Self comparison between before and after product consumption, [b]$P < 0.01$, [a]$P < 0.05$; compared with control group, [c]$P < 0.01$.

Results of blood, urine and stool routine assays, and liver and kidney function examinations were all in normal range before and after product consumption, and there were no clinical changes between the two groups. The chest X-ray, electrocardiogram and abdominal B-ultrasound examinations indicated that the subjects were healthy.

Stool frequency of the subjects

The stool frequency at baseline and after 1 and 2 wk of product consumption is shown in Table 2. Non-parametric rank sum tests were used to compare the difference between groups.

At baseline, no differences were found between groups. Compared to baseline, stool frequency was significantly increased after 2 wk of control product consumption (2.6 ± 1.0 *vs* 2.4 ± 0.6, $P < 0.05$) but no differences were found after 1 wk. In the test product group, stool frequency was significantly improved after 1 (3.5 ± 1.5 *vs* 2.4 ± 0.6, $P < 0.01$) and 2 wk (4.1 ± 1.7 *vs* 2.4 ± 0.6, $P < 0.01$), respectively. Compared with control group, stool frequency was also significantly increased after 1 and 2 wk of product consumption (3.5 ± 1.5 *vs*

Table 3 Defecation condition score (mean ± SD)

	n	Baseline	Week 1	Week 2
Control group	63	1.9 ± 1.2	1.6 ± 1.1[a]	1.6 ± 1.1
Test group	63	1.9 ± 1.2	1.1 ± 0.9[b,d]	0.8 ± 1.0[b,d]

Note: Self comparison before and after test, [a]$P < 0.05$, [b]$P < 0.01$; compared with control group, [d]$P < 0.01$.

Table 4 Stool consistency score (mean ± SD)

	n	Baseline	Week 1	Week 2
Control group	63	1.6 ± 1.1	1.4 ± 1.0	1.3 ± 1.0[a]
Test group	63	1.5 ± 1.1	1.0 ± 0.8[b,c]	0.6 ± 0.8[b,d]

Note: Self comparison before and after test, [a]$P < 0.05$, [b]$P < 0.01$; compared with control group, [c]$P < 0.05$, [d]$P < 0.01$.

Table 5 Food intake amount of the subjects (g, mean ± SD)

	day 1-2		day 6-7		day 13-14	
	Control group	Test group	Control group	Test group	Control group	Test group
Staple food	341 ± 110	324 ± 117	350 ± 109	334 ±115	351 ± 117	353 ± 123
Fruits and vegetables	287 ± 227	341 ± 235	296 ± 212	322 ± 223	305 ± 244	318 ± 201
Meat and egg	118 ± 120	119 ± 100	114 ± 104	123 ± 103	121 ± 95	111 ± 105
Total daily intake	745 ± 343	784 ± 322	761 ± 294	779 ± 316	776 ± 344	783 ± 315

2.5 ± 0.9, $P < 0.01$ and 4.1 ± 1.7 *vs* 2.6 ± 1.0, $P < 0.01$, respectively) as shown in Table 2.

Defecation condition scores of the subjects

Defecation condition scores at baseline and after 1 and 2 wk of product consumption are shown in Table 3. Non-parametric rank sum tests were used to compare the difference between groups.

At baseline, no differences were found between groups. Compared to baseline, defecation condition score was significantly improved after 1 wk of control product consumption (1.6 ± 1.1 *vs* 1.9 ± 1.2, $P < 0.05$), but no differences were found after 2 wk. In the test product group, defecation condition score was significantly improved after 1 (1.1 ± 0.9 *vs* 1.9 ± 1.2, $P < 0.01$) and 2 wk (0.8 ± 1.0 *vs* 1.9 ± 1.2, $P < 0.01$), respectively. Compared with control group, defecation condition scores were also significantly improved (1.1 ± 0.9 *vs* 1.6 ± 1.1, $P < 0.01$ and 0.8 ± 1.0 *vs* 1.6 ± 1.1, $P < 0.01$), respectively, after 1 and 2 wk of product consumption.

Stool consistency scores of the subjects

Stool consistency at baseline and after 1 and 2 wk of product consumption is showed in Table 4. Non-parametric rank sum tests were used to compare the difference between groups.

At baseline, no differences were found between groups. Compared to baseline, stool consistency score was significantly decreased after 2 wk of control product consumption (1.3 ± 1.0 *vs* 1.6 ± 1.1, $P < 0.05$), but no differences were found after 1 wk. In the test product group, stool consistency score was significantly improved after 1 (1.0 ± 0.8 *vs* 1.5 ± 1.1, $P < 0.01$) and 2 wk (0.6 ± 0.8 *vs* 1.5 ± 1.1, $P < 0.01$), respectively. Compared with control group, stool consistency score was also significantly decreased after 1 and 2 wk of product consumption, (1.0 ± 0.8 *vs* 1.4 ± 1.0, $P < 0.05$ and 0.6 ± 0.8 *vs* 1.3± 1.0, $P < 0.01$), respectively.

Food intake of the subjects during product consumption

Food intakes of the subjects was surveyed by 48 h dietary recall at initial stage (1st-2nd day), intermediate stage (6th-7th day) and end stage (13th-14th day) of product consumption. The mean daily intakes of staple food, fruits and vegetables, and meat and eggs of the three times were calculated and statistically analyzed by *t* test between the two groups (Table 5). Food intake throughout the study did not differ between groups.

DISCUSSION

Consumption of the fermented product tested in this study was well tolerated by all the participants, and no adverse effects were reported. An acidified milk was used as the control material in this study. Lactose in milk can cause intolerance characterized by rugitus, abdominal distension, abdominal pain, even diarrhea as a severe symptom. The incidence rate of lactose intolerance reaches 90% in Chinese adults. For example, Yang *et al*[21] have shown that lactose intolerance occurred in 87% of the 7-8 and 11-13 years old Chinese children. In order to prevent diarrhea, due to milk intake in the control group, extrinsic lactase was added to control milk samples during the manufacturing process. This resulted in an equal lactose content between the test and control products.

Several reviews[22-25] have described that some probiotics could improve lactose digestion and eliminate the symptoms of intolerance. A recent study has shown that a yogurt enriched with *B. lactis* DN-173010 and *B. longum* in capsules modifies the composition and metabolic activities of the colonic microbiota and alleviates symptoms in Chinese lactose-intolerant subjects[26].

BIO® is a fermented milk product which contains a mixture of live bacterial cultures; *B. lactis* DN-173010 (1.25×10^{10} cfu/pot) and yogurt starters *L. bulgaricus* and *S. thermophilus* (1.2×10^{9} cfu/pot). It has been shown to increase slow transit and, therefore, was tested in women with constipation to determine whether it would modulate bowel habits. Food intake and blood parameters remained constant throughout the study.

The results of this study indicated that stool frequency was significantly increased by 40% and 58% after 1 and 2 wk of product consumption, respectively ($P < 0.01$). In addition, after 1 wk of consumption, defecation condition scores (31% and 50%, $P < 0.01$) and stool consistency (29%, $P < 0.05$ and 25%, $P < 0.01$) were also significantly improved from baseline values in women consuming test product.

Three separate clinical studies have already demonstrated that daily consumption of a fermented milk containing *B. lactis* DN-173010 improves gastrointestinal transit time in elderly subjects[15,16], and in a group of healthy women with slow transit time[17]. Stool frequency was also significantly improved after product consumption in IBS subjects compared to controls with respect to subjects with a stool frequency < 4 stools per week at baseline[18]. A fifth study performed on fermented milk containing *B. lactis* DN-173010 alone also demonstrated a reduced transit time in healthy men and women with slow transit time[19]. Our finding that probiotics may normalize bowel movements is in line with those in some previous studies[27-30]. Some studies have shown that milk or yogurt fermented with different types of probiotics may increase the daily stool number in constipated subjects. In a double-blind, placebo-controlled study performed in 70 subjects with chronic constipation, a probiotic beverage containing *Lactobacillus casei Shirota* administered for a 4-wk period was significantly better than placebo in improving severity of constipation, stool frequency and consistency[27]. Likewise, a preparation containing *Escherichia coli* Nissle 1917 (a probiotic strain) was compared to placebo in a double-blind clinical trial in 70 subjects with chronic constipation, showing that the *E. coli* preparation was significantly better than placebo in increasing stool frequency[28]. Moreover, intake of a fermented milk product containing *L. casei* strain Shirota for 2 wk in a placebo-controlled double-blind cross-over design improved the state of bowel movements and stool quality in healthy subjects with a stronger tendency to constipation[29]. Finally, in an open trial in elderly subjects, a commercial mixture of *Lactobacillus rhamnosus* and *Propionibacterium freudenreichii* improved defecation frequency by 24%, but no reduction in laxative use was observed[30]. To date, no clinical studies have been performed to measure defecation conditions/straining on constipated subjects. Few studies have been focused on the effects of probiotics on the intestinal function in healthy people, and the observed effects depended on the strain used. Three randomized, double-blind, placebo-controlled human clinical trials[31-33] have been performed to investigate the effect of a fermented product containing probiotic strains. In these clinical studies, product consumption exerted a beneficial effect on the bowel functions, but with no significant effect compared to the placebo. Findings in previous studies are inconsistent possibly due to lesser statistical power, the use of different probiotic strains and different subject population. Thus, probiotics may be effective in subjects with mild to moderate constipation and controlled and well-designed studies in this type of subjects are warranted[34]. In any case, our study is the first showing the significant efficacy of fermented milk consumption on stool frequency and consistency, as well as defecation conditions in constipated subjects.

The patients' ability to achieve normal bowel habits without being in pain, and to control bowel movements, are important elements of physical well-being. This was shown by studies investigating the relationships between quality of life and gastrointestinal symptoms in persons with constipation[35,36]. The surveys revealed an impaired quality of life in constipated individuals in comparison with healthy persons, depending on the severity of the constipation. In addition, Guyonnet *et al*[18] have shown that daily consumption of a fermented milk containing *B. lactis* DN-173010 improves quality of life and symptoms in IBS compared to heat-treated yogurt. These results support the hypothesis of a relationship between improved stool frequency, transit time, quality of life and a fermented milk containing *B. lactis* DN-173010, and indicate that further research should be carried out to investigate the potential use of this fermented milk product in improving quality of life in subjects with constipation.

In conclusion, the present large-scale study showed a beneficial effect of a fermented milk containing *B. lactis* DN-173010 on stool frequency and consistency, as well as on defecation conditions of women with constipation. Further studies are required to elucidate mechanisms of such effects to provide additional scientific evidence to support the use of such probiotic food to relieve constipation.

COMMENTS

Background

In recent years, probiotics have been studied for their efficacy on gastrointestinal disorders. *Bifidobacterium lactis* DN-173010, a probiotic strain, has already demonstrated health benefit on the gastrointestinal transit. Positive results have been obtained with consumption of a fermented milk containing *B. lactis* DN-173010 and yogurt strains on gut transit time in healthy people with normal to slow transit time. Equally, encouraging positive results have been obtained on stool frequency in Irritable Bowel Syndrome subjects with predominant constipation. These results indicate that research should be carried out to investigate the potential use of this fermented milk product in improving stool parameters in subjects with constipation.

Research frontiers

Constipation is reported in many regions in the world. An epidemiological study conducted in Beijing concluded that 6.1% of the adult population was suffering from the symptoms of functional constipation but no clinical study has been carried out in this population. This is why we decided to investigate the effect of a fermented milk (BIO®) combining *B. lactis* DN-173010 and yogurt strains on constipation functional parameters of adult women in Beijing.

Innovations and breakthroughs

This research demonstrates the first positive results on the ability of the *B. lactis* DN-173010 to improve stool frequency, stool consistency and defecation conditions in adult constipated women. It is believed to be the first study to investigate the effect of *B. lactis* DN-173010 leaded in another ethnic population with a different diet than European populations. Finally, this study shows the first evidence of the positive effect of *B. lactis* DN-173010 on constipated subjects.

Peer review

The contribution by Yue-Xin Yang *et al* studied the effect of a fermented milk containing *B. lactis* DN-173010 and yogurt strains (BIO®) in adult constipated women in Bejing China. The authors determined that the fermented milk containing *B. lactis* DN-173010 had a beneficial effect on stool frequency, stool consistency, and defecation of women with constipation. This is a well-written report.

REFERENCES

1 **Lenoir-Wijnkoop I**, Sanders ME, Cabana MD, Caglar E, Corthier G, Rayes N, Sherman PM, Timmerman HM, Vaneechoutte M, Van Loo J, Wolvers DA. Probiotic and prebiotic influence beyond the intestinal tract. *Nutr Rev* 2007; **65**: 469-489

2 **Sanders ME**, Gibson G, Harsharnjit SG, Guarner F. Probiotics: Their Potential to Impact Human Health. Council for Agricultural Science and Technology 2007; **36**. Available from URL: http://www.cast-science.org

3 **Heller KJ**. Probiotic bacteria in fermented foods: product characteristics and starter organisms. *Am J Clin Nutr* 2001; **73**: 374S-379S

4 **Cheng C**, Chan AO, Hui WM, Lam SK. Coping strategies, illness perception, anxiety and depression of patients with idiopathic constipation: a population-based study. *Aliment Pharmacol Ther* 2003; **18**: 319-326

5 **Brandt LJ**, Prather CM, Quigley EM, Schiller LR, Schoenfeld P, Talley NJ. Systematic review on the management of chronic constipation in North America. *Am J Gastroenterol* 2005; **100** Suppl 1: S5-S21

6 **Garrigues V**, Galvez C, Ortiz V, Ponce M, Nos P, Ponce J. Prevalence of constipation: agreement among several criteria and evaluation of the diagnostic accuracy of qualifying symptoms and self-reported definition in a population-based survey in Spain. *Am J Epidemiol* 2004; **159**: 520-526

7 **Johanson JF**. Review of the treatment options for chronic constipation. *MedGenMed* 2007; **9**: 25

8 **Guo XK**, Pan MY, Han G, Fang S, Lu XC, Guo H. A cluster, stratified, randomized epidemiologic survey and analysis of related factors on adult chronic constipation in Beijing area. *Chin J Dig Dis* 2002; **22**: 637-638

9 **Lembo A**, Camilleri M. Chronic constipation. *N Engl J Med* 2003; **349**: 1360-1368

10 **Nakaji S**, Tokunaga S, Sakamoto J, Todate M, Shimoyama T, Umeda T, Sugawara K. Relationship between lifestyle factors and defecation in a Japanese population. *Eur J Nutr* 2002; **41**: 244-248

11 **Gill HS**, Guarner F. Probiotics and human health: a clinical perspective. *Postgrad Med J* 2004; **80**: 516-526

12 **Berrada N**, Lemeland JF, Laroche G, Thouvenot P, Piaia M. Bifidobacterium from fermented milks: survival during gastric transit. *J Dairy Sci* 1991; **74**: 409-413

13 **Pochart P**, Marteau P, Bouhnik Y, Goderel I, Bourlioux P, Rambaud JC. Survival of bifidobacteria ingested via fermented milk during their passage through the human small intestine: an in vivo study using intestinal perfusion. *Am J Clin Nutr* 1992; **55**: 78-80

14 **Duez H**, Pelletier C, Cools S, Aissi E, Cayuela C, Gavini F, Bouquelet S, Neut C, Mengaud J. A colony immunoblotting method for quantitative detection of a Bifidobacterium animalis probiotic strain in human faeces. *J Appl Microbiol* 2000; **88**: 1019-1027

15 **Meance S**, Cayuela C, Turchet P, Raimondi A, Lucas C, Antoine JM. A fermented milk with a Bifidobacterium probiotic strain DN-173 010 shortened oro-faecal gut transit time in elderly. *Microb Ecol Health Dis* 2001; **13**: 217-222

16 **Meance S**, Cayuela C, Raimondi A, Turchet P, Lucas C, Antoine JM. Recent advances in the use of functional foods: effects of the commercial fermented milk with Bifidobacterium animalis strain DN-173 010 and yoghurt strains on gut transit time in the elderly. *Microb Ecol Health Dis* 2003; **15**: 15-22

17 **Marteau P**, Cuillerier E, Meance S, Gerhardt MF, Myara A, Bouvier M, Bouley C, Tondu F, Bommelaer G, Grimaud JC. Bifidobacterium animalis strain DN-173 010 shortens the colonic transit time in healthy women: a double-blind, randomized, controlled study. *Aliment Pharmacol Ther* 2002; **16**: 587-593

18 **Guyonnet D**, Chassany O, Ducrotte P, Picard C, Mouret M, Mercier CH, Matuchansky C. Effect of a fermented milk containing Bifidobacterium animalis DN-173 010 on the health-related quality of life and symptoms in irritable bowel syndrome in adults in primary care: a multicentre, randomized, double-blind, controlled trial. *Aliment Pharmacol Ther* 2007; **26**: 475-486

19 **Bouvier M**, Meance S, Bouley C, Berta JL, Grimaud JC. Effects of consumption of a milk fermented by the probiotic strain Bifidobacterium animalis DN-173 010 on colonic transit times in healthy humans. *Bioscience and Microflora* 2001; **20**: 43-48

20 **Ministry of Health PR China**. Technical Standards for testing and Assessment of Health Food. Beijing, 2003: 203-204

21 **Yang Y**, He M, Cui H, Bian L, Wang Z. The prevalence of lactase deficiency and lactose intolerance in Chinese children of different ages. *Chin Med J* (Engl) 2000; **113**: 1129-1132

22 **Roberfroid MB**. Prebiotics and probiotics: are they functional foods? *Am J Clin Nutr* 2000; **71**: 1682S-1687S; discussion 1688S-1690S

23 **Rolfe RD**. The role of probiotic cultures in the control of gastrointestinal health. *J Nutr* 2000; **130**: 396S-402S

24 **de Vrese M**, Stegelmann A, Richter B, Fenselau S, Laue C, Schrezenmeir J. Probiotics--compensation for lactase insufficiency. *Am J Clin Nutr* 2001; **73**: 421S-429S

25 **Kopp-Hoolihan L**. Prophylactic and therapeutic uses of probiotics: a review. *J Am Diet Assoc* 2001; **101**: 229-238; quiz 239-241

26 **He T**, Priebe MG, Zhong Y, Huang C, Harmsen HJ, Raangs GC, Antoine JM, Welling GW, Vonk RJ. Effects of yogurt and bifidobacteria supplementation on the colonic microbiota in lactose-intolerant subjects. *J Appl Microbiol* 2008; **104**: 595-604

27 **Koebnick C**, Wagner I, Leitzmann P, Stern U, Zunft HJ. Probiotic beverage containing Lactobacillus casei Shirota improves gastrointestinal symptoms in patients with chronic constipation. *Can J Gastroenterol* 2003; **17**: 655-659

28 **Mollenbrink M**, Bruckschen E. [Treatment of chronic constipation with physiologic Escherichia coli bacteria. Results of a clinical study of the effectiveness and tolerance of microbiological therapy with the E. coli Nissle 1917 strain (Mutaflor)] *Med Klin* (Munich) 1994; **89**: 587-593

29 **Matsumoto K**, Takada T, Shimizu K, Kado Y, Kawakami K, Makino I, Yamaoka Y, Hirano K, Nishimura A, Kajimoto O, Nomoto K. The effects of a probiotic milk product containing Lactobacillus casei strain Shirota on the defecation frequency and the intestinal microflora of sub-optimal health state volunteers: a randomized placebo-controlled cross-over study. *Bioscience and Microflora* 2006; **25**: 39-48

30 **Ouwehand AC**, Lagstrom H, Suomalainen T, Salminen S. Effect of probiotics on constipation, fecal azoreductase activity and fecal mucin content in the elderly. *Ann Nutr Metab* 2002; **46**: 159-162

31 **Johansson ML**, Nobaek S, Berggren A, Nyman M, Bjorck I, Ahrne S, Jeppsson B, Molin G. Survival of Lactobacillus plantarum DSM 9843 (299v), and effect on the short-chain fatty acid content of faeces after ingestion of a rose-hip drink with fermented oats. *Int J Food Microbiol* 1998; **42**: 29-38

32 **Olivares M**, Diaz-Ropero MA, Gomez N, Lara-Villoslada F, Sierra S, Maldonado JA, Martin R, Lopez-Huertas E, Rodriguez JM, Xaus J. Oral administration of two probiotic strains, Lactobacillus gasseri CECT5714 and Lactobacillus

coryniformis CECT5711, enhances the intestinal function of healthy adults. *Int J Food Microbiol* 2006; **107**: 104-111
33 **Sairanen U**, Piirainen L, Grasten S, Tompuri T, Matto J, Saarela M, Korpela R. The effect of probiotic fermented milk and inulin on the functions and microecology of the intestine. *J Dairy Res* 2007; **74**: 367-373
34 **Fernandez-Banares F**. Nutritional care of the patient with constipation. *Best Pract Res Clin Gastroenterol* 2006; **20**: 575-587
35 **Rothbarth J**, Bemelman WA, Meijerink WJ, Stiggelbout AM, Zwinderman AH, Buyze-Westerweel ME, Delemarre JB. What is the impact of fecal incontinence on quality of life? *Dis Colon Rectum* 2001; **44**: 67-71
36 **Glia A**, Lindberg G. Quality of life in patients with different types of functional constipation. *Scand J Gastroenterol* 1997; **32**: 1083-1089

S- Editor Xiao LL **L- Editor** Rippe RA **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6244

World J Gastroenterol 2008 October 28; 14(40): 6244-6248
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Immunogenicity and immunoprotection of recombinant PEB1 in *Campylobacter-jejuni*-infected mice

Lian-Feng Du, Zhen-Jiang Li, Xian-Ying Tang, Jun-Qiong Huang, Wan-Bang Sun

Lian-Feng Du, Zhen-Jiang Li, Xian-Ying Tang, Jun-Qiong Huang, Wan-Bang Sun, Department of Immunology, Zhuhai Campus of Zunyi Medical College, Zhuhai 519041, Guangdong Province, China

Author contributions: Sun WB and Du LF designed the research; Du LF, Tang XY and Li ZJ performed the research and data collection; Du LF and Huang JQ performed the analysis; Du LF and Sun WB wrote the paper.

Supported by Grants from the Governor Foundation for Excellent Talents of Guizhou Province, No. 200607

Correspondence to: Dr. Wan-Bang Sun, Professor, Department of Immunology, Zhuhai Campus of Zunyi Medical College, Zhuhai 519041, Guangdong Province, China. sunwb7224@sina.com
Telephone: +86-756-7623360 Fax: +86-756-7623388

Received: July 8, 2008 Revised: October 6, 2008
Accepted: October 13, 2008
Published online: October 28, 2008

Abstract

AIM: To construct a prokaryotic expression vector carrying *Campylobacter jejuni peb1A* gene and express it in *Escherichia coli*. Immunoreactivity and antigenicity of rPEB1 were evaluated. The ability of rPEB1 to induce antibody responses and protective efficacy was identified.

METHODS: *peb1A* gene was amplified by PCR, target gene and prokaryotic expression plasmid pET28a (+) was digested with *Bam*HI and *Xho*I, respectively. DNA was ligated with T4 DNA ligase to construct recombinant plasmid pET28a(+)-*peb1A*. The rPEB1 was expressed in *E. coli* BL21 (DE3) and identified by SDS-PAGE. BALB/c mice were immunized with rPEB1. ELISA was used to detect the specific antibody titer and MTT method was used to measure the stimulation index of spleen lymphocyte transformation.

RESULTS: The recombinant plasmid pET28a (+)-*peb1A* was correctly constructed. The expression output of PEB1 protein in pET28a (+)-*peb1A* system was approximately 33% of total proteins in *E. coli*. The specific IgG antibody was detected in serum of BALB/c mice immunized with rPEB1 protein. Effective immunological protection with a lower sickness incidence and mortality was seen in the mice suffering from massive *C. jejuni* infection.

CONCLUSION: rPEB1 protein is a valuable candidate for *C. jejuni* subunit vaccine.

Key words: *Campylobacter jejuni*; Prokaryotic expression; Immunogenicity

Peer reviewer: Dr. Wang-Xue Chen, Institute for Biological Sciences, National Research Council of Canada, 100 Sussex Drive, Room 3100, Ottawa, Ontario K1A 0R6, Canada

Du LF, Li ZJ, Tang XY, Huang JQ, Sun WB. Immunogenicity and immunoprotection of recombinant PEB1 in *Campylobacter-jejuni*-infected mice. *World J Gastroenterol* 2008; 14(40): 6244-6248 Available from: URL: http://www.wjgnet.com/1007-9327/14/6244.asp DOI: http://dx.doi.org/10.3748/wjg.14.6244

INTRODUCTION

Campylobacter jejuni is one of the leading causes of bacterial diarrhea in travelers, children, and military personnel in regions where water and food sources are commonly contaminated[1]. Moreover, *C. jejuni* is an infectious agent most often associated with Guillain-Barre syndrome (GBS), a post-infectious polyneuropathy[2-5]. Campylobacter has been reported in many geographic regions and its incidence varies with the season. Campylobacter outbreak and sporadic cases occur in developed countries, but the risk of developing campylobacteriosis is greater in travelers, children, and military personnel in regions where water and food sources are commonly contaminated. Currently, no commercial vaccines are available for the prevention of campylobacter-induced diseases in humans or for the reduction/elimination of colonization in poultry.

The development of vaccines has been hampered because the pathogenesis of campylobacter infections is poorly understood. The live-attenuated or killed whole-cell campylobacter vaccine candidates have raised questions about its safety. The protein PEB1, encoded by *peb1A* genes, is considered a common antigen and a major cell adherence molecule of *C. jejuni*[6]. The *peb1A* gene contains 780 bases encoding a 259-residue polypeptide. The peptide sequence starting at residue 27 matches that determined from amino-terminal sequencing of mature PEB1 from *C. jejuni*. The molecular mass of mature PEBl (amino acids s 27-259) is 25.5 kDa. In this study, we constructed a prokaryotic expression vector carrying *C. jejuni peb1A* gene minus its signal sequence and expressed it in *E. coli*. These

vaccine candidates were evaluated in mice for their ability to induce antibody responses specific to rPEB1 immunization and to protect the candidates against oral challenge with *C. jejuni*.

MATERIALS AND METHODS

Animals

BALB/c mice, at the age of 6-8 wk, were purchased from Center of Experiment Animal of Sun Yat-sen University and housed in cages for 7 d before use.

Bacterial strains and culture conditions

C. jejuni was grown in brucella agar plates at 37℃ in a microaerobic environment. *E. coli* JM109 used for amplification of the recombinant plasmid pET28a (+) was grown in a LB medium supplemented with kanamycin (50 μg/mL) at 37℃.

Construction of pET28a (+)-peb1A

Primers were designed according to the sequence of the *C. jejuni peb1A* gene (Genbank, ATCC700819) minus its signal sequence. The sequence of up primer is 5'-GC GGATCCGCAGAAGGTAAACTTGAGTCTAT-3' and the sequence of down primer is 5'-CCGCTCGAGTTA TAAACCCCATTTTTTCGCT-3'. The restriction sites of *Bam*HI and *Xho*I (underline) were introduced into the sequences of up and down primers, respectively, for gene cloning.

As a first step in amplification of the *C. jejuni peb1A* gene, template DNA was extracted from the *C. jejuni* genome. In a 50-μL Eppendoff tube, 30.5 μL of ddH_2O, 5 μL of 2 mmol/L dNTP, 5 μL 10 × PCR buffer, 0.5 μL of Taq polymerase, 1 μL of template DNA were added. The PCR product was subjected to electrophoresis on 1.5% agarose, purified using a DNA purification kit and then subjected to digestion with *Bam*HI and *Xho*I. The digested PCR product was purified and inserted into pET28a (+) digested with the same restriction enzyme to construct pET28a (+)-*peb1A*. pET28a (+)-*peb1A* was transfected into *E. coli* JM109. After propagation, pET28a (+)-*peb1A* was identified with restriction enzyme by direct sequencing.

Protein expression and purification

The *peb1A* gene from *C. jejuni* was expressed in *E. coli* as hexahistidine tagged proteins in pET-28a (+). *E. coli* BL21 (DE3) containing *peb1A* clone was grown in LB broth containing 30 μg/mL kanamycin. Cells were incubated at 37℃ with shaking at 250 r/min for 3-4 h until the culture reached an OD of 0.3-0.4. Then, IPTG was added to the LB broth at a final concentration of 1 mmol/L to induce expression of the target protein PEB1. Culture was continued for 6 h and BL21(DE3) cells were harvested at 1, 2, 3, 4 and 6 h, respectively, by centrifugation. The pellet of BL21(DE3) cells was resuspended in 1 × LEW buffer containing 50 mmol/L NaH_2PO_4, 300 mmol/L NaCl, pH 8, and subjected to ultrasound in ice water. To evaluate the solubility and inclusion body formation, the resulting supernatant and sediments were separated by centrifugation at 12000 r/min for 10 min at 4℃, and subjected to SDS-PAGE for expression of recombinant PEB1 (rPEB1), which was purified by nickel chromatography under native conditions.

Vaccination

BALB/c mice were injected with 100 μL of PBS or with PBS containing 25, 50 or 100 μg of rPEB1 protein emulsified with an equal volume of CFA or IFA. Mice in each vaccination group (n =10 mice) were immunized four times at 1-wk intervals by intramuscular and subcutaneous injection. Following vaccination, the mice were monitored for adverse effects. Blood was collected from mice at various time points before and after immunization, and allowed to clot. The tubes were spun at 3000 r/min for 10 min, and the serum was collected into a clean microcentrifuge tube. Serum samples were logged in and stored at -20℃.

ELISA was used to evaluate the level of antibody response to anti-PEB1. Briefly, rPEB1 was used as the solid phase. After blocking with PBST supplemented with 10% fetal calf serum, the serum from mice was added. After extensive washing, bound antibodies were detected with goat anti-mouse IgG labeled with horseradish peroxidase. Antibody titers were determined by the serial end-point dilution method. The titer of serum was expressed as group geometric mean ± SD of the mean of individual animal values, which represented the average of duplicate assays.

T-cell proliferation assays

BALB/c mice immunized with rPEB1 or PBS (control) were sacrificed on day 60 after the first immunization. Splenocytes were harvested from the mice, co-cultured with rPEB1 (2 μg/mL) or with PHA in RPMI1640 for 54 h before addition of MTT (10 μL per well), and incubated at 37℃ for 3 h. The supernatant was transferred into a new Eppendorf tube. Absorbance of the converted dye was measured at a wavelength of 570 nm with a spectrophotometer.

Protective efficacy of oral challenge with C. jejuni

BALB/c mice at the age of 7-9 wk without specific pathogen were used in the study. The vaccinated mice were challenged with *C. jejuni* strain 81-176 in the oral model. We compared the protective efficacy of rPEB1 in immunized and non-immunized mice. Deaths occurred in challenged and control mice were recorded for more than 7 d. Illness index was scored as follows: 2 = dead, 1 = lethargic with ruffled fur and lower activity, and 0 = healthy.

RESULTS

Construction of pET28a (+)-peb1A

A single band at the 720-bp site was well shown in *C. jejuni* genome amplified by PCR. Recombinant plasmid pET28a (+)-*peb1A* analyzed by restriction enzyme digestion and DNA sequence was correctly constructed.

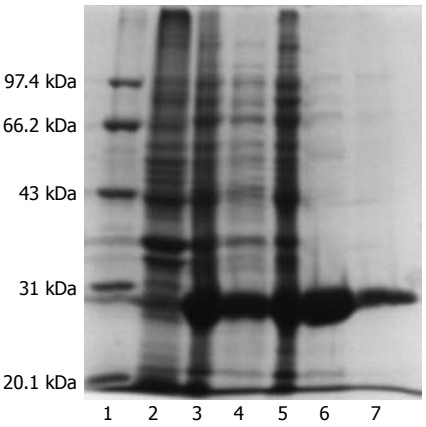


Figure 1 Analysis of expression pattern of recombinant protein by SDS-PAGE. Lane 1: protein marker; lanes 2-3: *E. coli* BL (DE3) transformed with pET28a(+) and pET28a(+)-*peb1A* respectively after induced with IPTG for 4 h; lanes 4-5: supernatant and precipitate of sonicated broken *E. coli* BL (DE3) transformed with pET28a(+)-*peb1A*; lanes 6-7: Purified recombinant protein.

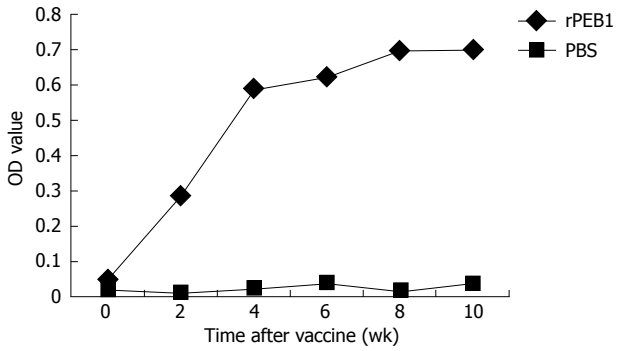


Figure 2 OD values of specific serum IgG antibody levels after immunization with rPEB1.

Expression and purification of recombinant protein

A rPEB1 protein with an expected molecular weight of 29kD was efficiently expressed in *E. coli* BL (DE3). The rPEB1 was mainly observed in supernatant of the *E. coli* BL (DE3) lysate and purified to approximately 96% purity by Ni-NTA resin after ultrasonication. The expression output of PEB1 protein in pET28a (+)-*peb1A* system was approximately 33% of total proteins of *E. coli* (Figure 1).

Strong immune response of BALB/c mice immunized with rPEB1

In subcutaneous and intramuscular injection groups, no apparent side effects were noted in mice and delivery of rPEB1 with CFA caused a ruffled fur appearance in all mice that lasted < 24 h, suggesting that injection of rPEB1 was safe. The mice in subcutaneous and intramuscular injection groups were immunized with rPEB1 interfused in CFA or IFA. PBS was substituted for rPEB1 in the control group. Anti- rPEB1 serum was detected 2 wk after the first immunization in both subcutaneous and intramuscular injection groups (Figure 2). Compared to the PBS group, significantly higher levels of serum IgG were detected in ⩾ 90% of the animals when 50 μg or higher rPEB1 was delivered with the adjuvant. Vaccination with 100 μg rPEB1 with the adjuvant induced antigen specific serum IgG, which was indistinguishable from that in 200 μg recipients (Table 1). A clear vaccine dose-dependent response was seen for the response magnitude and a strong immune response was observed in mice after immunization with rPEB1. The highest end point dilution titer of anti-rPEB1 serum was 1:5600.

Table 1 OD values of specific serum IgG antibody levels in BALB/c mice after immunization with recombinant PEB1 protein vaccine

Groups	Immune route	Serum IgG (mean ± SD)
Control	Subcutaneous	0.157 ± 0.010
P50 μg	Subcutaneous	0.365 ± 0.019[1]
P100 μg	Subcutaneous	0.521 ± 0.024[1]
P200 μg	Subcutaneous	0.619 ± 0.028[1]
Control	Muscular	0.157 ± 0.010
J50 μg	Muscular	0.350 ± 0.016[1]
J100 μg	Muscular	0.641 ± 0.019[1]
J200 μg	Muscular	0.638 ± 0.023[1]

[1]$P < 0.01$ *vs* control group.

Table 2 Illness index of BALB/c mice after oral challenge with wild-type *C. jejuni*

Groups	Cases	Healthy	Sickness	Dead	Illness index	Protective rate (%)
Control	6	0	2	4	9.14 ± 0.90	0
J50 μg	6	2	1	3	5.71 ± 0.49	33.3
J100 μg	6	4	1	1	3.00 ± 0.82	75
J200 μg	6	3	2	1	3.14 ± 0.90	66.7

T-cell proliferation assay of splenocytes in BALB/c mice

The proliferating response of splenocytes was generated in immunized mice when they were stimulated by PHA or rPEB1 protein. The stimulation index (SI) value for the immunized group was significantly higher than that for the control group, suggesting that proliferation of T cells from immunized mice could be stimulated by rPEB1. No difference in SI was observed in different groups immunized with different doses of rPEB1 compared with the PHA control group (Figure 3).

Protective efficacy of oral challenge with wild-type C. jejuni

Fourteen days following vaccination, animals immunized with different doses of rPEB1 were challenged with wild-type *C. jejuni*. The results are summarized in Table 2. Fifty micrograms rPEB1 failed to protect mice against *C. jejuni* infection and no significant difference was observed in illness pattern of PBS recipients. The efficacy of rPEB1 vaccine was significantly higher in animals challenged with *C. jejuni* than in those of the control group, indicating that rPEB1 vaccine could eradicate *C. jejuni* infection (Figure 4).

DISCUSSION

PEB1, a surface-exposed conserved antigen in *C. jejuni*,

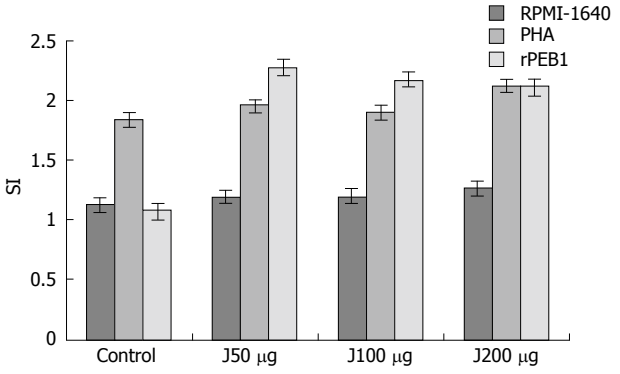


Figure 3 Stimulation index of immunized BALB/c mice spleen lymphocytes stimulated by intramuscular injection of rRPEB1.

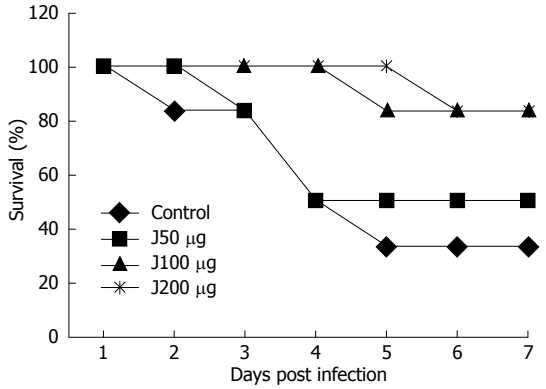


Figure 4 Survival of BALB/c mice immunized with rPEB1 after oral challenge with wild-type *C. jejuni* in control and vaccination groups.

is commonly recognized in convalescent sera from infected patients and involves binding of *C. jejuni* to eukaryotic cells[7]. Pei *et al*[8] have reported that PEB1 is a homolog of cluster 3 binding proteins of bacterial ABC transporters and a *C. jejuni* adhesion cell-binding factor 1. They determined the role of PEB1 in *C. jejuni* adherence and noted that the rate and duration of intestinal colonization by its mutants are significantly lower and shorter than those of the wild-type strain in mouse challenge test[8]. The adherence to epithelial cells is essential for the establishment of colonization in the gastrointestinal tract. It has also been shown that the PEB1 can adhere to Hela cells[9]. Inactivation of the *peb1A* locus significantly reduces *C. jejuni* adherence to Hela cells[10,11]. Moreover, the particulate PEB1 is the only antigen, known to elicit a prominent immune response[12,13]. We believe that PEB1 may be used as a vaccine for *C. jejuni* infection, which was confirmed by the fact that we successfully constructed a fusion gene containing *C. jejuni peb1A* gene and expressed rPEB1 protein in *E. coil* BL21 (DE3). rPEB1 with adjuvant CFA/IFA was used to immunize BALB/c mice, in which strong specific humoral immune responses were induced. High specific anti-rPEB1 and significantly higher specific T-cell proliferation were detected in BALB/c mice 3 wk after their first immunization. Furthermore, rPEB1 vaccination was found to have an effect on reducing the illness index of BALB/c mice after oral challenge with wild-type *C. jejuni*.

Sizemore *et al*[14] have reported that live and attenuated *Salmonella Typhimurium* strains expressing PEB1 can induce antibody responses specific to PEB1 following oral immunization, and have the ability to protect mice against infection with *S. Typhimurium* strains by reducing or eliminating systemic dissemination and intestinal colonization of wild-type *C. jejuni* strain 81-176. However, they noted that attenuated salmonella can stimulate production of serum IgG in mice and cannot protect mice against challenge with wild-type *C. jejuni*[14]. They believe that a small amount of antigen, available at the time of vaccination, may play a role in the absence of serum IgG[14]. Our results indicated that immunization with a low dose (50 μg) of rPEB1 could stimulate specific humoral immune responses and could not protect mice against challenge with wild-type *C. jejuni*.

The results of the animal protection test using 100 μg rPEB1 showed that most immunized mice remained healthy after *C. jejuni* challenge. Eighty percent of the control mice were lethargic with ruffled fur and lower activity and died 2 d post-challenge. The protective rate of rPEB1 immunization was 75% in the 100 μg rPEB1 group.

In conclusion, rPEB1, as a candidate vaccine, offers several advantages. It can lead to strong immune response, and provide a protective efficacy. Further study is needed to address its mechanism.

COMMENTS

Background

Despite the growing importance and widespread recognition of campylobacter enteritis as a major international public health problem, no commercial vaccines are available for the control of campylobacter-associated enteric disease in humans, or for the reduction/elimination of colonization in poultry.

Research frontiers

This study looked for the best induction of protective immune responses when the immunogenic campylobacter protein PEB1 was expressed.

Innovations and breakthroughs

The results of this study showed that rPEB1 was successfully expressed in *Escherichia coli* and immunization of mice through systemic routes could induce strong and specific serum IgG responses and splenocyte proliferation.

Applications

Based on the results of our study, further investigation should be focused on mucosal immune responses, which may be more important to *Campylobacter jejuni* subunit vaccines.

Peer review

The results of this study are interesting. The authors evaluated immunoreactivity and antigenicity of rPEB1, identified the ability of rPEB1 to induce systemic immune responses and its protective efficacy.

REFERENCES

1 **Stutman HR**. Salmonella, Shigella, and Campylobacter: common bacterial causes of infectious diarrhea. *Pediatr Ann* 1994; **23**: 538-543
2 **Hughes R**. Campylobacter jejuni in Guillain-Barre syndrome. *Lancet Neurol* 2004; **3**: 644
3 **Tsugawa T**, Nikaido K, Doi T, Koga M, Susuki K, Kubota T, Tsutsumi H. Guillain-Barre syndrome with meningoencephalitis after Campylobacter jejuni infection.

Pediatr Infect Dis J 2004; **23**: 966-968
4 **Jacobs BC**, van Doorn PA, Schmitz PI, Tio-Gillen AP, Herbrink P, Visser LH, Hooijkass H, van der Meche FG. Campylobacter jejuni infections and anti-GM1 antibodies in Guillain-Barre syndrome. *Ann Neurol* 1996; **40**: 181-187
5 **Yuki N**, Ang CW, Koga M, Jacobs BC, van Doorn PA, Hirata K, van der Meche FG. Clinical features and response to treatment in Guillain-Barre syndrome associated with antibodies to GM1b ganglioside. *Ann Neurol* 2000; **47**: 314-321
6 **Pei Z**, Blaser MJ. PEB1, the major cell-binding factor of Campylobacter jejuni, is a homolog of the binding component in gram-negative nutrient transport systems. *J Biol Chem* 1993; **268**: 18717-18725
7 **Pei ZH**, Ellison RT 3rd, Blaser MJ. Identification, purification, and characterization of major antigenic proteins of Campylobacter jejuni. *J Biol Chem* 1991; **266**: 16363-16369
8 **Pei Z**, Burucoa C, Grignon B, Baqar S, Huang XZ, Kopecko DJ, Bourgeois AL, Fauchere JL, Blaser MJ. Mutation in the peb1A locus of Campylobacter jejuni reduces interactions with epithelial cells and intestinal colonization of mice. *Infect Immun* 1998; **66**: 938-943
9 **Kervella M**, Pages JM, Pei Z, Grollier G, Blaser MJ, Fauchere JL. Isolation and characterization of two Campylobacter glycine-extracted proteins that bind to HeLa cell membranes. *Infect Immun* 1993; **61**: 3440-3448
10 **Baqar S**, Applebee LA, Bourgeois AL. Immunogenicity and protective efficacy of a prototype Campylobacter killed whole-cell vaccine in mice. *Infect Immun* 1995; **63**: 3731-3735
11 **Ang CW**, Endtz HP, Jacobs BC, Laman JD, de Klerk MA, van der Meche FG, van Doorn PA. Campylobacter jejuni lipopolysaccharides from Guillain-Barre syndrome patients induce IgG anti-GM1 antibodies in rabbits. *J Neuroimmunol* 2000; **104**: 133-138
12 **Baqar S**, Bourgeois AL, Schultheiss PJ, Walker RI, Rollins DM, Haberberger RL, Pavlovskis OR. Safety and immunogenicity of a prototype oral whole-cell killed Campylobacter vaccine administered with a mucosal adjuvant in non-human primates. *Vaccine* 1995; **13**: 22-28
13 **Baqar S**, Bourgeois AL, Applebee LA, Mourad AS, Kleinosky MT, Mohran Z, Murphy JR. Murine intranasal challenge model for the study of Campylobacter pathogenesis and immunity. *Infect Immun* 1996; **64**: 4933-4939
14 **Sizemore DR**, Warner B, Lawrence J, Jones A, Killeen KP. Live, attenuated Salmonella typhimurium vectoring Campylobacter antigens. *Vaccine* 2006; **24**: 3793-3803

S- Editor Xiao LL **L- Editor** Wang XL **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6249

World J Gastroenterol 2008 October 28; 14(40): 6249-6253
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Midkine accumulated in nucleolus of HepG2 cells involved in rRNA transcription

Li-Cheng Dai, Jian-Zhong Shao, Li-Shan Min, Yong-Tao Xiao, Li-Xin Xiang, Zhi-Hong Ma

Li-Cheng Dai, Li-Shan Min, Zhi-Hong Ma, Huzhou key laboratory of Molecular Medicine, Huzhou Central Hospital, Huzhou 313000, Zhejiang Province, China
Yong-Tao Xiao, Li-Xin Xiang, Jian-Zhong Shao, College of Life Sciences, Zhejiang University, Hangzhou 310031, Zhejiang Province, China
Author contributions: Dai LC and Shao JZ contributed equally to this work; Dai LC and Shao JZ designed research; Min LS, Xiao YT and Ma ZH performed research; Xiang LX contributed new reagents/analytic tools; Dai LC, Shao JZ, Min LS, Xiao YT and Ma ZH analyzed data; Dai LC, Min LS and Xiao YT wrote the paper.
Supported by The Medical Science Research Foundation of Zhejiang Province, No. 2004A083
Correspondence to: Li-Cheng Dai, Professor, Huzhou key laboratory of Molecular Medicine, Huzhou Central Hospital, Huzhou 313000, China. dlc@hzhospital.com
Telephone: +86-572-2033020 Fax: +86-572-2033020
Received: July 11, 2008 Revised: September 19, 2008
Accepted: September 26, 2008
Published online: October 28, 2008

Abstract

AIM: To investigate the ultrastructural location of midkine (MK) in nucleolus and function corresponding to its location.

METHODS: To investigate the ultrastructural location of MK in nucleolus with immunoelectronic microscopy. To study the role that MK plays in ribosomal biogenesis by real-time PCR. The effect of MK on anti-apoptotic activity of HepG2 cells was studied with FITC-conjugated annexin V and propidium iodide PI double staining through FACS assay.

RESULTS: MK mainly localized in the granular component (GC), dense fibrillar component (DFC) and the border between the DFC and fibrillar center (FC). The production of 45S precursor rRNA level was decreased significantly in the presence of MK antisense oligonucleotide in the HepG2 cells. Furthermore, it was found that exogenous MK could protect HepG2 from apoptosis significantly.

CONCLUSION: MK was constitutively translocated to the nucleolus of HepG2 cells, where it accumulated and mostly distributed at DFC, GC components and at the region between FC and DFC, MK played an important role in rRNA transcription, ribosome biogenesis, and cell proliferation in HepG2 cells. MK might serve as a molecular target for therapeutic intervention of human carcinomas.

Key words: 45S rRNA; Anti-apoptosis; Proliferation; Midkine

Peer reviewer: Xin-Yuan Guan, Professor, Department of Clinical Oncology, The University of Hong Kong, Room L10-56, 10/F, Laboratory Block, 21 Sassoon Road, Pokfulam, Hong Kong, China

Dai LC, Shao JZ, Min LS, Xiao YT, Xiang LX, Ma ZH. Midkine accumulated in nucleolus of HepG2 cells involved in rRNA transcription. *World J Gastroenterol* 2008; 14(40): 6249-6253 Available from: URL: http://www.wjgnet.com/1007-9327/14/6249.asp DOI: http://dx.doi.org/10.3748/wjg.14.6249

INTRODUCTION

Midkine (MK) is a cysteine-rich basic protein with a molecular weight of 13 Ku, which is strongly expressed during mid-gestation embryogenesis[1]. MK can be detected in most carcinoma specimens at a high level in a tissue type-independent manner, including those of esophageal, gastric, gall bladder, pancreas, colorectal, breast, and lung carcinomas, and Wilms' tumors[2-7]. Furthermore, MK exhibits several cancer-related activities, which include fibrinolytic, anti-apoptotic, mitogenic, transforming, angiogenic, and chemotactic activity[8-10]. Recently, Shibata *et al*[11] have shown that exogenous MK is endocytosed by cultured mouse L cells and then transported to the nucleus. However, more detailed information on the location and biological mechanism of MK within cells still remains to be elucidated. In a previous study, using green fluorescent protein (GFP) as a tracking molecule we have found that MK is exclusively localized to the nucleus and nucleolus in HepG2 cells[12]. We have also found that MK both with and without signal peptide is exclusively localized to the nucleus and accumulats in the nucleolus of DU145 and MCF7 cell lines[13].

In the present study, we demonstrated the ultrastructural location of MK in the nucleolus with immunoelectron microscopy. As is known, the nucleolus is a region of the nucleus that is known to be the locus for ribosomal biogenesis. This prompted us to hypothesize that MK may be involved in RNA

transcription and processing. We studied the effect of MK on rRNA synthesis and found 45S rRNA production level decreased in response to down-regulation of MK expression. Since cell proliferation and cancer survival require continuous protein synthesis that depends on a constant supply of ribosomes[14], 45S rRNA production may be affected by endogenous MK level, suggesting that cell proliferation is directly related to MK level. Investigating this possibility, we demonstrated that cell proliferation was inhibited by down-regulation of MK. Moreover, we reported that exogenous human MK is involved in anti-apoptotic activity of HepG2 cells.

MATERIALS AND METHODS

Immunoelectron microscopy

HepG2 cells were fixed with 3% paraformaldehyde and 1% glutaraldehyde at 4 degree for 2 h, and then sequentially dehydrated with 30%, 50% and 100% ethanol and embedded in Lowicryl K4M. Sections of 50 nm were cut and mounted on nickel grids. Non-specific binding was blocked with 1% BL (50 mmol/L PBS, pH 7.0, 1% BSA, 0.02% PEG20000, 100 mmol/L NaCl, 1% NaN_3) for 30 min at room temperature. Then, sections were incubated for 1 h at room temperature with anti-MK primary antibody (rabbit polyclonal to human MK, Abcam, UK) at a dilution of 1:100. After another treatment with BL, sections were incubated with 15 nm colloidal gold-labeled second antibody (goat polyclonal antibody to rabbit IgG, 15nm gold; Abcam, UK). Finally, sections were stained sequentially with uranyl acetate for 15 min and lead nitrate for 10 min. The ultrastructural distribution of MK was examined and photographed with a Hitachi H-800 transmission electron microscopy.

Antisense treatment

The sequence of MK morpholino antisense oligomer was as follows: MK-As (5'-AGGAAGCCTCGGTGCTGCA TCTCGC-3'). The sequence for MK-Sen was as follows: (5'-CGCTCTACGTCGTGGCTCCG AAGGA-3'). MK-Sen is a control oligonucleotide that has the same base composition as MK-As, but in the reverse sequence, and thus does not hybridize with MK mRNA. MK-As and MK-Sen were transfected into HepG2 cells in the presence of Lipofectamine-Plus (Life Technologies, Inc) in accordance with the manufacturer's instructions.

Real time PCR assay

The real-time PCR was performed with an RT-PCR kit (Takara, Japan) according to the manufacturer's instructions using GAPDH primers (5'-AACGACCCCTT CATTGAC-3' and 5'-TCCACGACA TACTCAGCAC-3'), MK primers (5'-AAACCGAACTCCAGGACCAGAGA C-3' and 5'-AACACTCGCTGCCCTTCTTCAC-3') and 45S primers (5'-CGCCGCTAGAGGTGAAATTC-3' and 5'-CATTCTTGGCAAATGCTTTCG-3'). Samples were amplified in a 7500 Real Time PCR system for 40 cycles using the following PCR parameters: 95℃ for 30 s, 58℃ for 1 min, and 72℃ for 1 min.

Cell proliferation assay

A total of 1.0×10^4 HepG2 cells were added to each well of a 96-well microtiter plate and allowed to attach overnight. Oligonucleotides at concentrations of 0.2, 0.4 and 0.6 μmol/L were transfected into HepG2 cells with Lipofectamine Plus (Life Technologies, Inc) following the manufacturer's instructions. The effects of antisense oligodeoxynucleotide on cellular viability were measured using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.

Analysis of apoptosis through FACS

1.0×10^5 HepG2 cells were seeded in six-well plates. Three wells were pre-treated with 500 ng/mL MK and three wells were treated with PBS as control for 3 h. Cells then were treated with 10^{-6} mol/L adriamycin, harvested 20 h later through trypsinization, and washed twice with cold PBS. The cells were centrifuged at 3000 r/min for 5 min, then the supernatant was discarded and the pellet was resuspended and incubated with FITC-conjugated annexin V and propidium iodide (Pharmingen) for 15 min at room temperature in the dark, and then analyzed by FACS.

RESULTS

The ultrastructural location of MK in HepG2 cells nucleolus

In a previous study, we demonstrated that MK exclusively localizes to the nucleus and nucleolus in HepG2 cells, using GFP as a tracking molecule[12]. At the ultrastructural level, the nucleolus includes three components: the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component (GC)[15]. Although we have found that MK accumulats in the nucleolus, the exact actions that MK performed on the nucleolus are still unclear. Therefore, we employed immunogold-labeling electron microscopy to investigate the ultrastructural location of MK, and found MK mainly localized in the GC, DFC and the border between the DFC and FC (Figure 1).

MK involved in rRNA transcription

In order to make clear that the function of MK corresponds to its ultrastructural location in the nucleolus, we investigated the role that MK plays in ribosomal biogenesis by real-time PCR. We found the production of 45S precursor rRNA in the HepG2 cells was decreased significantly in the presence of MK antisense oligonucleotide (Figure 2). This suggests that endogenous MK plays an important role in cancer cell proliferation.

MK promotes cell proliferation

Due to the central importance of rRNA transcription in cell growth, decreased rRNA transcription will slow cell proliferation. In this study, we showed that MK-As reduced cell proliferation rates by 41%, 48%, 58% after transfection of 0.2, 0.4 and 0.6 μmol/L MK antisense

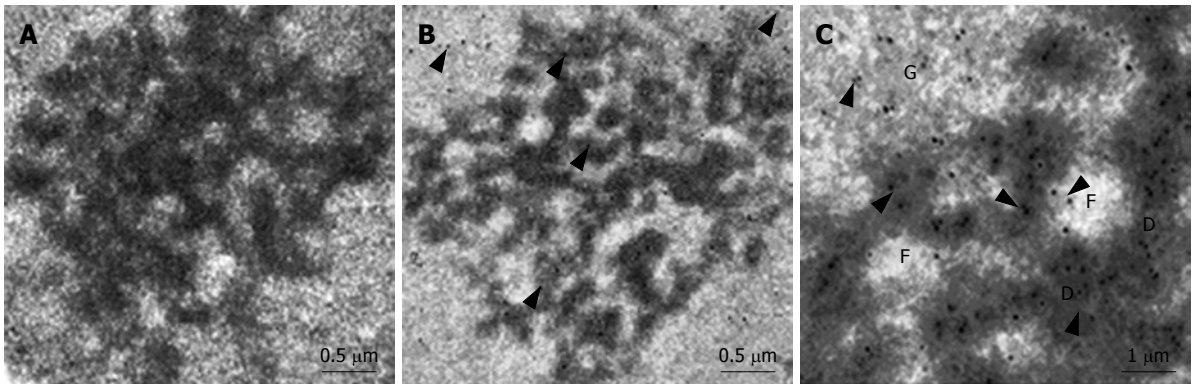


Figure 1 The location of MK in HepG2 cells nucleolus using immunogold labeling electron microscopy. HepG2 cells labelled without MK antibody were performed as the control. No immunogold particles of MK were seen (A). The MK protein (arrow) mostly localized to the DFC, GC and the region between FC and DFC (B, C) [scale bar represents 0.5 μm (A, B) and 1 μm (C)].

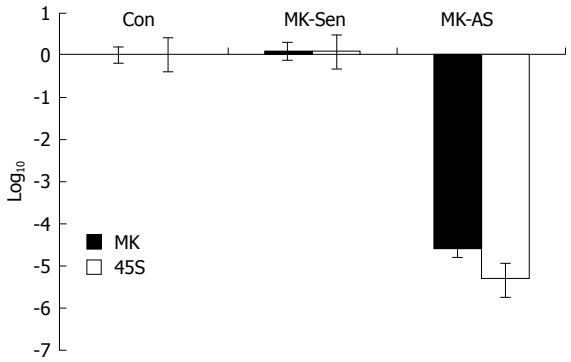


Figure 2 45S rRNA transcription could be regulated by endogenous MK level. It was shown that 45S rRNA transcription was decreased significantly in response to downregulation of MK expression, through real-time PCR analysis ($P < 0.05$).

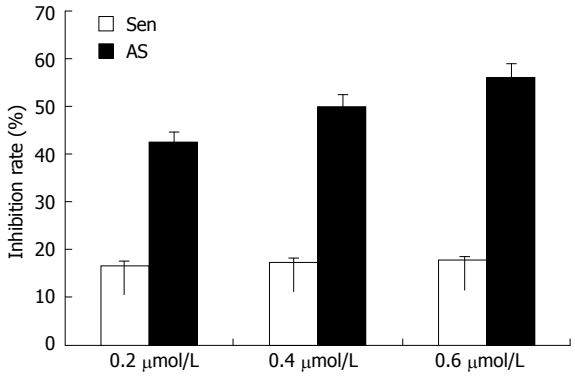


Figure 3 Effect of MK on proliferation of HepG2 cells. HepG2 cells were transfected with 0.2, 0.4 and 0.6 μmol/L MK-As or MK-Sen for 24 h, and were analyzed by MTT assay. Data show that HepG2 cell proliferation and growth were inhibited by downregulating the MK expression with antisense MK transfection ($P < 0.05$).

oligonucleotide into 10^4 HepG2 cells. The control MK-Sen did not inhibit cell proliferation significantly (Figure 3).

MK mediates anti-apoptotic activity

In order to investigate whether MK plays a role in the apoptosis of cancer cells, we added exogenous MK to HepG2 cells in the presence of adriamycin, inducing apoptosis. The results showed that exogenous MK could protect HepG2 from apoptosis significantly (Figure 4). This led us to suggest that MK may be a drug target for curing cancer.

DISCUSSION

In this study, we showed that MK was constitutively translocated to the nucleus of HepG2 cells, where it accumulated (Figure 1) and mostly distributed at the region between the FC and DFC, and GC components of the nucleolus (Figure 2). In a previous study, it has been demonstrated that each component of the nucleolus corresponds to a special biological function: the nascent transcripts appear in the junction region between the FC and DFC and accumulate in the DFC, and continue during the intranucleolar migration of the rRNA towards the GC. This implies that the role of the MK in the nucleolus is possibly related to control of rRNA gene transcription, pre-rRNA processing, and nascent ribosome subunit assembly, which could be the downstream elements of controlling cell proliferation. The finding that MK is involved in rRNA transcription in HepG2 cells in our study is significant for understanding cancer biology. One of the hallmarks of cancer is sustained cell growth and this can only be achieved by increased protein synthesis. To accommodate this need, there must be an increase in ribosome biogenesis. The role MK plays in rRNA transcription in cancer cells suggests that up-regulation of MK expression in various cancer cells not only induces tumor growth, but also directly contributes to cell proliferation. Thus, inhibitors targeting MK will be more effective than those that inhibit cancer cell proliferation alone. rRNA transcription regulates ribosome production and consequently, the translation potential of a cell, and increasingly expressed ribosomal proteins and rRNA transcription is an important factor in cancer transformation[16]. It is conceivable that deregulation of rRNA transcription may be an important determinant in transformation of cancer cells. Continuous nuclear translocation of MK in cancer cells can be possible one of the contributing factors in determinant of transformation. Indeed, inhibiting

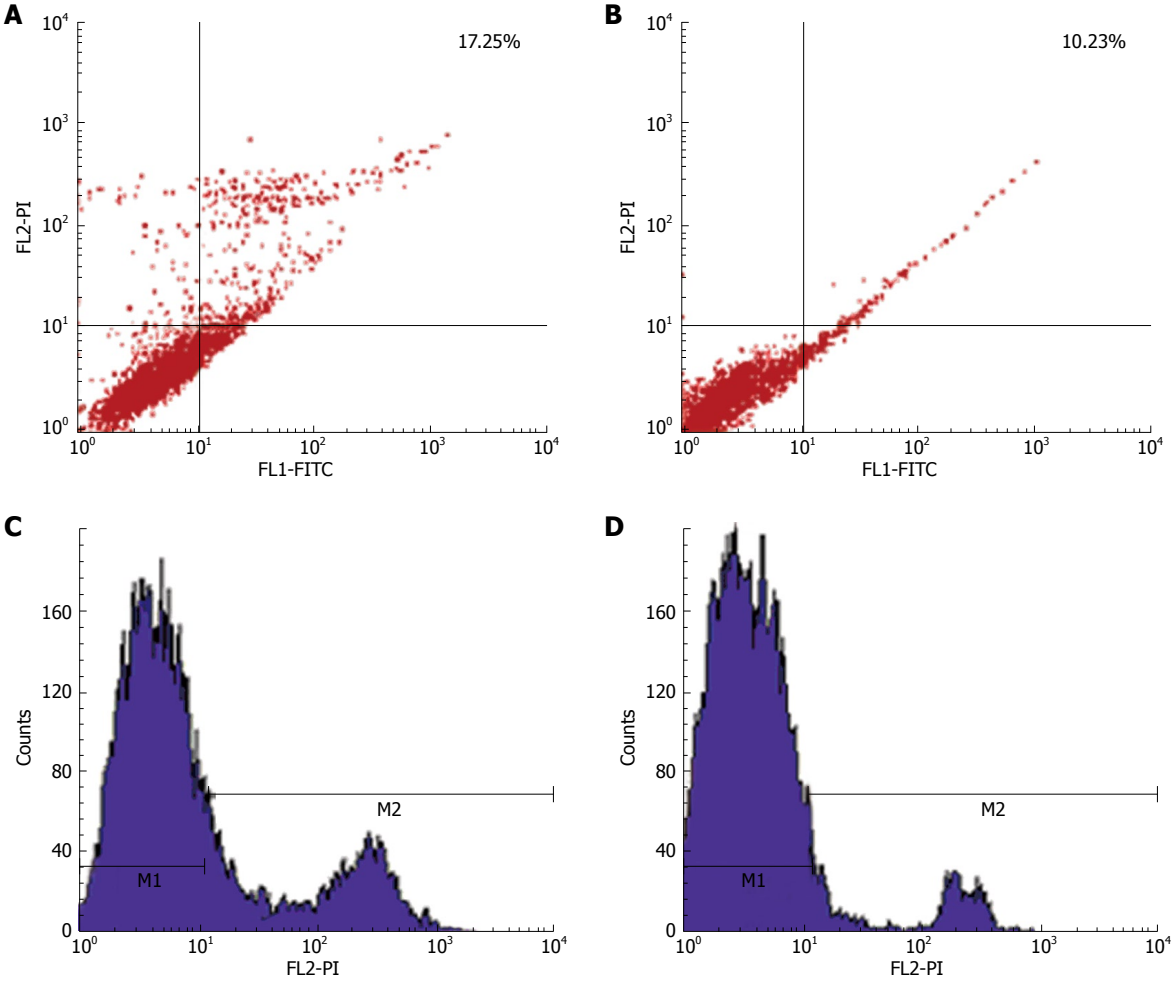


Figure 4 Exogenous MK mediates its anti-apoptotic activity. HepG2 cells are induced to apoptosis by 10^{-6} mol/L adriamycin for 20 h. It shows that about 17.25% of cells enter apoptosis (A, C), while 500 ng/mL exogenous MK showed its anti-apoptotic of activity (B, D).

MK expression reduced tumorigenicity and reversed the malignant phenotype of cancer cells, by antisense oligodeoxynucleotide targeted to MK. It is therefore possible that other effects such as repression of rRNA transcription of cancer cells also contributed to the marked anticancer activity. In conclusion, the results showed that MK played an important role in rRNA transcription, ribosome biogenesis, and cell proliferation in HepG2 cells. However, the mechanism by which MK stimulates rRNA transcription is still unclear at present. More extensive work is needed to understand how MK is translocated to the nucleus, whether it interacts with the RNA polymerase I machinery or binds to DNA.

COMMENTS

Background

Midkine (MK) has been found to play important roles in carcinogenesis, including mitogenic, anti-apoptotic, transforming, fibrinolytic, chemotactic, and angiogenic cancer-related activities. In a previous study, it has been demonstrated that each component of the nucleolus corresponds to a special biological function: the nascent transcripts appear in the junction region between the fibrillar center (FC) and dense fibrillar component (DFC) and accumulate, in the DFC, and continues during the intranucleolar migration of the rRNA towards the granular component (GC). This implies that the role of the MK in the nucleolus is possibly related to control of rRNA gene transcription, pre-rRNA processing, and nascent ribosome subunit assembly, which could be the downstream elements of controlling cell proliferation. In a previous study, we found that MK exclusively localized to the nucleus and nucleolus in HepG2 cells. However, it is unclear what is the function of MK in the nucleus and nucleolus.

Research frontiers

MK expression is increased in many human carcinomas, such as esophageal, stomach, colon, pancreatic, thyroid, lung, breast, urinary bladder, uterine, ovarian, prostate and hepatocellular carcinomas, osteosarcoma, neuroblastoma and glioblastoma. This phenomenon is observed in about 80% of cases in many types of carcinomas. Furthermore, MK exhibits several cancer-related activities, which include fibrinolytic, anti-apoptotic, mitogenic, transforming, angiogenic, and chemotactic activity.

Innovations and breakthroughs

In our study, we demonstrated that MK may be involved in 45S rRNA transcription. Since rRNA transcription is essential for tumor growth and proliferation, inhibition of MK-stimulated transcription of rRNA may be developed into a novel method to inhibit the growth of tumors.

Applications

The results may provide valuable evidence for the further study on the functions of MK in the nuclus and its mechanisms, in which rRNA transcription and ribosome assembly are involved. MK might serve as a molecular target for therapeutic intervention in human carcinomas.

Terminology

FC, DFC and GC are three components of the nucleolus. The nucleolus can be observed with the light microscope and its structure has more recently been clarified using the electron microscope.

Peer review

In this manuscript, authors report that the oncogenic function of MK could be associated with its role in 45S rRNA transcription. This finding is interesting.

REFERENCES

1 **Muramatsu T**. [Midkine (MK): a retinoic acid-responsive, heparin-binding growth factor in relationship with differentiation, development, cancer and neural function] *Seikagaku* 1993; **65**: 1494-1504
2 **Michikawa M**, Kikuchi S, Muramatsu H, Muramatsu T, Kim SU. Retinoic acid responsive gene product, midkine, has neurotrophic functions for mouse spinal cord and dorsal root ganglion neurons in culture. *J Neurosci Res* 1993; **35**: 530-539
3 **Michikawa M**, Xu RY, Muramatsu H, Muramatsu T, Kim SU. Midkine is a mediator of retinoic acid induced neuronal differentiation of embryonal carcinoma cells. *Biochem Biophys Res Commun* 1993; **192**: 1312-1318
4 **Garver RI Jr**, Radford DM, Donis-Keller H, Wick MR, Milner PG. Midkine and pleiotrophin expression in normal and malignant breast tissue. *Cancer* 1994; **74**: 1584-1590
5 **Take M**, Tsutsui J, Obama H, Ozawa M, Nakayama T, Maruyama I, Arima T, Muramatsu T. Identification of nucleolin as a binding protein for midkine (MK) and heparin-binding growth associated molecule (HB-GAM). *J Biochem* 1994; **116**: 1063-1068
6 **Kojima S**, Muramatsu H, Amanuma H, Muramatsu T. Midkine enhances fibrinolytic activity of bovine endothelial cells. *J Biol Chem* 1995; **270**: 9590-9596
7 **Adachi Y**, Matsubara S, Pedraza C, Ozawa M, Tsutsui J, Takamatsu H, Noguchi H, Akiyama T, Muramatsu T. Midkine as a novel target gene for the Wilms' tumor suppressor gene (WT1). *Oncogene* 1996; **13**: 2197-2203
8 **Kadomatsu K**, Muramatsu T. Midkine and pleiotrophin in neural development and cancer. *Cancer Lett* 2004; **204**: 127-143
9 **Kawai H**, Sato W, Yuzawa Y, Kosugi T, Matsuo S, Takei Y, Kadomatsu K, Muramatsu T. Lack of the growth factor midkine enhances survival against cisplatin-induced renal damage. *Am J Pathol* 2004; **165**: 1603-1612
10 **Friedrich C**, Holtkamp N, Cinatl J Jr, Sakuma S, Mautner VF, Wellman S, Michaelis M, Henze G, Kurtz A, Driever PH. Overexpression of Midkine in malignant peripheral nerve sheath tumor cells inhibits apoptosis and increases angiogenic potency. *Int J Oncol* 2005; **27**: 1433-1440
11 **Shibata Y**, Muramatsu T, Hirai M, Inui T, Kimura T, Saito H, McCormick LM, Bu G, Kadomatsu K. Nuclear targeting by the growth factor midkine. *Mol Cell Biol* 2002; **22**: 6788-6796
12 **Dai L**, Xu D, Yao X, Lu Y, Xu Z. Conformational determinants of the intracellular localization of midkine. *Biochem Biophys Res Commun* 2005; **330**: 310-317
13 **Dai LC**, Xu DY, Yao X, Min LS, Zhao N, Xu BY, Xu ZP, Lu YL. Construction of a fusion protein expression vector MK-EGFP and its subcellular localization in different carcinoma cell lines. *World J Gastroenterol* 2006; **12**: 7649-7653
14 **Hanahan D**, Weinberg RA. The hallmarks of cancer. *Cell* 2000; **100**: 57-70
15 **Cmarko D**, Verschure PJ, Rothblum LI, Hernandez-Verdun D, Amalric F, van Driel R, Fakan S. Ultrastructural analysis of nucleolar transcription in cells microinjected with 5-bromo-UTP. *Histochem Cell Biol* 2000; **113**: 181-187
16 **Ruggero D**, Pandolfi PP. Does the ribosome translate cancer? *Nat Rev Cancer* 2003; **3**: 179-192

S- Editor Xiao LL **L- Editor** Li M **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6254

World J Gastroenterol 2008 October 28; 14(40): 6254-6260
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Inhibition of pancreatic carcinoma cell growth *in vitro* by DPC4 gene transfection

Wei Shen, Guo-Qing Tao, De-Chun Li, Xing-Guo Zhu, Xia Bai, Bing Cai

Wei Shen, Guo-Qing Tao, Bing Cai, Department of General Surgery, Wuxi People's Hospital, Wuxi 214023, Jiangsu Province, China
De-Chun Li, Xing-Guo Zhu, General surgery department of the first affiliated hospital to Suzhou University, Suzhou 215007, Jiangsu Province, China
Xia Bai, Jiangsu Institute of Hematology, first affiliated hospital to Suzhou University, Suzhou, 215007, Jiangsu Province, China
Author contributions: Shen W, Tao GQ, Li DC and Cai B designed research; Shen W, Zhu XG, Bai X performed research; Cai B contributed with the EM; Shen W wrote the paper.
Correspondence to: Wei Shen, Department of General Surgery, Wuxi People's Hospital, Wuxi 214023, Jiangsu Province, China. shenweico@163.com
Telephone: +86-510-82700778 Fax: +86-510-85350555
Received: May 3, 2008 Revised: July 14, 2008
Accepted: July 21, 2008
Published online: October 28, 2008

Abstract

AIM: To detect the expression of DPC4 in malignant and non-malignant specimens of human pancreas, and observe the inhibition of retroviral pLXSN containing DPC4 on pancreatic carcinoma cells *in vitro*.

METHODS: The expression of DPC4 was determined in 40 pancreatic adenocarcinoma and 36 non-malignant pancreatic specimens by reverse-transcriptase polymerase chain reaction (RT-PCR) and immunohisto-chemistry. Furthermore, we constructed retroviral vectors containing DPC4, which then infected the pancreatic carcinoma cell line BxPC-3. Cell growth *in vitro* after being infected was observed, and the vascular endothelial growth factor (VEGF) mRNA level in the daughter cells was determined by semi-quantitative PCR assay.

RESULTS: The RT-PCR assay showed a positive rate of DPC4 mRNA in 100% (36/36) of normal specimens, compared to 40% (16/40) in adenocarcinoma specimens. The regional and intense positive cases of DPC4 expression in adenocarcinoma detected by immunohistochemistry were 10 and four, whereas it was all positive expression in normal tissues. There was a significant difference of DPC4 expression between them. The stable expression of DPC4 in the pancreatic carcinoma cells BxPC-3 could be resumed by retroviral vector pLXSN transfection, and could inhibit cell growth *in vitro*. Rather, DPC4 could decrease VEGF mRNA transcription levels.

CONCLUSION: The deletion of DPC4 expression in pancreatic carcinoma suggests that loss of DPC4 may be involved in the development of pancreatic carcinoma. The retroviral vector pLXSN containing DPC4 can inhibit the proliferation of pancreatic carcinoma cells, and down-regulate the level of VEGF.

Key words: Gene therapy; Pancreatic carcinoma; Retroviral vector

Peer reviewer: MA Hernandez Bartolome, General Surgery, Hospital Universitario Getafe, Getafe (Madrid) 28904, Spain

Shen W, Tao GQ, Li DC, Zhu XG, Bai X, Cai B. Inhibition of pancreatic carcinoma cell growth *in vitro* by DPC4 gene transfection. *World J Gastroenterol* 2008; 14(40): 6254-6260 Available from: URL: http://www.wjgnet.com/1007-9327/14/6254.asp DOI: http://dx.doi.org/10.3748/wjg.14.6254

INTRODUCTION

Chromosome 18q is lost in a high proportion of colorectal and pancreatic cancers. Three candidate tumor suppressor genes, DCC (deleted in colorectal carcinoma), DPC4 (deleted in pancreatic carcinoma, locus 4) and Smad2 have been identified in this chromosome region. The tumor suppressor DPC4, which was identified by Scott Kern in 1996[1], is frequently lost in many tumor cells, especially in pancreatic cells. DPC4, also named as Smad4, belongs to the evolutionarily conserved family of Smad proteins that are crucial intracellular mediators of signals from transforming growth factor-β (TGF-β)[2]. TGF-β regulates a wide variety of biological activities. Smad proteins can transduce the TGF-β signal at the cell surface into gene regulation in the nucleus.

Here, we detected the expression of DPC4 in 40 pancreatic adenocarcinoma and 36 non-malignant pancreatic specimens by RT-PCR and immunohistochemistry; then, we reintroduced the DPC4 gene in the pancreatic carcinoma cell line BxPC-3 (null for DPC4[3]), by transferring the retroviral vector pLXSN containing the DPC4 gene, in order to study inhibition of DPC4 gene expression in the pancreatic carcinoma cells *in vitro*.

MATERIALS AND METHODS

Patients and methods

Forty malignant pancreatic carcinoma and 36 corresponding non-cancerous tissues were obtained from the First Affiliated Hospital of Suzhou University and the Wuxi's People Hospital from 2003 to 2006. The clinical and pathological data from this patient population were readily available from pathology reports and a regularly updated clinical database. There were 28 males and 12 females with pancreatic carcinomas, and the average age of the patients was 55.18 ± 11.29 years old (mean ± SD). Tumor fragments were obtained in sterile conditions from different areas of the specimen and immediately placed in supplemented RPMI-1640 medium.

Reverse-transcriptase polymerase chain reaction (RT-PCR) for DPC4 mRNA

Total RNA was extracted from tissues with a single-step method. Randomly primed cDNAs were reverse-transcribed from 4 μg total RNA, which was extracted from about 1 g fresh specimen, using a cDNA synthesis kit in a 20-μL mixture. The 2-μL mixture was increased to 100 μL by adding 10 mmol/L Tris-HCI (pH 8.3), 50 mmol/L KCl_2, 1.5 nmol/L $MgCl_2$, 200 μL of each deoxynucleotide triphosphate, 6 U Taq polymerase, and 50 pmol of each of the specific oligonucleotide primers for DPC4. PCR amplification was performed in a DNA thermal cycler and consisted of 30 or 40 cycles of denaturation for 30 s at 94℃, annealing for 30 s at 58℃, and extension for 90 s 68℃. Final extension proceeded for 1 min at 68℃, Internal control for RNA quality was obtained with β-actin, which was amplified at 94℃ for 1 min, 55℃ for 1 min, and 72℃ for 2 min for 30 cycles. PCR amplification was performed by using primers 5'-CGGAATTCATGGACAATATGTCTATTACG-3' and 5'-GCGGATCCTCAGTCTAAAGGTTGTGG-3' for the DPC4 cDNA fragment. The product was about 1.6 kb. The PCR primers for β-actin were 5'-ACACTGTGCCCATCTACGAGG-3' and 5'-AGGGGCCGGACTCGTCATACT-3'. The product was 621 bp. All the products were run on a 1% agarose gel and visualized by ethidium bromide staining.

Immunohistochemical analysis for DPC4

Tissues were routinely fixed in neutral formalin and embedded in paraffin. After being deparaffinized, the slides were placed in a solution of 3% hydrogen peroxide for 5-10 min to block the activity of endogenous peroxidase. After being washed with distilled water and then dipped in PBS for 5 min, the slides were closed in normal sheep blood serum and then heated to room temperature for 10 min. Non-specific binding was blocked with a protein solution for 10 min, and then each slide was labeled with a 1:100 dilution of monoclonal antibody to DPC4 (murine anti-human; LAB Vision). Anti-DPC4 antibody was detected by adding secondary antibodies (rabbit anti-murine; Maxim Biotech, Fuzhou). After being incubated at 37℃ for 1 h, the slides were washed by PBS. The sections were counterstained with hematoxylin. Positive cells were stained dark brown in the nuclei and/or cytoplasm, and the staining was graded into three categories: no staining, weak staining, or heavy staining. Positive staining was considered as expression of DPC4. Normal pancreatic ducts, islets of Langerhans, acini, lymphocytes, and stromal fibroblasts showing moderate to strong expression of DPC4 served as positive internal controls for each section.

Construction of the retroviral vectors, cell culture and transfection

The human DPC4 cDNA was amplified from Smad4/DPC4-pBluescript plasmid (as a gift from Scott Kern) by PCR. The identity of the amplified fragments was confirmed by cycle sequencing using the manufacturer's directions (Shanghai Sangong Biological Engineering Technology and Servical Co., Ltd), then subcloned to the retroviral vector pLXSN to obtain pLXSN/DPC4+ recombinant with direct insertion, and packaged with GP+E86 and PA317 amphotropic packaging cells. AntiG418 clones were acquired and named as PA317/pLXSN DPC4+ cells. As a control, the empty vector pLXSN also was packaged with GP+E86 and PA317 cells and the antiG418 clones were named as PA317/pLXSN. The virus titer was elevated through cross infection from GP+E86 to PA317 cells and reached 6.0×10^5 pfu/L. DPC4 gene integration in PA317/pLXSN DPC4+ or PA317/pLXSN cells was confirmed by PCR assay.

Retroviral supernatant was obtained from the producer cell lines and maintained at 32℃ in 5% CO_2 atmosphere for 24-48 h. The BxPC-3 lines (purchased from Shanghai Institute for Biological Science, Chinese Academy Science) were transduced using the following protocol. One milliliter of the filtered supernatant was added to 4×10^5 target cells in the presence of 8 μg/mL polybrene. Cells and retroviral supernatant were incubated at 37℃ for 4 h. Medium from producer cells was then replaced by RPMI-1640 medium supplemented with 20% fetal calf serum and then in the presence of 2 μg/mL polybrene at 24-h intervals. After the last infection, the daughter cells were subjected to an initial period of selection in 0.2 mg/mL G418 for 4 d and then in 0.5 mg/mL for 1 wk. The positive cells were named as BxPC-3/DPC4. As a positive control, daughter cells transduced by empty vector were named as BxPC-3/pLXSN. As a negative control, mother cells were named as BxPC-3/-.

Western blot analysis

10^7 cells were lysed at 4℃ in a lysis buffer containing 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO, USA), and 25 mmol/L Tris (pH 7.5). The lysates were cleared by centrifugation and boiled for 5 min at 100℃ in Laemmli's SDS-PAGE sample buffer containing 100 mmol/L DTT. Proteins were resolved at 100 V on 10% polyacrylamide gels and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA, USA). The membranes were blocked with 5% non-fat dry milk, incubated with a Smad4/DPC4

monoclonal antibody (Neo Markers), and then incubated with the secondary clonal antibody. An enhanced chemiluminescence kit (Amersham, Arlington Heights, IL, USA) was used for detection.

MTT colorimetric growth assay for cells proliferation in vitro

Cell growth was determined by the MTT colorimetric growth assay as described previously. Cells were plated in three duplicate wells of a 96-well microtiter plate at 5×10^3 cells/well in 100 μL. After incubation at 37℃ in 5% CO_2, the cells were visually determined on each of the microtitration plates and 25 mL of RPMI-1640 containing 5 mg/mL of MTT was added to each well. Incubation was continued at 37℃ for 3 h. The content of each well was removed, and 200 mL of isopropanol containing 5% 1 mol/L HCl was added to extract the dye. After 30 min of incubation at room temperature and gentle agitation, the Absorbance (*A*) was measured with a microtitration plate spectrophotometer at 550 nm. The *A* of the blank, which consisted of an uninoculated plate incubated together with the inoculated plates, was subtracted from the *A* of the inoculated plates.

Semi-quantitative PCR assay

A semi-quantitative RT-PCR assay was performed to confirm the expression of vascular endothelial growth factor (VEGF) mRNA. Total RNA was extracted from the three cells, and 1 mg total RNA was reverse-transcribed into first strand cDNA in a reaction primed by oligo (dT) 12-18 primer using Superscript Ⅱ reverse transcriptase (Invitrogen). Two microliters of the first strand cDNA were used as template for the PCR reactions using Taq polymerase (Life Technologies, Inc.). The PCR reaction started at 94℃ for 2 min, followed by 35 cycles (94℃ for 30 s, 56℃ for 45 s, and 72℃ for 45 s), and ended with a 7-min incubation at 72℃. The primers of VEGF were 5'-GGGCCTCCGAAACCATGAACTT-3' and 5'-CGCATCAGGGGCACACAG-3'. The product size was 259 bp. Expression of β-actin was monitored as an internal control; the primers for β-actin were 5'-ACACTGTGCCCATCTACGAGG-3', 5'-AGGGGCCGGACTCGTCATACT-3', and the products was 621 bp. All RT-PCR products were separated by electrophoresis in 1.2% agarose gels and autoradiographed. All experiments were performed in triplicate.

Statistical analysis

Statistical analysis was performed using the Fisher's exact probability test and χ^2 analysis using the SAS statistical software. A two-tailed Student's *t* test was used for statistical analysis of comparative data. Values of $P < 0.05$ were considered significant.

RESULTS

RT-PCR for the DPC4 expression in the malignant pancreatic tissue

The RT-PCR assay showed a positive rate of DPC4 mRNA in 100% (36/36) in all normal specimens, compared to 40% (16/40) in adenocarcinoma specimens ($n = 76$, $\chi^2 = 31.5692$, $P < 0.0001$; Figure 1).

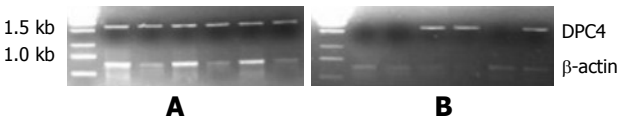


Figure 1 The mRNA of DPC4 by RT-PCR. A: Non-malignant pancreatic species; B: Malignant pancreatic species.

Immunohistochemical analysis for DPC4 expression in the malignant tissue

The regional and intense positive expression of DPC4 protein revealed by immunohistochemistry was 10 and four respectively, and all positive rate accounted for 35% (14/40), whereas there was all positive expression in normal tissues ($n = 76$, $\chi^2 = 35.568$, $P < 0.0001$; Figure 2).

PCR amplification of the DPC4 gene from Smad4/DPC4-pBluescript plasmid

The PCR product was about 1.6 kb in the electrophoresis gel as expected (Figure 3). pucm-T/DPC4 gene sequence was identified by Shanghai Sangong Biological Engineering Technology and Servical Co., Ltd., and agreed with our expected result.

Stable expression of DPC4 in the pancreatic carcinoma cell BxPC-3 after transfection demonstrated by RT-PCR and Western bloting

As an internal control, β-actin segments of about 838 bp were obtained in all three cell lines, which indicated that DNA was distilled effectually. DPC4 was expressed only in BxPC-3/DPC4 cells, but not the other control cells, BxPC-3/- and BxPC-3/pLXSN (Figure 4). An approximately 60-kDa protein blot, DPC4 protein, was obtained in BxPC-3/DPC4, but not in the BxPC-3/pLXSN or BxPC-3/- cells (Figure 5).

MTT for the cells grown in vitro

The BxPC-3/DPC4 cells grew much more slowly than the BxPC-3/pLXSN and BxPC-3/- cells. At day 7, the ratio of proliferation inhibition was about 50% ($F = 9.65$, $P = 0.0209$, BxPC-3/DPC4 *vs* BxPC-3/pLXSN; $F = 11.03$, $P = 0.0160$, BxPC-3/DPC4 *vs* BxPC-3/-; Figure 6).

Semi-quantitative PCR for VEGF mRNA

All three cell types had positive expression of VEGF and β-actin. However, semi-quantitative PCR assay showed the level of VEGF mRNA was much lower in BxPC-3/DPC4 than BxPC-3/pLXSN or BxPC-3/- (Table 1).

DISCUSSION

The majority of patients who present with pancreatic carcinoma have little chance of undergoing operative

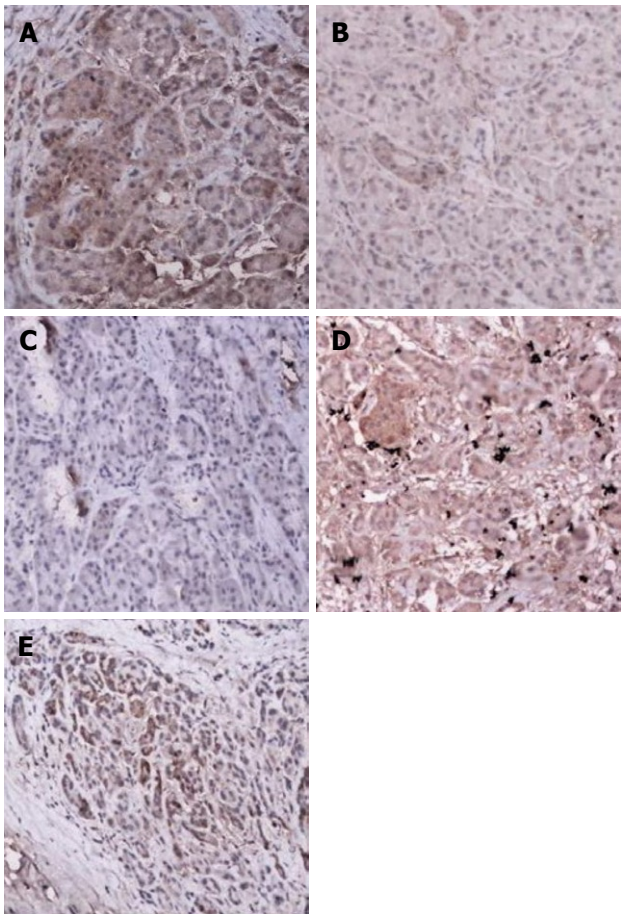


Figure 2 Immunohistochemical staining (x 100). A: Intense positive expression of DPC4 in the nuclei and/or cytoplasm in the normal pancreatic tissue; B: Weak expression of DPC4 in normal pancreatic tissue; C: Pancreatic carcinoma showed loss of DPC4 expression; D: DPC4 expression in an adenocarcinoma with a wild-type DPC4 gene; E: Intense positive expression of DPC4 in the malignant pancreatic tissue.

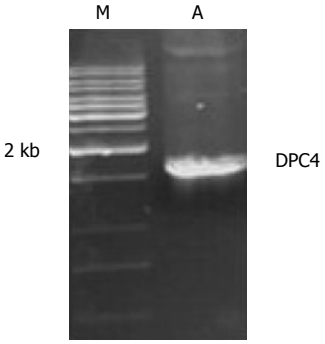


Figure 3 PCR amplification for the DPC4 gene. A: PCR product; M: Marker.

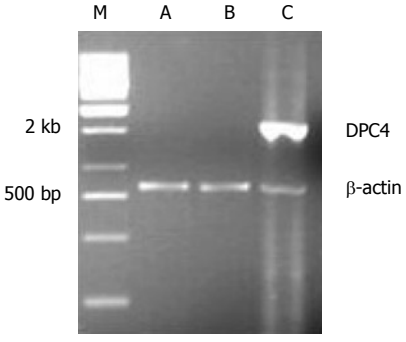


Figure 4 The expression of DPC4 mRNA in BxPC-3. M: Marker; A: BxPC-3/-; B: BxPC-3/pLXSN; C: BxPC-3/DPC4.

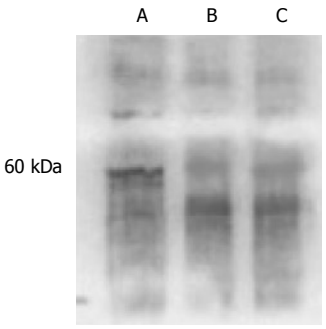


Figure 5 Westen blot for DPC4 in BxPC-3 cells. A: BxPC-3/DPC4; B: BxPC-3/pLXSN; C: BxPC-3/-.

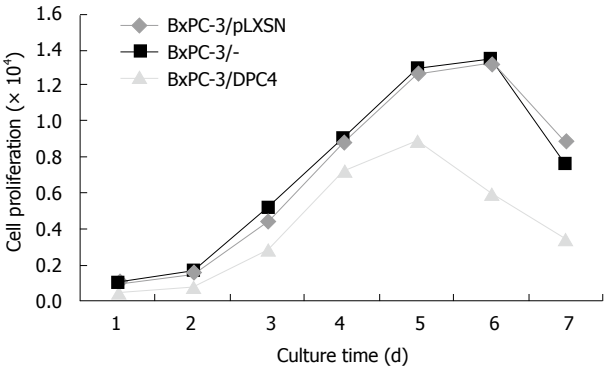


Figure 6 Cell growth curve. *P* = 0.0209, BxPC-3/DPC4 *vs* BxPC-3/pLXSN; *P* = 0.0160, BxPC-3/DPC4 *vs* BxPC-3/-.

Table 1 Semi-quantitative PCR for VEGF mRNA

	BxPC-3/DPC4	Bxpc-3/pLXSN	Bxpc-3/-	Ratio
VEGF	0.1887 ± 1.2399	0.3875 ± 2.0478	0.3910 ± 1.0714	2.0535
[1]*P*		0.035	0.021	

[1]*vs* BxPC-3/DPC4.

treatment, and non-operative treatments can offer little survival advantage. It is necessary to look for some other effective treatment for pancreatic carcinoma. TGF-β inhibits cell growth and/or induces apoptosis. In epithelium, disruption of the TGF-β signaling cascade is considered an important mechanism by which tumor cells can escape growth suppression. In a number of cancers, resistance to TGF-β growth inhibition is associated with mutations either in receptor Ⅱ or in the signal transducers. DPC4/Smad4 belongs to the evolutionarily conserved family of Smad proteins that are linked to the TGF-β superfamily of cytokines, forming a complex with R-Smads in response to ligand stimulation. Disruption of DPC4 can result in a TGF-β signal blackout and is involved in the regulation of cell differentiation, as well as the inhibition of cell proliferation. So, DPC4 is an essential signaling intermediate in the TGF-β receptor-mediated pathway. Abrogation of DPC4 function might cause a breakdown in this signaling pathway and loss of transcription of genes critical to cell-cycle control. Cells might therefore become TGF-β resistant and escape from TGF-β-mediated growth control and thereby contribute to tumorigenesis. C-terminal truncation of DPC4 protein

prevents DPC4 homomeric complex formation and heteromeric complex formation with activated Smad2. Furthermore, the mutant protein is unable to be recruited to DNA by transcription factors and hence cannot form transcriptionally active DNA-binding complexes[4]. Otherwise, oligo-ubiquitination positively regulates DPC4 function, whereas poly-ubiquitination primarily occurs in unstable cancer mutants and leads to protein degradation[5].

In the present study the relative contributions of three genes located at the 18q21 region (DCC, Smad2 and DPC4/Smad4) to progression and dissemination of human colorectal and pancreatic tumors were determined. DPC4 inactivation, always accompanied by alteration of all of the other three genes (K-ras, p53, p16)[6], has also been detected in colon, biliary tract, esophageal, gastric, ovarian, head and neck, lung and prostate cancer, especially in pancreatic carcinoma including one mutation and seven homozygous deletions[7-12]. Supporting evidence for the above observation was provided by Takaku and colleagues[13], who constructed knock-out mice with the DPC4 gene. Although DPC4-null mice were embryonically lethal, the heterozygotes of DPC4 were fertile and appeared normal up to the age of 1 year. However, gastric polyps developed in three of 15 heterozygous mice at the age of 50 wk, and in all heterozygous mice at the age of 100 wk. In addition, duodenal polyps were found in mice older than 50 wk. Morphologically, these polyps resembled those of human juvenile polypsis. These results suggest that inactivation of DPC4 is one of the early events in polyp formation in the DPC4 mice, which is analogous to human familial juvenile polyposis. Bartsch *et al*[14] have also revealed that the expression of DPC4 protein is associated with histopathological grades of pancreatic cancer. Meanwhile, DPC4 inactivation is associated with a poor prognosis[15-19]. Consistent with these reports, DPC4 was shown to be inactivated about in half of pancreatic cancer tissue in our study, whether mRNA or protein. Together with its deletion in pancreas carcinomas, these results suggest that DPC4 has the properties of a tumor suppressor gene, which indicates that it is involved in the carcinogenesis and development of pancreatic carcinoma and is a late event in pancreatic carcinogenesis. The present study can clarify the role of DPC4 in the development of pancreatic carcinoma. In a few substantial studies, significant prognostic markers for pancreatic carcinoma have been reported; markers such as tumor size, lymph node involvement, status of resection margins, DNA ploidy, degree of differentiation, and perineural invasion are inconclusive. In addition, preoperative estimation of tumor size and lymph node involvement is difficult. Deletion of DPC4 in pancreatic carcinoma and loss of DPC4 expression in those patients with poorly differentiated adenocarcinomas was significantly higher than that in those with well and moderately differentiated adenocarcinomas. Therefore, DPC4 gene might preserve phenotypic characteristics under normal conditions and control the malignant progression of pancreatic carcinoma. DPC4 may be proposed as a predictor of prognosis. Recently it was reported that expression of DPC4 can enhance the tumor response to drug treatment[20]. Hoever, it is regretable that we can not draw a consistent conclusion because of deficiency of the detailed clinical and survival data about these pancreatic carcinoma patients in our present study.

Since DPC4 plays a pivotal role in regulating all TGF-β superfamily signal pathways, it is reasonable to postulate that resumption of expression of DPC4 in pancreatic carcinoma cells can inhibit cell proliferation. In fact, DPC4 can induce growth inhibition in breast and colon tumor cells[21,22]**.** In order to develop an effective therapeutic intervention for patients with pancreatic cancer, we developed a new gene therapy that targets the genetic character of pancreatic cancer, using retroviruses that are selectively replication-competent in tumor cells. The DPC4 transcripts were cloned and subjected to sequence analysis. We performed reconstitution experiments of DPC4 in human pancreatic adenocarcinoma cell line BxPC-3. The wild-type DPC4 DNA was amplified from Smad4/DPC4-pBluescript plasmid by PCR and was enclosed successfully in the retroviral vector pLXSN. The pancreatic carcinoma cells BxPC-3 stably expressing DPC4 were obtained by retroviral transfection of DPC4 expression vectors and by selecting stable clones with G418. Stable transfection of BxPC-3 cells null for DPC4, accompanied by control vectors with DPC4 expression and an empty vector control, yielded similar numbers of G418-resistant clones. RT-PCR and Western blot analysis revealed restored expression of DPC4 in daughter clones derived from expression vector transfection. It was a feasible way to transfer the wild-type DPC4 gene to the DPC4-null cancer cells by pLXSN transfection. Some have reported that the DPC4 expression can inhibit growth of many tumor cells. In breast and colon carcinoma, DPC4 inhibited cell proliferation and induced anoikis[23,24]. Dai *et al*[25] have explored an inducible system in which DPC4 protein is activated by translocation to the nucleus, when cell lines that stably express wild-type or mutant DPC4 proteins fused to a murine estrogen receptor domain, are treated with 4-hydroxytamoxifen. This induced DPC4-mediated transcriptional activation and a decrease in growth rate, attributable to cell cycle arrest at the G1 phase and induction of apoptosis. In our study, MTT showed that the restored expression of DPC4 in the pancreatic cells can inhibit proliferation by approximately 50% *in vitro*. These data show that restored expression of functional DPC4 can be efficiently obtained via retrovirus-mediated gene transfer. Supporting our hypothesis, we found that restoration of DPC4 significantly delayed tumor growth *in vitro*.

The present study indicates that DPC4-inducible apoptosis has the greater consequence in growth control. Indeed, the period of the greatest growth suppression temporally was better correlated with apoptotic responses than with cell cycle arrest. This induced DPC4-mediated transcriptional activation and a decrease

in growth rate, attributable to cell cycle arrest at the G1 phase and induction of apoptosis in approximately 55% of pancreatic adenocarcinomas[24,25]. To date, two major apoptotic pathways, the death receptor and the mitochondrial pathway, have been well documented in mammalian cells. However, the involvement of these two apoptotic pathways, particularly the death receptor pathway, in TGF-β1-induced apoptosis is not well understood. Kim *et al*[21] have reported that apoptosis of human gastric SNU-620 carcinoma cells induced by TGF-β1 is caused by the Fas death pathway, in a Fas-ligand-independent manner, and that the Fas death pathway activated by TGF-β1 is linked to the mitochondrial apoptotic pathway.

Most solid tumor growth is dependent on angiogenesis, and the tumor growth and invasion can be inhibited through anti-angiogenesis. Serum levels of VEGF can decrease significantly after radical resection of the tumor. Elevated preoperative serum VEGF level is a significant prognostic factor, although not independent of stage, for patient survival[26,27]. A decrease in the levels of VEGF could be observed upon restoration of DPC4 expression in cell lines. This effect of DPC4 was found in the present study, implicating DPC4 for the first time as an inhibitor of pancreatic tumor angiogenesis. We found the level of VEGF mRNA level was decreased in the BxPC-3 cells after DPC4 resumption, as demonstrated by the semiquantitative RT-PCR. The retrovirus transfer of DPC4 in DPC4-null cells restored its expression and function, and may be correlated with the suppression of angiogenesis and invasion. However, it is not clear how DPC4 controls VEGF. Other experimental evidence indicates that DPC4 regulates an angiogenic switch by decreasing the expression of VEGF and increasing the levels of angiogenesis inhibitor thrombospondin-1 (TSP-1). It has been reported that DPC4 downregulates VEGF transcription and the secreted matrix metalloproteinase-2 (MMP-2) and MMP-9 expression levels consistently in pancreatic adenocarcinoma cell lines. There was a significant reduction in the tissue immunoreactivity of MMP-2 (a protease activated in angiogenic vasculature), and MMP-9 in samples from mice bearing DPC4-transfected tumors, compared to those from control groups. MMPs have been implicated in primary and metastatic tumor growth and angiogenesis, as well as in tumor invasion and progression. VEGF is a strong inducer and activator of MMP-2, while MMP-9 has been shown to increase the availability of VEGF to its receptors and identifying TSP-1 and VEGF as relevant tumor targets[21].

In summary, our study describes the deletion of tumor suppressor DPC4 in pancreatic carcinoma, and restoration of expression of DPC4 in human cancer cell lines growing *in vitro* showed the expected results. DPC4 decreased the expression of VEGF. We demonstrated that DPC4 mediates growth inhibition in pancreatic tumour cells even without TGF-β present, and suggest that DPC4 has the potency of a tumor suppressor gene.

ACKNOWLEDGMENTS

We thank Xia Bai for expert technical assistance.

COMMENTS

Background

Pancreatic carcinoma patients have poor survival, even those who have undergone surgery. The tumor suppressor DPC4 belongs to the evolutionarily conserved family of Smad proteins that are crucial intracellular mediators of signals from TGF-β and is frequently lost in many tumor cells, especially in pancreatic cells. The deletion of DPC4 is involved in the carcinogenesis and development of pancreatic carcinoma. How to further study the role of DPC4 and resumption of the DPC4 gene expression in the PC cell line by the transferring of the vectors containing DPC4 gene in order to inhibit PC growth is becoming a hot topic.

Research frontiers

It is necessary to develop a new modality of treatment for pancreatic cancer. Gene therapy strategies may provide therapeutic benefits with a more favorable risk-benefit ratio than the current conventional treatments. With the advances in understanding the pathogenesis, progression, and metastasis of pancreatic carcinoma that have been achieved, studies on gene therapy for pancreatic carcinoma have been attempted in different ways, such as inhibiting oncogenes, and activating tumor suppressor genes. New specific target genes and further development of gene technology may bring a break-through in this field.

Innovations and breakthroughs

We demonstrated that DPC4 can mediate growth inhibition in pancreatic tumor cells even without TGF-β present and reestablish one of the key regulatory controls of cell proliferation. Although numerous attempts have been made and different approaches have been used to identify the target genes, only limited success has been achieved. Our data showed that VEGF may be one of the DPC4-regulated downstream target genes, which will extend our understanding of the mechanism for DPC4 as an inhibitor of pancreatic tumor angiogenesis.

Applications

DPC4 is an important tumor suppressor. Further study on the biological nature of DPC4 may contribute to the study of the etiology of pancreatic cancer, and offer a theoretical basis for gene therapy of pancreatic cancer.

Peer review

In this study the authors demonstrated that deletion of DPC4 in pancreatic carcinoma, and restoring DPC4 expression in pancreatic carcinoma cells could effectively inhibit cancer cell growth *in vitro,* even without the presence of TGF-β. Re-expression of DPC4 in pancreatic carcinoma cells can downregulate VEGF mRNA expression and anti-angiogenesis therapy may represent a promising therapeutic option.

REFERENCES

1 **Hahn SA**, Schutte M, Hoque AT, Moskaluk CA, da Costa LT, Rozenblum E, Weinstein CL, Fischer A, Yeo CJ, Hruban RH, Kern SE. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* 1996; **271**: 350-353

2 **Ijichi H**, Ikenoue T, Kato N, Mitsuno Y, Togo G, Kato J, Kanai F, Shiratori Y, Omata M. Systematic analysis of the TGF-beta-Smad signaling pathway in gastrointestinal cancer cells. *Biochem Biophys Res Commun* 2001; **289**: 350-357

3 **Bartsch D**, Barth P, Bastian D, Ramaswamy A, Gerdes B, Chaloupka B, Deiss Y, Simon B, Schudy A. Higher frequency of DPC4/Smad4 alterations in pancreatic cancer cell lines than in primary pancreatic adenocarcinomas. *Cancer Lett* 1999; **139**: 43-49

4 **Maurice D**, Pierreux CE, Howell M, Wilentz RE, Owen MJ, Hill CS. Loss of Smad4 function in pancreatic tumors: C-terminal truncation leads to decreased stability. *J Biol Chem* 2001; **276**: 43175-43181

5 **Moren A**, Hellman U, Inada Y, Imamura T, Heldin CH, Moustakas A. Differential ubiquitination defines the functional status of the tumor suppressor Smad4. *J Biol Chem* 2003; **278**: 33571-33582

6 **Moore PS**, Sipos B, Orlandini S, Sorio C, Real FX, Lemoine NR, Gress T, Bassi C, Kloppel G, Kalthoff H, Ungefroren H, Lohr M, Scarpa A. Genetic profile of 22 pancreatic carcinoma cell lines. Analysis of K-ras, p53, p16 and DPC4/Smad4. *Virchows Arch* 2001; **439**: 798-802

7 **Koyama M**, Ito M, Nagai H, Emi M, Moriyama Y. Inactivation of both alleles of the DPC4/SMAD4 gene in advanced colorectal cancers: identification of seven novel somatic mutations in tumors from Japanese patients. *Mutat Res* 1999; **406**: 71-77

8 **Ju HR**, Jung U, Sonn CH, Yoon SR, Jeon JH, Yang Y, Lee KN, Choi I. Aberrant signaling of TGF-beta1 by the mutant Smad4 in gastric cancer cells. *Cancer Lett* 2003; **196**: 197-206

9 **Woodford-Richens K**, Williamson J, Bevan S, Young J, Leggett B, Frayling I, Thway Y, Hodgson S, Kim JC, Iwama T, Novelli M, Sheer D, Poulsom R, Wright N, Houlston R, Tomlinson I. Allelic loss at SMAD4 in polyps from juvenile polyposis patients and use of fluorescence in situ hybridization to demonstrate clonal origin of the epithelium. *Cancer Res* 2000; **60**: 2477-2482

10 **Xie W**, Mertens JC, Reiss DJ, Rimm DL, Camp RL, Haffty BG, Reiss M. Alterations of Smad signaling in human breast carcinoma are associated with poor outcome: a tissue microarray study. *Cancer Res* 2002; **62**: 497-505

11 **Wang LH**, Kim SH, Lee JH, Choi YL, Kim YC, Park TS, Hong YC, Wu CF, Shin YK. Inactivation of SMAD4 tumor suppressor gene during gastric carcinoma progression. *Clin Cancer Res* 2007; **13**: 102-110

12 **Tarafa G**, Villanueva A, Farre L, Rodriguez J, Musulen E, Reyes G, Seminago R, Olmedo E, Paules AB, Peinado MA, Bachs O, Capella G. DCC and SMAD4 alterations in human colorectal and pancreatic tumor dissemination. *Oncogene* 2000; **19**: 546-555

13 **Takaku K**, Miyoshi H, Matsunaga A, Oshima M, Sasaki N, Taketo MM. Gastric and duodenal polyps in Smad4 (Dpc4) knockout mice. *Cancer Res* 1999; **59**: 6113-6117

14 **Bartsch D**, Barth P, Bastian D, Ramaswamy A, Gerdes B, Chaloupka B, Deiss Y, Simon B, Schudy A. Higher frequency of DPC4/Smad4 alterations in pancreatic cancer cell lines than in primary pancreatic adenocarcinomas. *Cancer Lett* 1999; **139**: 43-49

15 **Biankin AV**, Morey AL, Lee CS, Kench JG, Biankin SA, Hook HC, Head DR, Hugh TB, Sutherland RL, Henshall SM. DPC4/Smad4 expression and outcome in pancreatic ductal adenocarcinoma. *J Clin Oncol* 2002; **20**: 4531-4542

16 **Liu F**. SMAD4/DPC4 and pancreatic cancer survival. Commentary re: M. Tascilar et al., The SMAD4 protein and prognosis of pancreatic ductal adenocarcinoma. Clin. Cancer Res., 7: 4115-4121, 2001. *Clin Cancer Res* 2001; **7**: 3853-3856

17 **Maitra A**, Molberg K, Albores-Saavedra J, Lindberg G. Loss of Dpc4 expression in colonic adenocarcinomas correlates with the presence of metastatic disease. *Am J Pathol* 2000; **157**: 1105-1111

18 **Isaksson-Mettavainio M**, Palmqvist R, Forssell J, Stenling R, Oberg A. SMAD4/DPC4 expression and prognosis in human colorectal cancer. *Anticancer Res* 2006; **26**: 507-510

19 **Tascilar M**, Skinner HG, Rosty C, Sohn T, Wilentz RE, Offerhaus GJ, Adsay V, Abrams RA, Cameron JL, Kern SE, Yeo CJ, Hruban RH, Goggins M. The SMAD4 protein and prognosis of pancreatic ductal adenocarcinoma. *Clin Cancer Res* 2001; **7**: 4115-4121

20 **Boulay JL**, Mild G, Lowy A, Reuter J, Lagrange M, Terracciano L, Laffer U, Herrmann R, Rochlitz C. SMAD4 is a predictive marker for 5-fluorouracil-based chemotherapy in patients with colorectal cancer. *Br J Cancer* 2002; **87**: 630-634

21 **Kim SG**, Jong HS, Kim TY, Lee JW, Kim NK, Hong SH, Bang YJ. Transforming growth factor-beta 1 induces apoptosis through Fas ligand-independent activation of the Fas death pathway in human gastric SNU-620 carcinoma cells. *Mol Biol Cell* 2004; **15**: 420-434

22 **Duda DG**, Sunamura M, Lefter LP, Furukawa T, Yokoyama T, Yatsuoka T, Abe T, Inoue H, Motoi F, Egawa S, Matsuno S, Horii A. Restoration of SMAD4 by gene therapy reverses the invasive phenotype in pancreatic adenocarcinoma cells. *Oncogene* 2003; **22**: 6857-6864

23 **Schwarte-Waldhoff I**, Volpert OV, Bouck NP, Sipos B, Hahn SA, Klein-Scory S, Luttges J, Kloppel G, Graeven U, Eilert-Micus C, Hintelmann A, Schmiegel W. Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis. *Proc Natl Acad Sci USA* 2000; **97**: 9624-9629

24 **Ramachandra M**, Atencio I, Rahman A, Vaillancourt M, Zou A, Avanzini J, Wills K, Bookstein R, Shabram P. Restoration of transforming growth factor Beta signaling by functional expression of smad4 induces anoikis. *Cancer Res* 2002; **62**: 6045-6051

25 **Dai JL**, Bansal RK, Kern SE. G1 cell cycle arrest and apoptosis induction by nuclear Smad4/Dpc4: phenotypes reversed by a tumorigenic mutation. *Proc Natl Acad Sci USA* 1999; **96**: 1427-1432

26 **Karayiannakis AJ**, Bolanaki H, Syrigos KN, Asimakopoulos B, Polychronidis A, Anagnostoulis S, Simopoulos C. Serum vascular endothelial growth factor levels in pancreatic cancer patients correlate with advanced and metastatic disease and poor prognosis. *Cancer Lett* 2003; **194**: 119-124

27 **Khorana AA**, Hu YC, Ryan CK, Komorowski RA, Hostetter G, Ahrendt SA. Vascular endothelial growth factor and DPC4 predict adjuvant therapy outcomes in resected pancreatic cancer. *J Gastrointest Surg* 2005; **9**: 903-911

S- Editor Zhong XY **L- Editor** Li M **E- Editor** Yin DH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6261

World J Gastroenterol 2008 October 28; 14(40): 6261-6264
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Solitary fibrous tumor of the liver expressing CD34 and vimentin: A case report

Dimitris P Korkolis, Katerina Apostolaki, Chrysanthi Aggeli, George Plataniotis, Emmanuel Gontikakis, Dimitra Volanaki, Maria Sebastiadou, Dimitris Dimitroulopoulos, Dimitris Xinopoulos, George N Zografos, Perikles P Vassilopoulos

Dimitris P Korkolis, Chrysanthi Aggeli, George Plataniotis, Emmanuel Gontikakis, Dimitra Volanaki, Perikles P Vassilopoulos, Department of Surgical Oncology, Hellenic Anticancer Institute, "Saint Savvas" Hospital, Athens, Greece
Katerina Apostolaki, Maria Sebastiadou, Department of Surgical Pathology, Hellenic Anticancer Institute, "Saint Savvas" Hospital, Athens, Greece
Dimitris Dimitroulopoulos, Dimitris Xinopoulos, Department of Gastroenterology, Hellenic Anticancer Institute, "Saint Savvas" Hospital, Athens, Greece
George N Zografos, Department of Surgery, General Hospital of Athens, "G. Gennimatas", Athens, Greece
Author contributions: Korkolis DP and Aggeli C designed the research; Korkolis DP, Aggeli C, Plataniotis G, Gontikakis E, Volanaki D, Vassilopoulos PP performed the research; Apostolaki K and Sebastiadou M performed the data analysis; Dimitroulopoulos D and Xinopoulos D contributed to the interpretation of data and Zografos GN had important intellectual contributions.
Correspondence to: Dimitris P Korkolis, MD, PhD, Department of Surgical Oncology, Hellenic Anticancer Institute, "Saint Savvas" Hospital, 22 Socratous Street, 1st Floor, Kifissia, 14561 Athens, Greece. dkorkolis_2000@yahoo.com
Telephone: +30-210-8083743 Fax: +30-210-8012689
Received: June 28, 2008 Revised: October 16, 2008
Accepted: October 23, 2008
Published online: October 28, 2008

Abstract

A case of a successfully treated solitary fibrous tumor (SFT) of the liver is reported. An 82-year-old female presented with left upper abdominal discomfort, a firm mass on palpation, and imaging studies revealed a large tumor, 15 cm in diameter, arising from the left lobe of the liver. A formal left hepatectomy was performed. Microscopic evaluation showed spindle and fibroblast-like cells within the collagenous stroma. Immunohistochemistry disclosed diffuse CD34 and positive vimentin, supporting the diagnosis of a benign SFT. The patient remained well 21 months after surgery. SFT of the liver is a very rare neoplasm of mesenchymal origin. In most cases it is a benign lesion, although some may have malignant histological features and recur locally or metastasize. With less than 30 reported cases in the literature, little can be said regarding its natural history or the benefits of adjuvant radiochemotherapy. Complete surgical resection remains the cornerstone of its treatment.

Key words: Liver neoplasm; Solitary fibrous tumor; CD34; Vimentin

Peer reviewers: Barry Rosser, MD, Department of Transplantation, Mayo Clinic Jacksonville, Suite 1100, 4205 Belfort Road, Jacksonville, Florida 32216, United States; Michael Torbenson, MD, Associate Professor of Pathology, Room B314, 1503 E Jefferson (Bond Street Building), The Johns Hopkins University School of Medicine, Baltimore, MD 21231, United States

Korkolis DP, Apostolaki K, Aggeli C, Plataniotis G, Gontikakis E, Volanaki D, Sebastiadou M, Dimitroulopoulos D, Xinopoulos D, Zografos GN, Vassilopoulos PP. Solitary fibrous tumor of the liver expressing CD34 and vimentin: A case report. *World J Gastroenterol* 2008; 14(40): 6261-6264 Available from: URL: http://www.wjgnet.com/1007-9327/14/6261.asp DOI: http://dx.doi.org/10.3748/wjg.14.6261

INTRODUCTION

Solitary fibrous tumor (SFT) is a rare neoplasm of mesenchymal origin that occurs preferentially in the pleura, meninges, orbit, upper respiratory tract, thyroid and peritoneum. In extremely unusual cases, SFT may arise from the liver parenchyma of adult patients[1]. In the English literature, less than 30 cases of SFTs of the liver have been reported[2-5].

Clinical or radiological findings are not specific and cannot exclude malignancy. Preoperative cytology may be inconclusive or misleading. Immunohistologically, CD34, vimentin and desmin should be used as markers to precisely diagnose an SFT of the liver[3]. In most cases, there is low cellularity with minimal atypia or necrosis, making this a benign entity. Occasionally, a large size, high mitotic rate, cellular pleomorphism, atypia and central necrosis are interpreted as features suggestive of malignant behavior. As a result of its rarity, overall experience is insignificant. The outcome of an SFT of the liver is mostly related to resectability,

although correlated with neither pathological grade nor tumor size[4]. Thus, complete surgical removal of the neoplasm is most commonly proposed. We describe a new case of SFT of the liver and review the literature.

CASE REPORT

An 82-year-old female patient was referred to our institution on account of persistent abdominal discomfort and a palpable firm mass in the left upper quadrant. She had a past medical history of severe sleep-apnea syndrome and was recovering from a recent episode of mild gallstone pancreatitis. Laboratory tests, including liver biochemical profile and tumor markers were within normal range. Ultrasonography revealed an ovoid mass in the left lobe of the liver. Dynamic computed tomography (CT) and magnetic resonance imaging demonstrated a large, space-occupying lesion arising from the left lobe and compressing the stomach, pancreas and hepatoduodenal ligament. The lesion showed an early arterial enhancement and delayed venous washing out. The tumor developed in a polycystic liver parenchyma. It was well defined and measured about 15 cm in diameter. No direct invasion of the great vessels or adjacent intraperitoneal structures was seen (Figure 1). Preoperative fine needle aspiration biopsy under CT guidance, performed in another institution, was suggestive of a primary hepatocellular carcinoma. The patient underwent formal left hepatectomy and cholecystectomy. On gross examination, the resected specimen measured 18 cm × 15 cm × 8 cm and weighed 1275 g (Figure 2A). On the cut section, the tumor was firm, lobulated, well-demarcated, greyish-white, with a whorled and fasciculated surface and focal myxoid degeneration (Figure 2B). No cirrhosis or fibrosis was observed in the peripheral liver parenchyma. Resection margins were free of tumor. Pathological examination showed a highly cellular neoplasm composed of monomorphic spindle and fibroblast-like cells arranged in a storiform pattern. They were intermingled with dense bundles of collagen. In some areas, tumor cells were arranged around ectatic vessels in a hemangiopericytoma-like pattern (Figure 3A). No mitotic figures or nuclear polymorphism were noted. Immunohistochemical studies revealed a strong cytoplasmic positivity for CD34 (Figure 3B), vimentin (Figure 3C), desmin and Bcl-2. An absence of staining for HHF-35, S-100, CD117, muscle-specific actin and cytokeratin was observed. The percentage of ki-67 positive tumor cells was less than 5%. On the basis of the aforementioned results, pathological evaluation ascertained the presence of a benign SFT of the liver.

The patient recovered uneventfully after the procedure. She was discharged home on the 6th postoperative day and followed-up on an outpatient basis. Twenty-one months after surgery, the patient was stll doing well with no evidence of recurrence.

DISCUSSION

SFTs of the liver are unusual neoplasms with fewer than 30 previous cases reported in the world literature. There is a 2:1 female-to-male predominance with the ages of affected individuals ranging from 16 to 83 years. The mean age at diagnosis is 55 years and the average follow-up period reaches 27 months[1]. Most SFTs of the liver are usually found as giant lesions growing in either the right or the left lobe of a non-cirrhotic liver, causing non-specific symptoms of fullness and pressure, gastrointestinal obstruction, weight loss or hypoglycemia[3,5]. In addition, the development of a primary SFT on a background of polycystic liver disease, as in the present case, has never been reported before.

As a result of their extreme rarity, overall experience of SFTs is limited. In addition, the difficulty of

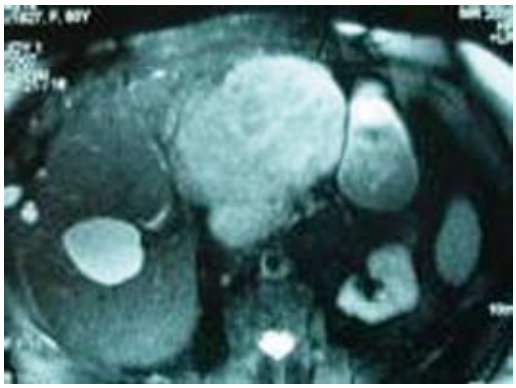


Figure 1 Magnetic resonance imaging demonstrating a large, 15 cm in size, well-circumscribed lesion in the left hepatic lobe, compressing the stomach, pancreas and hepatoduodenal ligament.

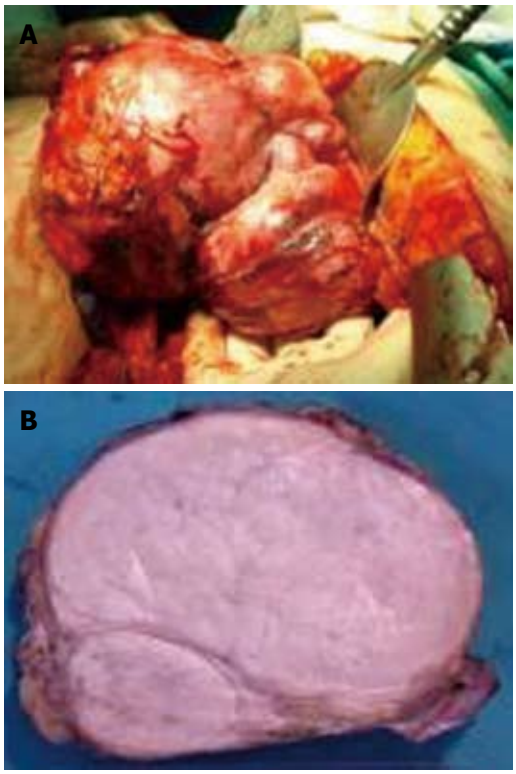


Figure 2 Gross appearance of a firm, well-demarcated and lobulated SFT of the liver (A) and greyish-white SFT of the liver with whorled and fasciculated surface and focal myxoid degeneration on cut section (B).

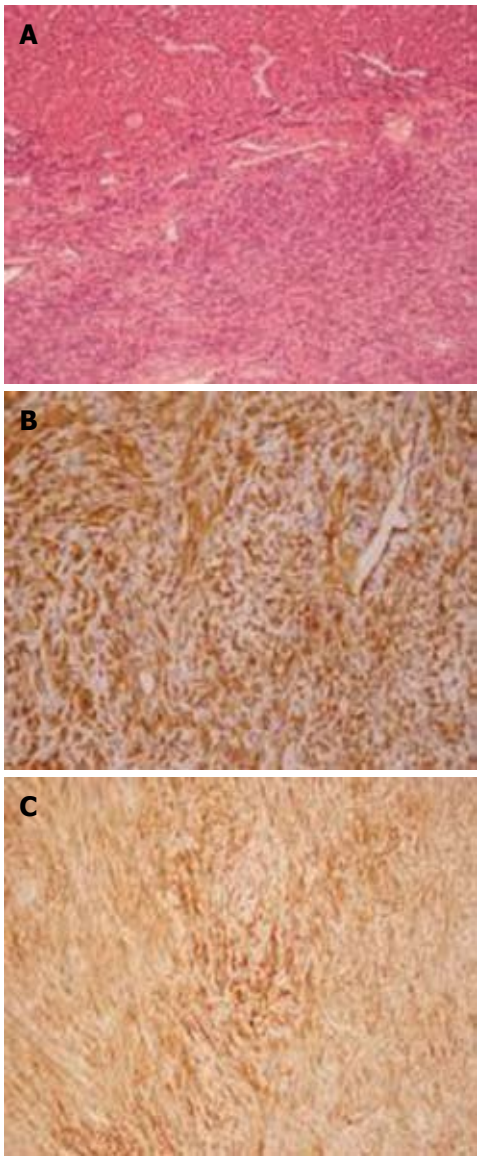


Figure 3 Tumor cells. A: Microscopy showing a tumor composed of uniform collagen-forming spindle cells arranged in interlacing fascicles and well-encapsulated and differentiated from the adjacent non-cirrhotic liver parenchyma (HE, x 100); B: CD34 immunohistochemical staining demonstrating diffusely strong reactivity (x 200); C: Tumor cells showing diffuse immunohistochemical positivity for vimentin (x 200).

interpretation of histological pictures and the huge volume at presentation still raise problems in terms of correct preoperative diagnosis and proper clinical management[6]. In the present case, the diagnosis of SFT of the liver was based on the association of characteristic histological and immunohistochemical features, i.e. high cellular proliferation of spindle cells arranged in a storiform pattern, together with the immunohistochemical staining profile of CD34 (+), vimentin (+), Bcl-2 (+) and cytokeratin (-), which is highly suggestive for SFTs. These characteristics differentiate it from other liver tumors, such as primary hepatocellular carcinoma (CD34-negative), leiomyoma (smooth-muscle actin-positive and CD34-negative), inflammatory pseudotumor (forms fibrous tissue made by collagen fibers with fibro and myofibroblast and plasma cells), fibrosarcoma (forms a "herring bone" pattern), and epithelioid hemangioendothelioma (factor Ⅷ positivity)[7]. The pathological features described for the presented case (low mitotic rate, no nuclear atypia and cellular pleomorphism) are in keeping with those of a benign SFT of the liver. This was confirmed by the favorable course of the patient, who was alive and disease-free 21 mo after surgery.

Radical surgical removal of the tumor with clear margins of resection is the mainstay of treatment. Since most SFTs of the liver are usually found to represent large, well-circumscribed lesions, complete removal necessitates the performance of major hepatectomies (≥ 3 segments). Owing to the size of most SFTs of the liver and their tendency to displace surrounding structures, they are virtually altering the intra-abdominal anatomy in an unpredictable manner. Although an SFT is not a primarily malignant disease, it seems to be of utmost importance that tumor-free resection margins be achieved in order that locoregional recurrence is prevented[8,9]. In light of this, surgical treatment of a hepatic SFT poses a tremendous challenge to the liver surgeon.

Little can be written about the possible benefits of adjuvant radio- and/or chemotherapy in these patients, as reported data are scarce. As SFT of the liver is often a benign neoplasm, postoperative chemotherapy or radiotherapy should not be necessary and is reserved for when resection is incomplete or pathological examination reveals features of malignancy[3,10].

In conclusion, SFTs of the liver are extremely rare neoplasms in adult patients and their incidence is unknown. Precise diagnosis is solely based on the correct interpretation of unique pathological and immunohistochemical features. Aggressive surgical management remains the treatment of choice. The outcome of SFTs is mostly related to resectability, rather than on pathologic grade or tumor size. Since the current number of reported cases is very limited, all efforts should be made to ensure careful follow-up of all identified patients. Only then we can reliably comment on the behavior and definite prognosis of SFTs of the liver.

REFERENCES

1 **Neeff H**, Obermaier R, Technau-Ihling K, Werner M, Kurtz C, Imdahl A, Hopt UT. Solitary fibrous tumour of the liver: case report and review of the literature. *Langenbecks Arch Surg* 2004; **389**: 293-298
2 **Changku J**, Shaohua S, Zhicheng Z, Shusen Z. Solitary fibrous tumor of the liver: retrospective study of reported cases. *Cancer Invest* 2006; **24**: 132-135
3 **Vennarecci G**, Ettorre GM, Giovannelli L, Del Nonno F, Perracchio L, Visca P, Corazza V, Vidiri A, Visco G, Santoro E. Solitary fibrous tumor of the liver. *J Hepatobiliary Pancreat Surg* 2005; **12**: 341-344
4 **Ji Y**, Fan J, Xu Y, Zhou J, Zeng HY, Tan YS. Solitary fibrous tumor of the liver. *Hepatobiliary Pancreat Dis Int* 2006; **5**: 151-153
5 **Guglielmi A**, Frameglia M, Iuzzolino P, Martignoni G, De Manzoni G, Laterza E, Veraldi GF, Girlanda R. Solitary

fibrous tumor of the liver with CD 34 positivity and hypoglycemia. *J Hepatobiliary Pancreat Surg* 1998; **5**: 212-216

6 **Saint-Marc O**, Pozzo A, Causse X, Heitzmann A, Debillon G. [Solitary fibrous liver tumor: clinical, radiological and pathological characteristics] *Gastroenterol Clin Biol* 2002; **26**: 171-173

7 **Barnoud R**, Arvieux C, Pasquier D, Pasquier B, Letoublon C. Solitary fibrous tumour of the liver with CD34 expression. *Histopathology* 1996; **28**: 551-554

8 **Nath DS**, Rutzick AD, Sielaff TD. Solitary fibrous tumor of the liver. *AJR Am J Roentgenol* 2006; **187**: W187-W190

9 **Yilmaz S**, Kirimlioglu V, Ertas E, Hilmioglu F, Yildirim B, Katz D, Mizrak B. Giant solitary fibrous tumor of the liver with metastasis to the skeletal system successfully treated with trisegmentectomy. *Dig Dis Sci* 2000; **45**: 168-174

10 **Kwak SY**, Gwak GY, Yun WK, Kim HJ, Do IG, Joh JW, Park CK. [A case of solitary fibrous tumor of the liver] *Korean J Hepatol* 2007; **13**: 560-564

S- Editor Xiao LL **L- Editor** Wang XL **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.14.6265

World J Gastroenterol 2008 October 28; 14(40): 6265-6267
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Acute ulcerative jejunal diverticulitis: Case report of an uncommon entity

Wojciech Staszewicz, Michel Christodoulou, Stefania Proietti, Nicolas Demartines

Wojciech Staszewicz, Michel Christodoulou, Nicolas Demartines, Service de Chirurgie Viscerale, Centre Hospitalier Universitaire Vaudois, Lausanne 1011, Switzerland
Stefania Proietti, Service de Radiologie, Centre Hospitalier Universitaire Vaudois, Lausanne 1011, Switzerland
Author contributions: Staszewicz W and Christodoulou M contributed equally to this work; Proietti S contributed to the imaging processing and analysis; Staszewicz W and Christodoulou M drafted the paper; Staszewicz W wrote and Demartines N critically revised the paper with an important conceptual and editorial input.
Correspondence to : Wojciech Staszewicz, Service de Chirurgie Viscerale, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland. wojciech.staszewicz@rsv-gnw.ch
Telephone: +41-27-6039826 Fax: +41-27-6039606
Received: January 22, 2008 Revised: October 13, 2008
Accepted: October 20, 2008
Published online: October 28, 2008

Abstract

Jejunal diverticulosis is a rare entity with variable clinical and anatomical presentations. Its reported incidence varies from 0.05% to 6%. Although there is no consensus on the management of asymptomatic jejunal diverticular disease, some complications are potentially life threatening and require early surgical treatment. We report a case of an 88-year-old man investigated for acute abdominal pain with a high biological inflammatory syndrome. Inflammation of multiple giant jejunal diverticulum was discovered at abdominal computed tomography (CT). As a result of the clinical and biological signs of early peritonitis, an emergency surgical exploration was performed. The first jejunal loop showed clear signs of jejunal diverticulitis. Primary segmental jejunum resection with end-to-end anastomosis was performed. Histopathology report confirmed an ulcerative jejunal diverticulitis with imminent perforation and acute local peritonitis. The patient made an excellent rapid postoperative recovery. Jejunal diverticulum is rare but may cause serious complications. It should be considered a possible etiology of acute abdomen, especially in elderly patients with unusual symptomatology. Abdominal CT is the diagnostic tool of choice. The best treatment is emergency surgical management.

Key words: Jejunal diverticulum; Diverticulitis; Surgery; Tomography

Peer reviewer: Amado Salvador Pena, Professor, Department of Pathology, VU University Medical Centre, De Bolelaan 117, Boelelaan 1117, Po Box 7057, Amsterdam 1007 MB, The Netherlands

Staszewicz W, Christodoulou M, Proietti S, Demartines N. Acute ulcerative jejunal diverticulitis: Case report of an uncommon entity. *World J Gastroenterol* 2008; 14(40): 6265-6267 Available from: URL: http://www.wjgnet.com/1007-9327/14/6265.asp DOI: http://dx.doi.org/10.3748/wjg.14.6265

INTRODUCTION

The majority of jejunal diverticulosis cases are discovered incidentally during radiological investigations. Symptomatic presentations are rare and generally complicated. Jejunal and jejuno-ileal localization is nearly three times less frequent than duodenal, but about four times likely to develop complications[1]. Asymptomatic cases require neither medical nor surgical treatment. Rarity of mild or chronic presentations explains the absence of clear consensus on therapeutic strategy and conservative management[2]. Complicated presentations remain a diagnostic challenge because of non-specific and ambiguous symptomatology. Surgical exploration is the treatment of choice for almost all acute complicated cases[3].

CASE REPORT

An 88-year-old male patient with a 6-year history of hypertensive terminal renal failure managed by hemodialysis was admitted for acute abdominal pain. Physical examination revealed right lower quadrant tenderness. C-reactive protein was 260 mg/L and white blood cell count was 19 g/L. There were no clinical or biological signs of bleeding. Abdominal computed tomography (CT) scan showed multiple small bowel giant diverticula, initially attributed to the ileum because of their localization in right iliac fossa. The diameters of these diverticula were between 3.5 cm and 6 cm and some showed clearly inflammatory infiltration

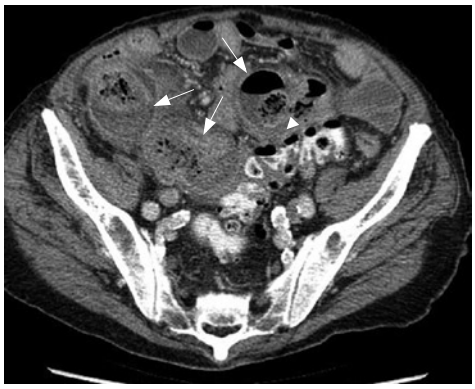


Figure 1 Axial CT image illustrating multiple diverticula (white arrows) developed on the mesenteric border of the jejunum, surrounded by inflammatory mesenteric fat. The arrowhead shows the communication between the diverticula and jejunal lumen.

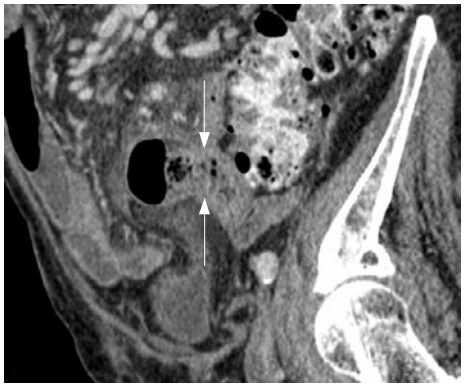


Figure 2 Multiplanar reconstructions on a sagittal view demonstrating a communication between the diverticula and jejunum (white arrow).

(Figures 1 and 2). There was no pneumoperitoneum or intraperitoneal fluid. Emergency laparotomy confirmed the presence of inflamed diverticula on the proximal jejunal portion (Figure 3). Treatment was the resection of a 35-cm jeujunal segment, removing all visible diverticula. End-to-end anastomosis was performed. Histopathology report confirmed an ulcerative jejunal diverticulitis with some areas of perforation and acute local peritonitis. The patient made an excellent postoperative recovery with normalization of inflammatory parameters and rapid intestinal transit recovery. He was discharged on the 12th postoperative day.

DISCUSSION

The present case shows an exemplary preoperative diagnosis and the surgical management of jejunal diverticula in its typical presentation.

The difficulty is that diverticular small bowel disease is an uncommon entity with an incidence between 0.05% and 6%. Jejuno-ileal diverticulosis remains asymptomatic in about 80% of cases, but chronic clinical manifestations may be under-or misdiagnosed with dyspepsia and irritable bowel syndrome. The mean age

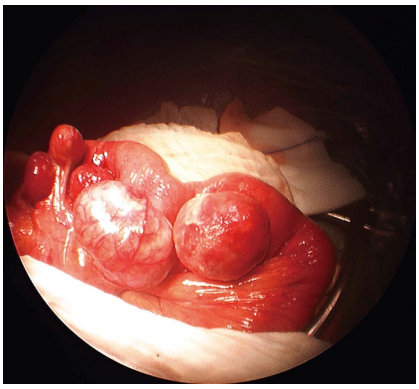


Figure 3 Inflamed giant jejunal diverticula on macroscopic operator view.

of symptomatic patients is up to 60 years in all reported studies. Only less than 10% of the affected individuals develop acute complications such as inflammation, perforation, hemorrhage and obstruction[4].

Complicated jejuno-ileal diverticulosis is generally present in elderly males with non-specific unexplained central abdominal pain, associated with clinical and biological septic syndrome. Some cases have a history of chronic symptoms such as vague abdominal discomfort, fullness, recurrent central and upper abdominal cramping pain caused by pseudo-obstruction or bacterial overgrowth. Yet the diagnostic accuracy of complicated acute jejunal diverticulosis based on simple clinical evaluation is still extremely poor[5,6].

Abdominal CT with double-oral and intravenous contrast may allow the diagnosis based on the following findings: focal area of out-pouching of the mesenteric side of the bowel filled or not filled with feces-like materials, focal asymmetric wall thickening, or inflammatory process adjacent to a loop of jejunum[7,8]. In some cases, use of coronal or sagittal reformatted images is helpful in identifying the bowel segment with diverticula (Figure 2). Today, multi-slice CT is very helpful in diagnosing jejunal diverticulosis and appears clearly superior to conventional enteroclysis for small intestine diseases[9].

Radiology-proven complicated presentations of jejuno-ileal diverticulosis require emergency surgical management. Emergency resection of perforated, inflamed, bleeding or obstructed bowel segment with primary anastomosis is safe and gives the best outcome.

Jejunal diverticulum is rare and generally discovered in complicated presentations only. Interpretation of abdominal CT findings is the key to its correct diagnosis. The treatment of choice is surgical excision of the affected jejunum segment with primary end-to-end anastomosis.

REFERENCES

1 **Miller RE**, McCabe RE, Salomon PF, Knox WG. Surgical complications of small bowel diverticula exclusive of Meckel's. *Ann Surg* 1970; **171**: 202-210

2 **Liu CY**, Chang WH, Lin SC, Chu CH, Wang TE, Shih SC.

Analysis of clinical manifestations of symptomatic acquired jejunoileal diverticular disease. *World J Gastroenterol* 2005; **11**: 5557-5560

3 **Zager JS**, Garbus JE, Shaw JP, Cohen MG, Garber SM. Jejunal diverticulosis: a rare entity with multiple presentations, a series of cases. *Dig Surg* 2000; **17**: 643-645

4 **Akhrass R**, Yaffe MB, Fischer C, Ponsky J, Shuck JM. Small-bowel diverticulosis: perceptions and reality. *J Am Coll Surg* 1997; **184**: 383-388

5 **Lempinen M**, Salmela K, Kemppainen E. Jejunal diverticulosis: a potentially dangerous entity. *Scand J Gastroenterol* 2004; **39**: 905-909

6 **El-Haddawi F**, Civil ID. Acquired jejuno-ileal diverticular disease: a diagnostic and management challenge. *ANZ J Surg* 2003; **73**: 584-589

7 **Coulier B**, Maldague P, Bourgeois A, Broze B. Diverticulitis of the small bowel: CT diagnosis. *Abdom Imaging* 2007; **32**: 228-233

8 **Macari M**, Faust M, Liang H, Pachter HL. CT of jejunal diverticulitis: imaging findings, differential diagnosis, and clinical management. *Clin Radiol* 2007; **62**: 73-77

9 **Bitterling H**, Rock C, Reiser M. [Computed tomography in the diagnosis of inflammatory bowel disease -- methodology of MSCT and clinical results] *Radiologe* 2003; **43**: 17-25

S- Editor Tian L **L- Editor** Wang XL **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2008 October 28; 14(40): 6268
World Journal of Gastroenterology ISSN 1007-9327

ACKNOWLEDGMENTS

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

Kyoichi Adachi, MD
Department of Gastroenterology and Hepatology, Shimane University, School of Medicine Shimane, 89-1 Enya-cho, Izumo-shi Shimane 693-8501, Japan

Reinhard Buettner, Professor
Institute of Pathology, University Hospital Bonn, Sigmund-Freud-Str. 25, D-53127 Bonn, Germany

Sharon DeMorrow, Assistant Professor
Division of Research and Education, Scott and White Hospital and The Texas A&M University System, Health Science Center College of Medicine, Temple, Texas 76504, United States

Dr. Mitsuhiro Fujishiro
Department of Gastroenterology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan

Yoshihide Fujiyama, Professor
Internal Medicine, Division of Gastroenterol, Shiga University of Medical Science, Tsukinowa, Seta, Otsu 520-2192, Japan

Julia B Greer, MD, MPH
Department of Gastroenterology, Hepatology and Nutrition, University of Pittsburgh Medical Center, M2, Presbyterian University Hospital, 200 Lothrop Street, Pittsburgh, Pa 15213, United States

Anna S Gukovskaya, Professor
VA Greater Los Angeles Health Care System ,University of California, Los Angeles, 11301 Wilshire Blvd, Los Angeles 91301, United States

Dr. Serdar Karakose, Professor
Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey

Tom H Karlsen, MD
Institute of Immunology, Rikshospitalet University Hospital, N-0027 Oslo, Norway

Robert J Korst, MD
Department of Cardiothoracic Surgery, Weill Medical College of Cornell University, Room M404, 525 East 68th Street, New York 10032, United States

Richard A Kozarek, MD
Department of Gastroenterology, Virginia Mason Medical Center, 1100 Ninth Avenue, PO Box 900, Seattle 98111-0900, United States

Alyssa M Krasinskas, MD, Assistant Professor
Department of Pathology, University of Pittsburgh Medical Center, Presbyterian Hospital, A610, 200 Lothrop Street, Pittsburgh, PA 15213-2546, United States

Ton Lisman, PhD
Thrombosis and Haemostasis Laboratory, Department of Haematology G.03.550, University Medical Centre, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands

Laura Lladóa, PhD
Department of Surgery, Liver Transplant Unit, Hospital Universitari de Bellvitge, IDIBELL, 08907 Barcelona, Spain

Mercedes Susan Mandell, MD, PhD
Department of Anesthesiology, University of Colorado Health Sciences Ctr., 12401 E. 17th Ave, B113 Aurora, CO 80045, United States

Giulio Marchesini, Professor
Department of Internal Medicine and Gastroenterology, "Alma Mater Studiorum" University of Bologna, Policlinico S. Orsola, Via Massarenti 9, Bologna 40138, Italy

Roberto Mazzanti, Professor
Internal Medicine, University of Florence, Firenze 50134, Italy

Sri P Misra, Professor
Gastroenterology, Moti Lal Nehru Medical College, Allahabad 211001, India

Hiroki Nakamura, MD
Department of Gastroenterology and Hepatology, 1-1-1, Minami Kogushi, Ube, Yamaguchi 755-8505, Japan

Minna Nyström, PhD
Department of Biological and Environmental Sciences, PO Box 56 (Viikinkaari 5 D), University of Helsinki, FI-00014 Helsinki, Finland

Dr. Phillip S Oates
Department of Physiology, School of Biomedical and Chemical Sciences, The University of Western Australia, Perth, WA, Australia

Robert D Odze, MD, FRCPc, Chief
Gastrointestinal Pathology Service, Associate Professor of Pathology, Brigham and Women's Hospital , Harvard Medical School, Boston MA, United States

George Papatheodoridis, MD, Assistant Professor
Department of Internal Medicine, Athens University Medical School, Hippokration General Hospital of Athens, 114 Vas. Sophias Ave., 115 27 Athens, Greece

Dr. Jean Rosenbaum
Inserm E362, Universite Victor Segalen Bordeaux 2, Bordeaux 33076, France

Dr. Shawn David Safford
Department of Surgery, Duke University Medical Center, 994 West Ocean View Avenue, Norfolk VA23503, United States

Dr. Didier Samuel, Professor
Head Liver Unit and Liver ICU Medical, Director Liver Transplant Program, Head Research Inserm-Paris XI Unit 785, Centre Hepatobiliaire Hopital Paul Brousse, 94800, Villejuif, France

Reinhold W Stockbrugger, Professor
Department of Gastroenterology and Hepatology, University Hospital Maastricht, Debyelaan 25, Maastricht NL-6206 AZ, The Netherlands

Wing-Kin Syn, MD
Division of Gastroenterology, GSRB-1, Suite 1073, DUMC 3256, 595 LaSalle Street, Durham, NC27710, United States

Kiichi Tamada, MD
Department of Gastroenterology, Jichi Medical Shool, 3311-1 Yakushiji, Minamika wachi,Kawachigun, Tochigi 329-0498, Japan

Ian D Wallace, MD
Shakespeare Specialist Group, 181 Shakesperare Rd, Milford, Auckland 1309, New Zealand

Jian Wu, Associate Professor of Medicine
Internal Medicine/Transplant Research Program, University of California, Davis Medical Center, 4635 2nd Ave. Suite 1001, Sacramento CA 95817, United States

Katsutoshi Yoshizato, PhD, Academic Advisor
Phoenix Bio Co., Ltd., 3-4-1 Kagamiyama, Higashihiroshima, 739-0046, Japan

Silvana Zanlungo, Professor
Departamento de Gastroenterología, Pontificia Universidad Católica de Chile, Marcoleta 367, Casilla 114-D, Santiago, Chile

Michael A Zimmerman, MD, Assistant Professor
Division of Transplant Surgery, University of Colorado Health Sciences Center, 1635 N. Ursula St, Po Box 6510, 7th floor, Aurora, Colorado 80045, United States

World J Gastroenterol 2008 October 28; 14(40): 6269
World Journal of Gastroenterology ISSN 1007-9327

Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
January 24-25, Frankfurt, Germany
Falk Workshop: Perspectives in Liver
Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
E-mail: education@asge.org

June 4-7, Helsinki, Finland
The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
ESGAR 2008 19th Annual Meeting and Postgraduate Course
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden
16th International Congress of the European Association for Endoscopic Surgery
E-mail: info@eaes-eur.org

June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
10th World Congress on Gastrointestinal Cancer
Imedex and ESMO
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland
Joint Meeting of the European Pancreatic Club (EPC)
and the International Association of Pancreatology (IAP)
E-mail: office@epc-iap2008.org
www.e-p-c.org
www.pancreatology.org

June 26-28, Bratislava, Slovakia
5th Central European Gastroenterology Meeting
www.ceurgem2008.cz

July 9-12, Paris, France
ILTS 14th Annual International Congress
www.ilts.org

September 10-13, Budapest, Hungary
11th World Congress of the International Society for Diseases of the Esophagus
E-mail: isde@isde.net

September 13-16, New Delhi, India
Asia Pacific Digestive Week
E-mail: apdw@apdw2008.net

APDW 2008
September 13-16, New Delhi, Indian
Organized: Indian Society of Gastroenterology

III FALK GASTRO-CONFERENCE
September 17, Mainz, Germany
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
Falk Symposium 166:
GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
Falk Symposium 167:
Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
Third Annual Meeting
European Society of Coloproctology
www.escp.eu.com

October 8-11, Istanbul, Turkey
18th World Congress of the International Association of Surgeons,
Gastroenterologists and Oncologists
E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria
16th United European Gastroenterology Week
www.negf.org
www.acv.at

October 22-25, Minnesota, USA
Anstralian Gastroenterology Week 2008
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia
71st Annual Colon and Rectal Surgery Conference
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA
59th AASLD Annual Meeting and Postgraduate Course
The Liver Meeting
Information: www.aasld.org

November 6-9, Lucerne, Switzerland
Neurogastroenterology & Motility Joint International Meeting 2008
E-mail: ngm2008@mci-group.com
www.ngm2008.com

November 12, Santiago de Chile, Chile
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt
1st Hepatology and Gastroenterology Post Graduate Course
www.egyptgastrohep.com

December 7-9, Seoul, Korea
6th International Meeting
Hepatocellular Carcinoma: Eastern and Western Experiences
E-mail: sglee@amc.seoul.kr

INFORMATION FOR ALL
FALK FOUNDATION e.V.
E-mail: symposia@falkfoundation.de
www.falkfoundation.de

Advanced Courses - European Institute of Telesurgery EITS - 2008
Strasbourg, France
January 18-19, March 28-29, June 6-7, October 3-4

N.O.T.E.S
April 3-5, November 27-29
Laparoscopic Digestive Surgery

June 27-28, November 7-8
Laparoscopic Colorectal Surgery

July 3-5
Interventional GI Endoscopy Techniques
Contact address for all courses:
E-mail: info@eits.fr

International Gastroenterological Congresses 2009
March 23-26, Glasgow, Scotland
Meeting of the British Society of Gastroenterology (BSG)
E-mail: bsg@mailbox.ulcc.ac.uk

May 17-20, Denver, Colorado, USA
Digestive Disease Week 2009

November 21-25, London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2008 October 28; 14(40): 6270-6272
World Journal of Gastroenterology ISSN 1007-9327

Instructions to authors

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol* ISSN 1007-9327 CN 14-1219/R) is a weekly open access peer-reviewed journal supported by an editorial board consisting of 1208 experts in gastroenterology and hepatology from 60 countries. The aim of the journal is to deliver the most clinically relevant original and commentary articles to readers, and to make the full text publicly available to all clinicians, scientists, patients and biomedical students on an unrestricted platform, so that they can access and learn about the most recent key advances in the field.

In addition to the open access nature, another key characteristic of *WJG* is its reading guidance for each article which includes background, research frontier, related reports, breakthroughs, applications, terminology, and comments of peer reviewers for the general readers.

WJG publishes articles on esophageal, gastrointestinal, hepatobiliary and pancreatic tumors, and other esophageal, gastrointestinal, hepatic-biliary and pancreatic diseases in relation to epidermiology, immunology, microbiology, motility & nerve-gut interaction, endocrinology, nutrition & obesity, endoscopy, imaging and advanced hi-technology.

The main goal of *WJG* is to publish high quality commentary articles contributed by leading experts in gastroenterology and hepatology and original articles that combine the clinical practice and advanced basic research, to provide an interactive platform for clinicians and researchers in internal medicine, surgery, infectious diseases, traditional Chinese medicine, oncology, integrated Chinese and Western medicine, imaging, endoscopy, interventional therapy, pathology and other basic medical specialities, and thus eventually improving the clinical practice and healthcare for patients.

Indexed and abstracted in

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, Index Medicus, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology* and *Hepatology*, CAB Abstracts and Global Health. ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

Published by

The WJG Press

SUBMISSION OF MANUSCRIPTS

Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of The WJG Press, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the International Committee of Medical Journal Editors to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now available, to our knowledge, is http://www. clinicaltrials.gov sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

Authors should retain one copy of the text, tables, photographs and illustrations because rejected manuscripts will not be returned to the author(s) and the editors will not be responsible for loss or damage to photographs and illustrations sustained during mailing.

Online submissions

Manuscripts should be submitted through the Online Submission System at: http://wjg.wjgnet.com/wjg. Authors are highly recommended to consult the ONLINE INSTRUCTIONS TO AUTHORS (http://www.wjgnet.com/wjg/help/instructions.jsp) before attempting to submit online. For assistance, authors encountering problems with the Online Submission System may send an email describing the problem to submission@wjgnet.com, or by telephone: +86-10-85381892. If you submit your manuscript online, do not make a postal contribution. Repeated online submission for the same manuscript is strictly prohibited.

MANUSCRIPT PREPARATION

All contributions should be written in English. All articles must be submitted using word-processing software. All submissions must be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Style should conform to our house format. Required information for each of the manuscript sections is as follows:

Title page

Full manuscript title, running title, all author(s) name(s), affiliations, institution(s) and/or department(s) where the work was carried out; author contributions; disclosure of any financial support for the research; and the name, full address, telephone and fax numbers and email address of the corresponding author should be included. Titles should be concise and informative (remove all unnecessary words), emphasize what is new, and avoid abbreviations. A short running title of less than 40 letters should be provided. List the author(s)' name(s) as follows: initial and/or first name, middle name or initial(s), and full family name.

Author controbutions: The format of this section should be like this: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed research; Wang CL, Zou CC, Hong F and Wu XM performed research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed data; and Wang CL, Liang L and Fu JF wrote the paper.

Peer reviewers: All articles received are subject to peer review. Normally, three experts are invited for each article. Decision for acceptance is made only when at least two experts recommend an article for publication. Reviewers for accepted manuscripts are acknowledged in each manuscript, and reviewers of articles which were not accepted will be acknowledged at the end of each issue. To ensure the quality of the articles published in *WJG*, reviewers of accepted manuscripts will be announced by publishing the name, title/position and institution of the reviewer in the footnote accompanying the printed article. For example, reviewers: Professor Jing-Yuan Fang, Shanghai Institute of Digestive Disease, Shanghai, Affiliated Renji Hospital, Medical Faculty, Shanghai Jiaotong University, Shanghai, China; Professor Xin-Wei Han, Department of Radiology, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan Province, China; and Professor Anren Kuang, Department of Nuclear Medicine, Huaxi Hospital, Sichuan University, Chengdu, Sichuan Province, China.

Abstract

An informative, structured abstract of no more than 350 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections: AIM: Only the purpose should be included. METHODS: The materials, techniques, instruments and equipment, and the experimental procedures should be included. RESULTS: The observed and experimental results, including data, effects, outcome, *etc.* should be included. Authors should present *P* value where necessary, and also include any significant data. CONCLUSION: Accurate view and the value of the results should be included.

The format for structured abstracts can be found at: http://www.wjgnet.com/wjg/help/11.doc.

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles, rapid communication

and case reports, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the body text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, should be found at: http://www.wjgnet.com/wjg/help/instructions.jsp.

Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: …*etc.* It is our principle to publish high resolution-figures for the printed and E-versions.

Tables

Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of P values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscripts and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

Coding system

The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability[1,2]". If references are cited directly in the text, they should be put together within the text, for example, "From references[19,22-24], we know that..."

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

PMID requirement

PMID roots in the abstract serial number indexed by PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed). The author should supply the PMID for journal citation. For those references that have not been indexed by PubMed, a printed copy of the first page of the full reference should be submitted.

The accuracy of the information for journal citations is very important. Using the reference testing system, the authors and editor should check the authors name, title, journal title, publication date, volume number, start page, and end page. We will interlink all references with PubMed in an ASP file so that the readers can immediately access the abstract of the citations online.

DOI requirement

A CrossRef DOI® (Digital Object Identifier) name is a unique string created to identify a piece of scholarly content in the online environment. The author should supply the DOIs for journal citation(doi:10.3748/wjg.13.6458). This link (http://www.crossref.org/SimpleTextQuery/) allows you to retrieve Digital Object Identifiers (DOIs) for journal articles, books, and chapters by simply cutting and pasting the reference list into the box. You may use the form with any reference style, although the tool works most reliably if references are formatted in a standard style such as shown in this example: Assimakopoulos SF, Scopa CD, Vagianos CE. Pathophysiology of increased intestinal permeability in obstructive jaundice. *World J Gastroenterol* 2007; 13(48): 6458-6464

The accuracy of the information of journal citations is very important. We will interlink all references with DOI in ASP file so that readers can access the abstracts of cited articles online immediately.

Style for journal references

Authors: the name of the first author should be typed in bold-faced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

Style for book references

Authors: the name of the first author should be typed in bold-faced letters. The surname of all authors should be typed with the initial letter capitalized, followed by their abbreviated middle and first initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR) Book title. Publication number. Publication place: Publication press, Year: start page and end page.

Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Inappropriate references

Authors should always cite references that are relevant to their article, and avoid any inappropriate references. Inappropriate references include those linked with a hyphen when the difference between the two numbers is greater than five. For example, [1-6], [2-14] and [1, 3, 4-10, 22] are all considered inappropriate references. Authors should not cite their own unrelated published articles.

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/wjg/help/15.doc.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H pylori*, *E coli*, *etc.*

SUBMISSION OF THE REVISED MANUSCRIPTS AFTER ACCEPTED

Please revise your article according to the revision policies of *WJG*. The revised version including manuscript and high-resolution image figures (if any) should be copied on a floppy or compact disk. The author should send the revised manuscript, along with printed high-resolution color or black and white photos, copyright transfer letter, and responses to the reviewers by courier (such as EMS/DHL).

Editorial Office

World Journal of Gastroenterology
Editorial Department: Room 903
Ocean International Center, Building D
No. 62 Dongsihuan Zhonglu
Chaoyang District, Beijing 100025, China
E-mail: wjg@wjgnet.com
http://www.wjgnet.com
Telephone: +86-10-59080039
Fax: +86-10-85381893

Language evaluation

The language of a manuscript will be graded before it is sent for revision. (1) Grade A: priority publishing; (2) Grade B: minor language polishing; (3) Grade C: a great deal of language polishing needed; (4) Grade D: rejected. Revised articles should reach Grade A or B.

Copyright assignment form

Please download a Copyright assignment form from http://www.wjgnet.com/wjg/help/9.doc.

Responses to reviewers

Please revise your article according to the comments/suggestions provided by the reviewers. The format for responses to the reviewers' comments can be found at: http://www.wjgnet.com/wjg/help/10.doc.

Proof of financial support

For paper supported by a foundation, authors should provide a copy of the document and serial number of the foundation.

Links to documents related to the manuscript

WJG will be initiating a platform to promote dynamic interactions between the editors, peer reviewers, readers and authors. After a manuscript is published online, links to the PDF version of the submitted manuscript, the peer-reviewers' report and the revised manuscript will be put on-line. Readers can make comments on the peer reviewer's report, authors' responses to peer reviewers, and the revised manuscript. We hope that authors will benefit from this feedback and be able to revise the manuscript accordingly in a timely manner.

Science news releases

Authors of accepted manuscripts are suggested to write a science news item to promote their articles. The news will be released rapidly at EurekAlert/AAAS (http://www.eurekalert.org). The title for news items should be less than 90 characters; the summary should be less than 75 words; and main body less than 500 words. Science news items should be lawful, ethical, and strictly based on your original content with an attractive title and interesting pictures.

Publication fee

Authors of accepted articles must pay a publication fee.
EDITORIAL, TOPIC HIGHLIGHTS, BOOK REVIEWS and LETTERS TO THE EDITOR are published free of charge.