ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

World Journal of *Gastroenterology*

World J Gastroenterol 2017 February 14; 23(6): 931-1118

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

A peer-reviewed, online, open-access journal of gastroenterology and hepatology

Editorial Board

2014-2017

The *World Journal of Gastroenterology* Editorial Board consists of 1376 members, representing a team of worldwide experts in gastroenterology and hepatology. They are from 68 countries, including Algeria (2), Argentina (7), Australia (31), Austria (9), Belgium (11), Brazil (20), Brunei Darussalam (1), Bulgaria (2), Cambodia (1), Canada (26), Chile (4), China (164), Croatia (2), Cuba (1), Czech (6), Denmark (2), Egypt (9), Estonia (2), Finland (6), France (20), Germany (58), Greece (31), Guatemala (1), Hungary (15), Iceland (1), India (33), Indonesia (2), Iran (10), Ireland (9), Israel (18), Italy (194), Japan (149), Jordan (1), Kuwait (1), Lebanon (7), Lithuania (1), Malaysia (1), Mexico (11), Morocco (1), Netherlands (5), New Zealand (4), Nigeria (3), Norway (6), Pakistan (6), Poland (12), Portugal (8), Puerto Rico (1), Qatar (1), Romania (10), Russia (3), Saudi Arabia (2), Singapore (7), Slovenia (2), South Africa (1), South Korea (69), Spain (51), Sri Lanka (1), Sudan (1), Sweden (12), Switzerland (5), Thailand (7), Trinidad and Tobago (1), Tunisia (2), Turkey (55), United Kingdom (49), United States (180), Venezuela (1), and Vietnam (1).

Gordon Stanley Howarth, *Roseworthy*
Seungha Kang, *Brisbane*
Alfred King Lam, *Gold Coast*
Ian C Lawrance, *PerthFremantle*
Barbara Anne Leggett, *Brisbane*
Daniel A Lemberg, *Sydney*
Rupert W Leong, *Sydney*
Finlay A Macrae, *Victoria*
Vance Matthews, *Melbourne*
David L Morris, *Sydney*
Reme Mountifield, *Bedford Park*
Hans J Netter, *Melbourne*
Nam Q Nguyen, *Adelaide*
Liang Qiao, *Westmead*
Rajvinder Singh, *Adelaide*
Ross Cyril Smith, *StLeonards*
Kevin J Spring, *Sydney*
Debbie Trinder, *Fremantle*
Daniel R van Langenberg, *Box Hill*
David Ian Watson, *Adelaide*
Desmond Yip, *Garran*
Li Zhang, *Sydney*

Austria

Felix Aigner, *Innsbruck*
Gabriela A Berlakovich, *Vienna*
Herwig R Cerwenka, *Graz*
Peter Ferenci, *Wien*
Alfred Gangl, *Vienna*
Kurt Lenz, *Linz*
Markus Peck-Radosavljevic, *Vienna*
Markus Raderer, *Vienna*
Stefan Riss, *Vienna*

Belgium

Michael George Adler, *Brussels*
Benedicte Y De Winter, *Antwerp*
Mark De Ridder, *Jette*
Olivier Detry, *Liege*
Denis Dufrane Dufrane, *Brussels*
Sven M Francque, *Edegem*
Nikos Kotzampassakis, *Liège*
Geert KMM Robaeys, *Genk*
Xavier Sagaert, *Leuven*
Peter Starkel, *Brussels*
Eddie Wisse, *Keerbergen*

Brazil

SMP Balzan, *Santa Cruz do Sul*
JLF Caboclo, *Sao jose do rio preto*
Fábio Guilherme Campos, *Sao Paulo*
Claudia RL Cardoso, *Rio de Janeiro*
Roberto J Carvalho-Filho, *Sao Paulo*
Carla Daltro, *Salvador*
José Sebastiao dos Santos, *Ribeirao Preto*
Eduardo LR Mello, *Rio de Janeiro*
Sthela Maria Murad-Regadas, *Fortaleza*
Claudia PMS Oliveira, *Sao Paulo*
Júlio C Pereira-Lima, *Porto Alegre*
Marcos V Perini, *Sao Paulo*
Vietla Satyanarayana Rao, *Fortaleza*
Raquel Rocha, *Salvador*
AC Simoes e Silva, *Belo Horizonte*
Mauricio F Silva, *Porto Alefre*
Aytan Miranda Sipahi, *Sao Paulo*
Rosa Leonôra Salerno Soares, *Niterói*
Cristiane Valle Tovo, *Porto Alegre*
Eduardo Garcia Vilela, *Belo Horizonte*

Brunei Darussalam

Vui Heng Chong, *Bandar Seri Begawan*

Bulgaria

Tanya Kirilova Kadiyska, *Sofia*
Mihaela Petrova, *Sofia*

Cambodia

Francois Rouet, *Phnom Penh*

Canada

Brian Bressler, *Vancouver*
Frank J Burczynski, *Winnipeg*
Wangxue Chen, *Ottawa*
Francesco Crea, *Vancouver*
Mirko Diksic, *Montreal*
Jane A Foster, *Hamilton*
Hugh J Freeman, *Vancouver*
Shahrokh M Ghobadloo, *Ottawa*
Yuewen Gong, *Winnipeg*
Philip H Gordon, *Quebec*
Rakesh Kumar, *Edmonton*
Wolfgang A Kunze, *Hamilton*
Patrick Labonte, *Laval*
Zhikang Peng, *Winnipeg*
Jayadev Raju, *Ottawa*
Maitreyi Raman, *Calgary*
Giada Sebastiani, *Montreal*
Maida J Sewitch, *Montreal*
Eldon A Shaffer, *Alberta*
Christopher W Teshima, *Edmonton*
Jean Sévigny, *Québec*
Pingchang Yang, *Hamilton*
Pingchang Yang, *Hamilton*
Eric M Yoshida, *Vancouver*
Bin Zheng, *Edmonton*

Chile

Marcelo A Beltran, *La Serena*
Flavio Nervi, *Santiago*
Adolfo Parra-Blanco, *Santiago*
Alejandro Soza, *Santiago*

China

Zhao-Xiang Bian, *Hong Kong*
San-Jun Cai, *Shanghai*
Guang-Wen Cao, *Shanghai*
Long Chen, *Nanjing*
Ru-Fu Chen, *Guangzhou*
George G Chen, *Hong Kong*
Li-Bo Chen, *Wuhan*
Jia-Xu Chen, *Beijing*
Hong-Song Chen, *Beijing*
Lin Chen, *Beijing*
Yang-Chao Chen, *Hong Kong*
Zhen Chen, *Shanghai*
Ying-Sheng Cheng, *Shanghai*
Kent-Man Chu, *Hong Kong*
Zhi-Jun Dai, *Xi'an*
Jing-Yu Deng, *Tianjin*
Yi-Qi Du, *Shanghai*
Zhi Du, *Tianjin*
Hani El-Nezami, *Hong Kong*
Bao-Ying Fei, *Hangzhou*
Chang-Ming Gao, *Nanjing*
Jian-Ping Gong, *Chongqing*
Zuo-Jiong Gong, *Wuhan*
Jing-Shan Gong, *Shenzhen*
Guo-Li Gu, *Beijing*
Yong-Song Guan, *Chengdu*
Mao-Lin Guo, *Luoyang*
Jun-Ming Guo, *Ningbo*
Yan-Mei Guo, *Shanghai*
Xiao-Zhong Guo, *Shenyang*
Guo-Hong Han, *Xi'an*
Ming-Liang He, *Hong Kong*
Peng Hou, *Xi'an*
Zhao-Hui Huang, *Wuxi*
Feng Ji, *Hangzhou*
Simon Law, *Hong Kong*
Yu-Yuan Li, *Guangzhou*
Meng-Sen Li, *Haikou*
Shu-De Li, *Shanghai*
Zong-Fang Li, *Xi'an*
Qing-Quan Li, *Shanghai*
Kang Li, *Lasa*
Han Liang, *Tianjin*
Xing'e Liu, *Hangzhou*
Zheng-Wen Liu, *Xi'an*
Xiao-Fang Liu, *Yantai*
Bin Liu, *Tianjin*
Quan-Da Liu, *Beijing*
Hai-Feng Liu, *Beijing*
Fei Liu, *Shanghai*
Ai-Guo Lu, *Shanghai*
He-Sheng Luo, *Wuhan*
Xiao-Peng Ma, *Shanghai*
Yong Meng, *Shantou*
Ke-Jun Nan, *Xi'an*
Siew Chien Ng, *Hong Kong*
Simon SM Ng, *Hong Kong*
Zhao-Shan Niu, *Qingdao*
Di Qu, *Shanghai*
Ju-Wei Mu, *Beijing*
Rui-Hua Shi, *Nanjing*
Bao-Min Shi, *Shanghai*
Xiao-Dong Sun, *Hangzhou*
Si-Yu Sun, *Shenyang*
Guang-Hong Tan, *Haikou*
Wen-Fu Tang, *Chengdu*
Anthony YB Teoh, *Hong Kong*
Wei-Dong Tong, *Chongqing*
Eric Tse, *Hong Kong*
Hong Tu, *Shanghai*

Rong Tu, *Haikou*
Jian-She Wang, *Shanghai*
Kai Wang, *Jinan*
Xiao-Ping Wang, *Xianyang*
Xiu-Yan Wang, *Shanghai*
Dao-Rong Wang, *Yangzhou*
De-Sheng Wang, *Xi'an*
Chun-You Wang, *Wuhan*
Ge Wang, *Chongqing*
Xi-Shan Wang, *Harbin*
Wei-hong Wang, *Beijing*
Zhen-Ning Wang, *Shenyang*
Wai Man Raymond Wong, *Hong Kong*
Chun-Ming Wong, *Hong Kong*
Jian Wu, *Shanghai*
Sheng-Li Wu, *Xi'an*
Wu-Jun Wu, *Xi'an*
Qing Xia, *Chengdu*
Yan Xin, *Shenyang*
Dong-Ping Xu, *Beijing*
Jian-Min Xu, *Shanghai*
Wei Xu, *Changchun*
Ming Yan, *Jinan*
Xin-Min Yan, *Kunming*
Yi-Qun Yan, *Shanghai*
Feng Yang, *Shanghai*
Yong-Ping Yang, *Beijing*
He-Rui Yao, *Guangzhou*
Thomas Yau, *Hong Kong*
Winnie Yeo, *Hong Kong*
Jing You, *Kunming*
Jian-Qing Yu, *Wuhan*
Ying-Yan Yu, *Shanghai*
Wei-Zheng Zeng, *Chengdu*
Zong-Ming Zhang, *Beijing*
Dian-Liang Zhang, *Qingdao*
Ya-Ping Zhang, *Shijiazhuang*
You-Cheng Zhang, *Lanzhou*
Jian-Zhong Zhang, *Beijing*
Ji-Yuan Zhang, *Beijing*
Hai-Tao Zhao, *Beijing*
Jian Zhao, *Shanghai*
Jian-Hong Zhong, *Nanning*
Ying-Qiang Zhong, *Guangzhou*
Ping-Hong Zhou, *Shanghai*
Yan-Ming Zhou, *Xiamen*
Tong Zhou, *Nanchong*
Li-Ming Zhou, *Chengdu*
Guo-Xiong Zhou, *Nantong*
Feng-Shang Zhu, *Shanghai*
Jiang-Fan Zhu, *Shanghai*
Zhao-Hui Zhu, *Beijing*

Croatia
Tajana Filipec Kanizaj, *Zagreb*
Mario Tadic, *Zagreb*

Cuba
Damian Casadesus, *Havana*

Czech
Jan Bures, *Hradec Kralove*
Marcela Kopacova, *Hradec Kralove*
Otto Kucera, *Hradec Kralove*
Marek Minarik, *Prague*
Pavel Soucek, *Prague*
Miroslav Zavoral, *Prague*

Denmark
Vibeke Andersen, *Odense*
E Michael Danielsen, *Copenhagen*

Egypt
Mohamed MM Abdel-Latif, *Assiut*
Hussein Atta, *Cairo*
Ashraf Elbahrawy, *Cairo*
Mortada Hassan El-Shabrawi, *Cairo*
Mona El Said El-Raziky, *Cairo*
Elrashdy M Redwan, *New Borg Alrab*
Zeinab Nabil Ahmed Said, *Cairo*
Ragaa HM Salama, *Assiut*
Maha Maher Shehata, *Mansoura*

Estonia
Margus Lember, *Tartu*
Tamara Vorobjova, *Tartu*

Finland
Marko Kalliomäki, *Turku*
Thomas Kietzmann, *Oulu*
Kaija-Leena Kolho, *Helsinki*
Eija Korkeila, *Turku*
Heikki Makisalo, *Helsinki*
Tanja Pessi, *Tampere*

France
Armando Abergel Clermont, *Ferrand*
Elie K Chouillard, *Polssy*
Pierre Cordelier, *Toulouse*
Pascal P Crenn, *Garches*
Catherine Daniel, *Lille*
Fanny Daniel, *Paris*
Cedric Dray, *Toulouse*
Benoit Foligne, *Lille*
Jean-Noel Freund, *Strasbourg*
Hervé Guillou, *Toulouse*
Nathalie Janel, *Paris*
Majid Khatib, *Bordeaux*
Jacques Marescaux, *Strasbourg*
Jean-Claude Marie, *Paris*
Driffa Moussata, *Pierre Benite*
Hang Nguyen, *Clermont-Ferrand*
Hugo Perazzo, *Paris*
Alain L Servin, *Chatenay-Malabry*
Chang Xian Zhang, *Lyon*

Germany
Stavros A Antoniou, *Monchengladbach*
Erwin Biecker, *Siegburg*
Hubert E Blum, *Freiburg*
Thomas Bock, *Berlin*
Katja Breitkopf-Heinlein, *Mannheim*
Elke Cario, *Essen*
Güralp Onur Ceyhan, *Munich*
Angel Cid-Arregui, *Heidelberg*
Michael Clemens Roggendorf, *München*
Christoph F Dietrich, *Bad Mergentheim*
Valentin Fuhrmann, *Hamburg*
Nikolaus Gassler, *Aachen*
Andreas Geier, *Wuerzburg*
Markus Gerhard, *Munich*
Anton Gillessen, *Muenster*
Thorsten Oliver Goetze, *Offenbach*
Daniel Nils Gotthardt, *Heidelberg*
Robert Grützmann, *Dresden*
Thilo Hackert, *Heidelberg*
Claus Hellerbrand, *Regensburg*
Harald Peter Hoensch, *Darmstadt*
Jens Hoeppner, *Freiburg*
Richard Hummel, *Muenster*
Jakob Robert Izbicki, *Hamburg*
Gernot Maximilian Kaiser, *Essen*
Matthias Kapischke, *Hamburg*
Michael Keese, *Frankfurt*
Andrej Khandoga, *Munich*
Jorg Kleeff, *Munich*
Alfred Koenigsrainer, *Tuebingen*
Peter Christopher Konturek, *Saalfeld*
Michael Linnebacher, *Rostock*
Stefan Maier, *Kaufbeuren*
Oliver Mann, *Hamburg*
Marc E Martignoni, *Munic*
Thomas Minor, *Bonn*
Oliver Moeschler, *Osnabrueck*
Jonas Mudter, *Eutin*
Sebastian Mueller, *Heidelberg*
Matthias Ocker, *Berlin*
Andreas Ommer, *Essen*
Albrecht Piiper, *Frankfurt*
Esther Raskopf, *Bonn*
Christoph Reichel, *Bad Brückenau*
Elke Roeb, *Giessen*
Udo Rolle, *Frankfurt*
Karl-Herbert Schafer, *Zweibrücken*
Peter Schemmer, *Heidelberg*
Andreas G Schreyer, *Regensburg*
Manuel A Silva, *Penzberg*
Georgios C Sotiropoulos, *Essen*
Ulrike S Stein, *Berlin*
Dirk Uhlmann, *Leipzig*
Michael Weiss, *Halle*
Hong-Lei Weng, *Mannheim*
Karsten Wursthorn, *Hamburg*

Greece
Alexandra Alexopoulou, *Athens*
Nikolaos Antonakopoulos, *Athens*
Stelios F Assimakopoulos, *Patras*
Grigoris Chatzimavroudis, *Thessaloniki*
Evangelos Cholongitas, *Thessaloniki*
Gregory Christodoulidis, *Larisa*
George N Dalekos, *Larissa*
Urania Georgopoulou, *Athens*
Eleni Gigi, *Thessaloniki*

Stavros Gourgiotis, *Athens*
Leontios J Hadjileontiadis, *Thessaloniki*
Thomas Hyphantis, *Ioannina*
Ioannis Kanellos, *Thessaloniki*
Stylianos Karatapanis, *Rhodes*
Michael Koutsilieris, *Athens*
Spiros D Ladas, *Athens*
Theodoros K Liakakos, *Athens*
Emanuel K Manesis, *Athens*
Spilios Manolakopoulos, *Athens*
Gerassimos John Mantzaris, *Athens*
Athanasios D Marinis, *Piraeus*
Nikolaos Ioannis Nikiteas, *Athens*
Konstantinos X Papamichael, *Athens*
George Sgourakis, *Athens*
Konstantinos C Thomopoulos, *Patras*
Konstantinos Triantafyllou, *Athens*
Christos Triantos, *Patras*
Georgios Zacharakis, *Athens*
Petros Zezos, *Alexandroupolis*
Demosthenes E Ziogas, *Ioannina*

Guatemala

Carlos Maria Parellada, *Guatemala*

Hungary

Mihaly Boros, *Szeged*
Tamás Decsi, *Pécs*
Gyula Farkas, *Szeged*
Andrea Furka, *Debrecen*
Y vette Mandi, *Szeged*
Peter L Lakatos, *Budapest*
Pal Miheller, *Budapest*
Tamás Molnar, *Szeged*
Attila Olah, *Gyor*
Maria Papp, *Debrecen*
Zoltan Rakonczay, *Szeged*
Ferenc Sipos, *Budapest*
Miklós Tanyi, *Debrecen*
Tibor Wittmann, *Szeged*

Iceland

Tryggvi Bjorn Stefánsson, *Reykjavík*

India

Brij B Agarwal, *New Delhi*
Deepak N Amarapurkar, *Mumbai*
Shams ul Bari, *Srinagar*
Sriparna Basu, *Varanasi*
Runu Chakravarty, *Kolkata*
Devendra C Desai, *Mumbai*
Nutan D Desai, *Mumbai*
Suneela Sunil Dhaneshwar, *Pune*
Radha K Dhiman, *Chandigarh*
Pankaj Garg, *Mohali*
Uday C Ghoshal, *Lucknow*
Kalpesh Jani, *Vadodara*
Premashis Kar, *New Delhi*
Jyotdeep Kaur, *Chandigarh*
Rakesh Kochhar, *Chandigarh*
Pradyumna K Mishra, *Mumbai*
Asish K Mukhopadhyay, *Kolkata*
Imtiyaz Murtaza, *Srinagar*
P Nagarajan, *New Delhi*
Samiran Nundy, *Delhi*
Gopal Pande, *Hyderabad*
Benjamin Perakath, *Vellore*
Arun Prasad, *New Delhi*
D Nageshwar Reddy, *Hyderabad*
Lekha Saha, *Chandigarh*
Sundeep Singh Saluja, *New Delhi*
Mahesh Prakash Sharma, *New Delhi*
Sadiq Saleem Sikora, *Bangalore*
Sarman Singh, *New Delhi*
Rajeev Sinha, *Jhansi*
Rupjyoti Talukdar, *Hyderabad*
Rakesh Kumar Tandon, *New Delhi*
Narayanan Thirumoorthy, *Coimbatore*

Indonesia

David Handojo Muljono, *Jakarta*
Andi Utama, *Jakarta*

Iran

Arezoo Aghakhani, *Tehran*
Seyed Mohsen Dehghani, *Shiraz*
Ahad Eshraghian, *Shiraz*
Hossein Khedmat, *Tehran*
Sadegh Massarrat, *Tehran*
Marjan Mohammadi, *Tehran*
Roja Rahimi, *Tehran*
Farzaneh Sabahi, *Tehran*
Majid Sadeghizadeh, *Tehran*
Farideh Siavoshi, *Tehran*

Ireland

Gary Alan Bass, *Dublin*
David J Brayden, *Dublin*
Ronan A Cahill, *Dublin*
Glen A Doherty, *Dublin*
Liam J Fanning, *Cork*
Barry Philip McMahon, *Dublin*
RossMcManus, *Dublin*
Dervla O'Malley, *Cork*
Sinead M Smith, *Dublin*

Israel

Dan Carter, *Ramat Gan*
Jorge-Shmuel Delgado, *Metar*
Eli Magen, *Ashdod*
Nitsan Maharshak, *Tel Aviv*
Shaul Mordechai, *Beer Sheva*
Menachem Moshkowitz, *Tel Aviv*
William Bahij Nseir, *Nazareth*
Shimon Reif, *Jerusalem*
Ram Reifen, *Rehovot*
Ariella Bar-Gil Shitrit, *Jerusalem*
Noam Shussman, *Jerusalem*
Igor Sukhotnik, *Haifa*
Nir Wasserberg, *Petach Tiqwa*
Jacob Yahav, *Rehovot*
Doron Levi Zamir, *Gedera*
Shira Zelber-Sagi, *Haifa*
Romy Zemel, *Petach-Tikva*

Italy

Ludovico Abenavoli, *Catanzaro*
Luigi Elio Adinolfi, *Naples*
Carlo Virginio Agostoni, *Milan*
Anna Alisi, *Rome*
Piero Luigi Almasio, *Palermo*
Donato Francesco Altomare, *Bari*
Amedeo Amedei, *Florence*
Pietro Andreone, *Bologna*
Imerio Angriman, *Padova*
Vito Annese, *Florence*
Paolo Aurello, *Rome*
Salavtore Auricchio, *Naples*
Gian Luca Baiocchi, *Brescia*
Gianpaolo Balzano, *Milan*
Antonio Basoli, *Rome*
Gabrio Bassotti, *San Sisto*
Mauro Bernardi, *Bologna*
Alberto Biondi, *Rome*
Ennio Biscaldi, *Genova*
Massimo Bolognesi, *Padua*
Luigi Bonavina, *Milano*
Aldo Bove, *Chieti*
Raffaele Bruno, *Pavia*
Luigi Brusciano, *Napoli*
Giuseppe Cabibbo, *Palermo*
Carlo Calabrese, *Bologna*
Daniele Calistri, *Meldola*
Vincenza Calvaruso, *Palermo*
Lorenzo Camellini, *Reggio Emilia*
Marco Candela, *Bologna*
Raffaele Capasso, *Naples*
Lucia Carulli, *Modena*
Renato David Caviglia, *Rome*
Luigina Cellini, *Chieti*
Giuseppe Chiarioni, *Verona*
Claudio Chiesa, *Rome*
Michele Cicala, *Roma*
Rachele Ciccocioppo, *Pavia*
Sandro Contini, *Parma*
Gaetano Corso, *Foggia*
Renato Costi, *Parma*
Alessandro Cucchetti, *Bologna*
Rosario Cuomo, *Napoli*
Giuseppe Currò, *Messina*
Paola De Nardi, *Milano*
Giovanni D De Palma, *Naples*
Raffaele De Palma, *Napoli*
Giuseppina De Petro, *Brescia*
Valli De Re, *Aviano*
Paolo De Simone, *Pisa*
Giuliana Decorti, *Trieste*
Emanuele Miraglia del Giudice, *Napoli*
Isidoro Di Carlo, *Catania*
Matteo Nicola Dario Di Minno, *Naples*
Massimo Donadelli, *Verona*
Mirko D'Onofrio, *Verona*
Maria Pina Dore, *Sassari*
Luca Elli, *Milano*
Massimiliano Fabozzi, *Aosta*

Massimo Falconi, *Ancona*
Ezio Falletto, *Turin*
Silvia Fargion, *Milan*
Matteo Fassan, *Verona*
Gianfranco Delle Fave, *Roma*
Alessandro Federico, *Naples*
Francesco Feo, *Sassari*
Davide Festi, *Bologna*
Natale Figura, *Siena*
Vincenzo Formica, *Rome*
Mirella Fraquelli, *Milan*
Marzio Frazzoni, *Modena*
Walter Fries, *Messina*
Gennaro Galizia, *Naples*
Andrea Galli, *Florence*
Matteo Garcovich, *Rome*
Eugenio Gaudio, *Rome*
Paola Ghiorzo, *Genoa*
Edoardo G Giannini, *Genova*
Luca Gianotti, *Monza*
Maria Cecilia Giron, *Padova*
Alberto Grassi, *Rimini*
Gabriele Grassi, *Trieste*
Francesco Greco, *Bergamo*
Luigi Greco, *Naples*
Antonio Grieco, *Rome*
Fabio Grizzi, *Rozzano*
Laurino Grossi, *Pescara*
Simone Guglielmetti, *Milan*
Tiberiu Hershcovici, *Jerusalem*
Calogero Iacono, *Verona*
Enzo Ierardi, *Bari*
Amedeo Indriolo, *Bergamo*
Raffaele Iorio, *Naples*
Paola Iovino, *Salerno*
Angelo A Izzo, *Naples*
Loreta Kondili, *Rome*
Filippo La Torre, *Rome*
Giuseppe La Torre, *Rome*
Giovanni Latella, *L'Aquila*
Salvatore Leonardi, *Catania*
Massimo Libra, *Catania*
Anna Licata, *Palermo*
C armela Loguercio, *Naples*
Amedeo Lonardo, *Modena*
Carmelo Luigiano, *Catania*
Francesco Luzza, *Catanzaro*
Giovanni Maconi, *Milano*
Antonio Macrì, *Messina*
Mariano Malaguarnera, *Catania*
Francesco Manguso, *Napoli*
Tommaso Maria Manzia, *Rome*
Daniele Marrelli, *Siena*
Gabriele Masselli, *Rome*
Sara Massironi, *Milan*
Giuseppe Mazzarella, *Avellino*
Michele Milella, *Rome*
Giovanni Milito, *Rome*
Antonella d'Arminio Monforte, *Milan*
Fabrizio Montecucco, *Genoa*
Giovanni Monteleone, *Rome*
Mario Morino, *Torino*
Vincenzo La Mura, *Milan*
Gerardo Nardone, *Naples*
Riccardo Nascimbeni, *Brescia*
Gabriella Nesi, *Florence*
Giuseppe Nigri, *Rome*
Erica Novo, *Turin*
Veronica Ojetti, *Rome*
Michele Orditura, *Naples*
Fabio Pace, *Seriate*
Lucia Pacifico, *Rome*
Omero Alessandro Paoluzi, *Rome*
Valerio Pazienza, *San Giovanni Rotondo*
Rinaldo Pellicano, *Turin*
Adriano M Pellicelli, *Rome*
Nadia Peparini, *Ciampino*
Mario Pescatori, *Rome*
Antonio Picardi, *Rome*
Alberto Pilotto, *Padova*
Alberto Piperno, *Monza*
Anna Chiara Piscaglia, *Rome*
Maurizio Pompili, *Rome*
Francesca Romana Ponziani, *Rome*
Cosimo Prantera, *Rome*
Girolamo Ranieri, *Bari*
Carlo Ratto, *Tome*
Barbara Renga, *Perugia*
Alessandro Repici, *Rozzano*
Maria Elena Riccioni, *Rome*
Lucia Ricci-Vitiani, *Rome*
Luciana Rigoli, *Messina*
Mario Rizzetto, *Torino*
Ballarin Roberto, *Modena*
Roberto G Romanelli, *Florence*
Claudio Romano, *Messina*
Luca Roncucci, *Modena*
Cesare Ruffolo, *Treviso*
L ucia Sacchetti, *Napoli*
Rodolfo Sacco, *Pisa*
Lapo Sali, *Florence*
Romina Salpini, *Rome*
Giulio Aniello, *Santoro Treviso*
Armando Santoro, *Rozzano*
Edoardo Savarino, *Padua*
Marco Senzolo, *Padua*
Annalucia Serafino, *Rome*
Giuseppe S Sica, *Rome*
Pierpaolo Sileri, *Rome*
Cosimo Sperti, *Padua*
Vincenzo Stanghellini, *Bologna*
Cristina Stasi, *Florence*
Gabriele Stocco, *Trieste*
Roberto Tarquini, *Florence*
Mario Testini, *Bari*
Guido Torzilli, *Milan*
Guido Alberto Massimo, *Tiberio Brescia*
Giuseppe Toffoli, *Aviano*
Alberto Tommasini, *Trieste*
Francesco Tonelli, *Florence*
Cesare Tosetti Porretta, *Terme*
Lucio Trevisani, *Cona*
Guglielmo M Trovato, *Catania*
Mariapia Vairetti, *Pavia*
Luca Vittorio Valenti, *Milano*
Mariateresa T Ventura, *Bari*
Giuseppe Verlato, *Verona*
Marco Vivarelli, *Ancona*
Giovanni Li Volti, *Catania*
Giuseppe Zanotti, *Padua*
Vincenzo Zara, *Lecce*
Gianguglielmo Zehender, *Milan*
Anna Linda Zignego, *Florence*
Rocco Antonio Zoccali, *Messina*
Angelo Zullo, *Rome*

Japan

Yasushi Adachi, *Sapporo*
Takafumi Ando, *Nagoya*
Masahiro Arai, *Tokyo*
Makoto Arai, *Chiba*
Takaaki Arigami, *Kagoshima*
Itaru Endo,*Yokohama*
Munechika Enjoji, *Fukuoka*
Shunji Fujimori, *Tokyo*
Yasuhiro Fujino, *Akashi*
Toshiyoshi Fujiwara, *Okayama*
Yosuke Fukunaga, *Tokyo*
Toshio Fukusato, *Tokyo*
Takahisa Furuta, *Hamamatsu*
Osamu Handa, *Kyoto*
Naoki Hashimoto, *Osaka*
Yoichi Hiasa, *Toon*
Masatsugu Hiraki, *Saga*
Satoshi Hirano, *Sapporo*
Keiji Hirata, *Fukuoka*
Toru Hiyama, *Higashihiroshima*
Akira Hokama, *Nishihara*
Shu Hoteya, *Tokyo*
Masao Ichinose, *Wakayama*
Tatsuya Ide, *Kurume*
Masahiro Iizuka, *Akita*
Toshiro Iizuka, *Tokyo*
Kenichi Ikejima, *Tokyo*
Tetsuya Ikemoto, *Tokushima*
Hiroyuki Imaeda, *Saitama*
Atsushi Imagawa, *Kan-onji*
Hiroo Imazu, *Tokyo*
Shuji Isaji, *Tsu*
Toru Ishikawa, *Niigata*
Toshiyuki Ishiwata, *Tokyo*
Soichi Itaba, *Kitakyushu*
Yoshiaki Iwasaki, *Okayama*
Tatehiro Kagawa, *Isehara*
Satoru Kakizaki, *Maebashi*
Naomi Kakushima, *Shizuoka*
Terumi Kamisawa, *Tokyo*
Akihide Kamiya, *Isehara*
Osamu Kanauchi, *Tokyo*
Tatsuo Kanda, *Chiba*
Shin Kariya, *Okayama*
Shigeyuki Kawa, *Matsumoto*
Takumi Kawaguchi, *Kurume*
Takashi Kawai, *Tokyo*
Soo Ryang Kim, *Kobe*
Shinsuke Kiriyama, *Gunma*
Tsuneo Kitamura, *Urayasu*
Masayuki Kitano, *Osakasayama*
Hirotoshi Kobayashi, *Tokyo*
Hironori Koga, *Kurume*
Takashi Kojima, *Sapporo*
Satoshi Kokura, *Kyoto*
Shuhei Komatsu, *Kyoto*
Tadashi Kondo, *Tokyo*
Yasuteru Kondo, *Sendai*
Yasuhiro Kuramitsu, *Yamaguchi*
Yukinori Kurokawa, *Osaka*
Shin Maeda, *Yokohama*
Koutarou Maeda, *Toyoake*

Hitoshi Maruyama, *Chiba*
Atsushi Masamune, *Sendai*
Hiroyuki Matsubayashi, *Suntogun*
Akihisa Matsuda, *Inzai*
Hirofumi Matsui, *Tsukuba*
Akira Matsumori, *Kyoto*
Yoichi Matsuo, *Nagoya*
Y Matsuzaki, *Ami*
Toshihiro Mitaka, *Sapporo*
Kouichi Miura, *Akita*
Shinichi Miyagawa, *Matumoto*
Eiji Miyoshi, *Suita*
Toru Mizuguchi, *Sapporo*
Nobumasa Mizuno, *Nagoya*
Zenichi Morise, *Nagoya*
Tomohiko Moriyama, *Fukuoka*
Kunihiko Murase, *Tusima*
Michihiro Mutoh, *Tsukiji*
Akihito Nagahara, *Tokyo*
Hikaru Nagahara, *Tokyo*
Hidenari Nagai, *Tokyo*
Koichi Nagata, *Shimotsuke-shi*
Masaki Nagaya, *Kawasaki*
Hisato Nakajima, *Nishi-Shinbashi*
Toshifusa Nakajima, *Tokyo*
Hiroshi Nakano, *Kawasaki*
Hiroshi Nakase, *Kyoto*
Toshiyuki Nakayama, *Nagasaki*
Takahiro Nakazawa, *Nagoya*
Shoji Natsugoe, *Kagoshima City*
Tsutomu Nishida, *Suita*
Shuji Nomoto, *Naogya*
Sachiyo Nomura, *Tokyo*
Takeshi Ogura, *Takatsukishi*
Nobuhiro Ohkohchi, *Tsukuba*
Toshifumi Ohkusa, *Kashiwa*
Hirohide Ohnishi, *Akita*
Teruo Okano, *Tokyo*
Satoshi Osawa, *Hamamatsu*
Motoyuki Otsuka, *Tokyo*
Michitaka Ozaki, *Sapporo*
Satoru Saito, *Yokohama*
Naoaki Sakata, *Sendai*
Ken Sato, *Maebashi*
Toshiro Sato, *Tokyo*
Tomoyuki Shibata, *Toyoake*
Tomohiko Shimatani, *Kure*
Yukihiro Shimizu, *Nanto*
Tadashi Shimoyama, *Hirosaki*
Masayuki Sho, *Nara*
Ikuo Shoji, *Kobe*
Atsushi Sofuni, *Tokyo*
Takeshi Suda, *Niigata*
M Sugimoto, *Hamamatsu*
Ken Sugimoto, *Hamamatsu*
Haruhiko Sugimura, *Hamamatsu*
Shoichiro Sumi, *Kyoto*
Hidekazu Suzuki, *Tokyo*
Masahiro Tajika, *Nagoya*
Hitoshi Takagi, *Takasaki*
Toru Takahashi, *Niigata*
Yoshihisa Takahashi, *Tokyo*
Shinsuke Takeno, *Fukuoka*
Akihiro Tamori, *Osaka*
Kyosuke Tanaka, *Tsu*
Shinji Tanaka, *Hiroshima*
Atsushi Tanaka, *Tokyo*
Yasuhito Tanaka, *Nagoya*
Shinji Tanaka, *Tokyo*
Minoru Tomizawa, *Yotsukaido City*
Kyoko Tsukiyama-Kohara, *Kagoshima*
Takuya Watanabe, *Niigata*
Kazuhiro Watanabe, *Sendai*
Satoshi Yamagiwa, *Niigata*
Takayuki Yamamoto, *Yokkaichi*
Hiroshi Yamamoto, *Otsu*
Kosho Yamanouchi, *Nagasaki*
Ichiro Yasuda, *Gifu*
Yutaka Yata, *Maebashi-city*
Shin-ichi Yokota, *Sapporo*
Norimasa Yoshida, *Kyoto*
Hiroshi Yoshida, *Tama-City*
Hitoshi Yoshiji, *Kashihara*
Kazuhiko Yoshimatsu, *Tokyo*
Kentaro Yoshioka, *Toyoake*
Nobuhiro Zaima, *Nara*

Jordan
Khaled Ali Jadallah, *Irbid*

Kuwait
Islam Khan, *Kuwait*

Lebanon
Bassam N Abboud, *Beirut*
Kassem A Barada, *Beirut*
Marwan Ghosn, *Beirut*
Iyad A Issa, *Beirut*
Fadi H Mourad, *Beirut*
AIa Sharara, *Beirut*
Rita Slim, *Beirut*

Lithuania
Antanas Mickevicius, *Kaunas*

Malaysia
Huck Joo Tan, *Petaling Jaya*

Mexico
Richard A Awad, *Mexico City*
Carlos R Camara-Lemarroy, *Monterrey*
Norberto C Chavez-Tapia, *Mexico City*
Wolfgang Gaertner, *Mexico City*
Diego Garcia-Compean, *Monterrey*
Arturo Panduro, *Guadalajara*
OT Teramoto-Matsubara, *Mexico City*
Felix Tellez-Avila, *Mexico City*
Omar Vergara-Fernandez, *Mexico City*
Saúl Villa-Trevino, *Cuidad de México*

Morocco
Samir Ahboucha, *Khouribga*

Netherlands
Robert J de Knegt, *Rotterdam*
Tom Johannes Gerardus Gevers, *Nijmegen*
Menno Hoekstra, *Leiden*
BW Marcel Spanier, *Arnhem*
Karel van Erpecum, *Utrecht*

New Zealand
Leo K Cheng, *Auckland*
Andrew Stewart Day, *Christchurch*
Jonathan Barnes Koea, *Auckland*
Max Petrov, *Auckland*

Nigeria
Olufunmilayo Adenike Lesi, *Lagos*
Jesse Abiodun Otegbayo, *Ibadan*
Stella Ifeanyi Smith, *Lagos*

Norway
Trond Berg, *Oslo*
Trond Arnulf Buanes, *Krokkleiva*
Thomas de Lange, *Rud*
Magdy El-Salhy, *Stord*
Rasmus Goll, *Tromso*
Dag Arne Lihaug Hoff, *Aalesund*

Pakistan
Zaigham Abbas, *Karachi*
Usman A Ashfaq, *Faisalabad*
Muhammad Adnan Bawany, *Hyderabad*
Muhammad Idrees, *Lahore*
Saeed Sadiq Hamid, *Karachi*
Yasir Waheed, *Islamabad*

Poland
Thomas Brzozowski, *Cracow*
Magdalena Chmiela, *Lodz*
Krzysztof Jonderko, *Sosnowiec*
Anna Kasicka-Jonderko, *Sosnowiec*
Michal Kukla, *Katowice*
Tomasz Hubert Mach, *Krakow*
Agata Mulak, *Wroclaw*
Danuta Owczarek, *Kraków*
Piotr Socha, *Warsaw*
Piotr Stalke, *Gdansk*
Julian Teodor Swierczynski, *Gdansk*
Anna M Zawilak-Pawlik, *Wroclaw*

Portugal
Marie Isabelle Cremers, *Setubal*
Ceu Figueiredo, *Porto*
Ana Isabel Lopes, *LIsbon*
M Paula Macedo, *Lisboa*
Ricardo Marcos, *Porto*
Rui T Marinho, *Lisboa*
Guida Portela-Gomes, *Estoril*

Filipa F Vale, *Lisbon*

Puerto Rico

Caroline B Appleyard, *Ponce*

Qatar

Abdulbari Bener, *Doha*

Romania

Mihai Ciocirlan, *Bucharest*
Dan LucianDumitrascu, *Cluj-Napoca*
Carmen Fierbinteanu-Braticevici, *Bucharest*
Romeo G Mihaila, *Sibiu*
Lucian Negreanu, *Bucharest*
Adrian Saftoiu, *Craiova*
Andrada Seicean, *Cluj-Napoca*
Ioan Sporea, *Timisoara*
Letiţia Adela Maria Streba, *Craiova*
Anca Trifan, *Iasi*

Russia

Victor Pasechnikov, *Stavropol*
Vasiliy Ivanovich Reshetnyak, *Moscow*
Vitaly Skoropad, *Obninsk*

Saudi Arabia

Abdul-Wahed N Meshikhes, *Dammam*
M Ezzedien Rabie, *Khamis Mushait*

Singapore

Brian KP Goh, *Singapore*
Richie Soong, *Singapore*
Ker-Kan Tan, *Singapore*
Kok-Yang Tan, *Singapore*
Yee-Joo Tan, *Singapore*
Mark Wong, *Singapore*
Hong Ping Xia, *Singapore*

Slovenia

Matjaz Homan, *Ljubljana*
Martina Perse, *Ljubljana*

South Korea

Sang Hoon Ahn, *Seoul*
Seung Hyuk Baik, *Seoul*
Soon Koo Baik, *Wonju*
Soo-Cheon Chae, *Iksan*
Byung-Ho Choe, *Daegu*
Suck Chei Choi, *Iksan*
Hoon Jai Chun, *Seoul*
Yeun-Jun Chung, *Seoul*
Young-Hwa Chung, *Seoul*
Ki-Baik Hahm, *Seongnam*
Sang Young Han, *Busan*
Seok Joo Han, *Seoul*
Seung-Heon Hong, *Iksan*
Jin-Hyeok Hwang, *Seoungnam*
Jeong Won Jang, *Seoul*
Jin-Young Jang, *Seoul*
Dae-Won Jun, *Seoul*
Young Do Jung, *Kwangju*
Gyeong Hoon Kang, *Seoul*
Sung-Bum Kang, *Seoul*
Koo Jeong Kang, *Daegu*
Ki Mun Kang, *Jinju*
Chang Moo Kang, *Seodaemun-gu*
Gwang Ha Kim, *Busan*
Sang Soo Kim, *Goyang-si*
Jin Cheon Kim, *Seoul*
Tae Il Kim, *Seoul*
Jin Hong Kim, *Suwon*
Kyung Mo Kim, *Seoul*
Kyongmin Kim, *Suwon*
Hyung-Ho Kim, *Seongnam*
Seoung Hoon Kim, *Goyang*
Sang Il Kim, *Seoul*
Hyun-Soo Kim, *Wonju*
Jung Mogg Kim, *Seoul*
Dong Yi Kim, *Gwangju*
Kyun-Hwan Kim, *Seoul*
Jong-Han Kim, *Ansan*
Sang Wun Kim, *Seoul*
Ja-Lok Ku, *Seoul*
Kyu Taek Lee, *Seoul*
Hae-Wan Lee, *Chuncheon*
Inchul Lee, *Seoul*
Jung Eun Lee, *Seoul*
Sang Chul Lee, *Daejeon*
Song Woo Lee, *Ansan-si*
Hyuk-Joon Lee, *Seoul*
Seong-Wook Lee, *Yongin*
Kil Yeon Lee, *Seoul*
Jong-Inn Lee, *Seoul*
Kyung A Lee, *Seoul*
Jong-Baeck Lim, *Seoul*
Eun-Yi Moon, *Seoul*
SH Noh, *Seoul*
Seung Woon Paik, *Seoul*
Won Sang Park, *Seoul*
Sung-Joo Park, *Iksan*
Kyung Sik Park, *Daegu*
Se Hoon Park, *Seoul*
Yoonkyung Park, *Gwangju*
Seung-Wan Ryu, *Daegu*
Il Han Song, *Cheonan*
Myeong Jun Song, *Daejeon*
Yun Kyoung Yim, *Daejeon*
Dae-Yeul Yu *Daejeon*

Spain

Mariam Aguas, *Valencia*
Raul J Andrade, *Málaga*
Antonio Arroyo, *Elche*
Josep M Bordas, *Barcelona*
Lisardo Boscá, *Madrid*
Ricardo Robles Campos, *Murcia*
Jordi Camps, *Reus*
Carlos Cervera *Barcelona*
Alfonso Clemente, *Granada*
Pilar Codoner-Franch, *Valencia*
Fernando J Corrales, *Pamplona*
Fermin Sánchez de Medina, *Granada*
Alberto Herreros de Tejada, *Majadahonda*
Enrique de-Madaria, *Alicante*
JE Dominguez-Munoz, *Santiago de Compostela*
Vicente Felipo, *Valencia*
CM Fernandez-Rodriguez, *Madrid*
Carmen Frontela-Saseta, *Murcia*
Julio Galvez, *Granada*
Maria Teresa García, *Vigo*
MI Garcia-Fernandez, *Málaga*
Emilio Gonzalez-Reimers, *La Laguna*
Marcel Jimenez, *Bellaterra*
Angel Lanas, *Zaragoza*
Juan Ramón Larrubia, *Guadalajara*
Antonio Lopez-Sanroman, *Madrid*
Vicente Lorenzo-Zuniga, *Badalona*
Alfredo J Lucendo, *Tomelloso*
Vicenta Soledad Martinez-Zorzano, *Vigo*
José Manuel Martin-Villa, *Madrid*
Julio Mayol, *Madrid*
Manuel Morales-Ruiz, *Barcelona*
Alfredo Moreno-Egea, *Murcia*
Albert Pares, *Barcelona*
Maria Pellise, *Barcelona*
José Perea, *Madrid*
Miguel Angel Plaza, *Zaragoza*
María J Pozo, *Cáceres*
Enrique Quintero, *La Laguna*
Jose M Ramia, *Madrid*
Francisco Rodriguez-Frias, *Barcelona*
Silvia Ruiz-Gaspa, *Barcelona*
Xavier Serra-Aracil, *Barcelona*
Vincent Soriano, *Madrid*
Javier Suarez, *Pamplona*
Carlos Taxonera, *Madrid*
M Isabel Torres, *Jaén*
Manuel Vazquez-Carrera, *Barcelona*
Benito Velayos, *Valladolid*
Silvia Vidal, *Barcelona*

Sri Lanka

Arjuna Priyadarsin De Silva, *Colombo*

Sudan

Ishag Adam, *Khartoum*

Sweden

Roland G Andersson, *Lund*
Bergthor Björnsson, *Linkoping*
Johan Christopher Bohr, *Örebro*
Mauro D'Amato, *Stockholm*
Thomas Franzen, *Norrkoping*
Evangelos Kalaitzakis, *Lund*
Riadh Sadik, *Gothenburg*
Per Anders Sandstrom, *Linkoping*
Ervin Toth, *Malmö*
Konstantinos Tsimogiannis, *Vasteras*
Apostolos V Tsolakis, *Uppsala*

Switzerland

Gieri Cathomas, *Liestal*
Jean Louis Frossard, *Geneve*
Christian Toso, *Geneva*
Stephan Robert Vavricka, *Zurich*
Dominique Velin, *Lausanne*

Thailand

Thawatchai Akaraviputh, *Bangkok*
P Yoysungnoen Chintana, *Pathumthani*
Veerapol Kukongviriyapan, *Muang*
Vijittra Leardkamolkarn, *Bangkok*
Varut Lohsiriwat, *Bangkok*
Somchai Pinlaor, *Khaon Kaen*
D Wattanasirichaigoon, *Bangkok*

Trinidad and Tobago

B Shivananda Nayak, *Mount Hope*

Tunisia

Ibtissem Ghedira, *Sousse*
Lilia Zouiten-Mekki, *Tunis*

Turkey

Inci Alican, *Istanbul*
Mustafa Altindis, *Sakarya*
Mutay Aslan, *Antalya*
Oktar Asoglu, *Istanbul*
Yasemin Hatice Balaban, *Istanbul*
Metin Basaranoglu, *Ankara*
Yusuf Bayraktar, *Ankara*
Süleyman Bayram, *Adiyaman*
Ahmet Bilici, *Istanbul*
Ahmet Sedat Boyacioglu, *Ankara*
Züleyha Akkan Cetinkaya, *Kocaeli*
Cavit Col, *Bolu*
Yasar Colak, *Istanbul*
Cagatay Erden Daphan, *Kirikkale*
Mehmet Demir, *Hatay*
Ahmet Merih Dobrucali, *Istanbul*
Gülsüm Ozlem Elpek, *Antalya*
Ayse Basak Engin, *Ankara*
Eren Ersoy, *Ankara*
Osman Ersoy, *Ankara*
Yusuf Ziya Erzin, *Istanbul*
Mukaddes Esrefoglu, *Istanbul*
Levent Filik, *Ankara*
Ozgur Harmanci, *Ankara*
Koray Hekimoglu, *Ankara*
Abdurrahman Kadayifci, *Gaziantep*
Cem Kalayci, *Istanbul*
Selin Kapan, *Istanbul*
Huseyin Kayadibi, *Adana*
Sabahattin Kaymakoglu, *Istanbul*
Metin Kement, *Istanbul*
Mevlut Kurt, *Bolu*
Resat Ozaras, *Istanbul*
Elvan Ozbek, *Adapazari*
Cengiz Ozcan, *Mersin*
Hasan Ozen, *Ankara*
Halil Ozguc, *Bursa*
Mehmet Ozturk, *Izmir*
Orhan V Ozkan, *Sakarya*
Semra Paydas, *Adana*
Ozlem Durmaz Suoglu, *Istanbul*
Ilker Tasci, *Ankara*
Müge Tecder-ünal, *Ankara*
Mesut Tez, *Ankara*
Serdar Topaloglu, *Trabzon*
Murat Toruner, *Ankara*
Gokhan Tumgor, *Adana*
Oguz Uskudar, *Adana*
Mehmet Yalniz, *Elazig*
Mehmet Yaman, *Elazig*
Veli Yazisiz, *Antalya*
Yusuf Yilmaz, *Istanbul*
Ozlem Yilmaz, *Izmir*
Oya Yucel, *Istanbul*
Ilhami Yuksel, *Ankara*

United Kingdom

Nadeem Ahmad Afzal, *Southampton*
Navneet K Ahluwalia, *Stockport*
Yeng S Ang, *Lancashire*
Ramesh P Arasaradnam, *Coventry*
Ian Leonard Phillip Beales, *Norwich*
John Beynon, *Swansea*
Barbara Braden, *Oxford*
Simon Bramhall, *Birmingham*
Geoffrey Burnstock, *London*
Ian Chau, *Sutton*
Thean Soon Chew, *London*
Helen G Coleman, *Belfast*
Anil Dhawan, *London*
Sunil Dolwani, *Cardiff*
Piers Gatenby, *London*
Anil T George, *London*
Pasquale Giordano, *London*
Paul Henderson, *Edinburgh*
Georgina Louise Hold, *Aberdeen*
Stefan Hubscher, *Birmingham*
Robin D Hughes, *London*
Nusrat Husain, *Manchester*
Matt W Johnson, *Luton*
Konrad Koss, *Macclesfield*
Anastasios Koulaouzidis, *Edinburgh*
Simon Lal, *Salford*
John S Leeds, *Aberdeen*
JK K Limdi, *Manchester*
Hongxiang Liu, *Cambridge*
Michael Joseph McGarvey, *London*
Michael Anthony Mendall, *London*
Alexander H Mirnezami, *Southampton*
J Bernadette Moore, *Guildford*
Claudio Nicoletti, *Norwich*
Savvas Papagrigoriadis, *London*
Sylvia LF Pender, *Southampton*
David Mark Pritchard, *Liverpool*
James A Ross, *Edinburgh*
Kamran Rostami, *Worcester*
Xiong Z Ruan, *London*
Frank I Tovey, *London*
Dhiraj Tripathi, *Birmingham*
Vamsi R Velchuru, *Great Yarmouth*
Nicholas T Ventham, *Edinburgh*
Diego Vergani, *London*
Jack Westwood Winter, *Glasgow*
Terence Wong, *London*
Ling Yang, *Oxford*

United States

Daniel E Abbott, *Cincinnati*
Ghassan K Abou-Alfa, *New York*
Julian Abrams, *New York*
David William Adelson, *Los Angeles*
Jonathan Steven Alexander, *Shreveport*
Tauseef Ali, *Oklahoma City*
Mohamed R Ali, *Sacramento*
Rajagopal N Aravalli, *Minneapolis*
Hassan Ashktorab, *Washington*
Shashi Bala, *Worcester*
Charles F Barish, *Raleigh*
P Patrick Basu, *New York*
Robert L Bell, *Berkeley Heights*
David Bentrem, *Chicago*
Henry J Binder, *New Haven*
Joshua Bleier, *Philadelphia*
Wojciech Blonski, *Johnson City*
Kenneth Boorom, *Corvallis*
Brian Boulay, *Chicago*
Carla W Brady, *Durham*
Kyle E Brown, *Iowa City*
Adeel A Butt, *Pittsburgh*
Weibiao Cao, *Providence*
Andrea Castillo, *Cheney*
Fernando J Castro, *Weston*
Adam S Cheifetz, *Boston*
Xiaoxin Luke Chen, *Durham*
Ramsey Cheung, *Palo Alto*
Parimal Chowdhury, *Little Rock*
Edward John Ciaccio, *New York*
Dahn L Clemens, *Omaha*
Yingzi Cong, *Galveston*
Laura Iris Cosen-Binker, *Boston*
Joseph John Cullen, *Lowa*
Mark J Czaja, *Bronx*
Mariana D Dabeva, *Bronx*
Christopher James Damman, *Seattle*
Isabelle G De Plaen, *Chicago*
Punita Dhawan, *Nashville*
Hui Dong, *La Jolla*
Wael El-Rifai, *Nashville*
Sukru H Emre, *New Haven*
Paul Feuerstadt, *Hamden*
Josef E Fischer, *Boston*
Laurie N Fishman, *Boston*
Joseph Che Forbi, *Atlanta*
Temitope Foster, *Atlanta*
Amy E Foxx-Orenstein, *Scottsdale*
Daniel E Freedberg, *New York*
Shai Friedland, *Palo Alto*
Virgilio George, *Indianapolis*
Ajay Goel, *Dallas*
Oliver Grundmann, *Gainesville*
Stefano Guandalini, *Chicago*
Chakshu Gupta, *St. Joseph*
Grigoriy E Gurvits, *New York*

Xiaonan Han, *Cincinnati*
Mohamed Hassan, *Jackson*
Martin Hauer-Jensen, *Little Rock*
Koichi Hayano, *Boston*
Yingli Hee, *Atlanta*
Samuel B Ho, *San Diego*
Jason Ken Hou, *Houston*
Lifang Hou, *Chicago*
K-Qin Hu, *Orange*
Jamal A Ibdah, *Columbia*
Robert Thomas Jensen, *Bethesda*
Huanguang "Charlie" Jia, *Gainesville*
Rome Jutabha, *Los Angeles*
Andreas M Kaiser, *Los Angeles*
Avinash Kambadakone, *Boston*
David Edward Kaplan, *Philadelphia*
Randeep Kashyap, *Rochester*
Rashmi Kaul, *Tulsa*
Ali Keshavarzian, *Chicago*
Amir Maqbul Khan, *Marshall*
Nabeel Hasan Khan, *New Orleans*
Sahil Khanna, *Rochester*
Kusum K Kharbanda, *Omaha*
Hyun Sik Kim, *Pittsburgh*
Joseph Kim, *Duarte*
Jae S Kim, *Gainesville*
Miran Kim, *Providence*
Timothy R Koch, *Washington*
Burton I Korelitz, *New York*
Betsy Kren, *Minneapolis*
Shiu-Ming Kuo, *Buffalo*
Michelle Lai, *Boston*
Andreas Larentzakis, *Boston*
Edward Wolfgang Lee, *Los Angeles*
Daniel A Leffler, *Boston*
Michael Leitman, *New York*
Suthat Liangpunsakul, *Indianapolis*
Joseph K Lim, *New Haven*
Elaine Y Lin, *Bronx*
Henry C Lin, *Albuquerque*
Rohit Loomba, *La Jolla*
James David Luketich, *Pittsburgh*
Li Ma, *Stanford*
Mohammad F Madhoun, *Oklahoma City*
Thomas C Mahl, *Buffalo*
Ashish Malhotra, *Bettendorf*
Pranoti Mandrekar, *Worcester*
John Marks, *Wynnewood*
Wendy M Mars, *Pittsburgh*
Julien Vahe Matricon, *San Antonio*
Craig J McClain, *Louisville*
Tamir Miloh, *Phoenix*
Ayse Leyla Mindikoglu, *Baltimore*
Huanbiao Mo, *Denton*
Klaus Monkemuller, *Birmingham*
John Morton, *Stanford*
Adnan Muhammad, *Tampa*
Michael J Nowicki, *Jackson*
Patrick I Okolo, *Baltimore*
Giusepp Orlando, *Winston Salem*
Natalia A Osna, *Omaha*
Virendra N Pandey, *Newark*
Mansour A Parsi, *Cleveland*
Michael F Picco, *Jacksonville*
Daniel S Pratt, *Boston*
Xiaofa Qin, *Newark*
Janardan K Reddy, *Chicago*
Victor E Reyes, *Galveston*
Jon Marc Rhoads, *Houston*
Giulia Roda, *New York*
Jean-Francois Armand Rossignol, *Tampa*
Paul A Rufo, *Boston*
Madhusudana Girija Sanal, *New York*
Miguel Saps, *Chicago*
Sushil Sarna, *Galveston*
Ann O Scheimann, *Baltimore*
Bernd Schnabl, *La Jolla*
Matthew J Schuchert, *Pittsburgh*
Ekihiro Seki, *La Jolla*
Chanjuan Shi, *Nashville*
David Quan Shih, *Los Angeles*
Shadab A Siddiqi, *Orlando*
William B Silverman, *Iowa City*
Shashideep Singhal, *New York*
Bronislaw L Slomiany, *Newark*
Steven F Solga, *Bethlehem*
Byoung-Joon Song, *Bethesda*
Dario Sorrentino, *Roanoke*
Scott R Steele, *Fort Lewis*
Branko Stefanovic, *Tallahassee*
Arun Swaminath, *New York*
Kazuaki Takabe, *Richmond*
Naoki Tanaka, *Bethesda*
Hans Ludger Tillmann, *Durham*
George Triadafilopoulos, *Stanford*
John Richardson Thompson, *Nashville*
Andrew Ukleja, *Weston*
Miranda AL van Tilburg, *Chapel Hill*
Gilberto Vaughan, *Atlanta*
Vijayakumar Velu, *Atlanta*
Gebhard Wagener, *New York*
Kasper Saonun Wang, *Los Angeles*
Xiangbing Wang, *New Brunswick*
Daoyan Wei, *Houston*
Theodore H Welling, *Ann Arbor*
C Mel Wilcox, *Birmingham*
Jacqueline Lee Wolf, *Boston*
Laura Ann Woollett, *Cincinnati*
Harry Hua-Xiang Xia, *East Hanover*
Wen Xie, *Pittsburgh*
Guang Yu Yang, *Chicago*
Michele T Yip-Schneider, *Indianapolis*
Sam Zakhari, *Bethesda*
Kezhong Zhang, *Detroit*
Huiping Zhou, *Richmond*
Xiao-Jian Zhou, *Cambridge*
Richard Zubarik, *Burlington*

Venezuela
Miguel Angel Chiurillo, *Barquisimeto*

Vietnam
Van Bang Nguyen, *Hanoi*

World Journal of Gastroenterology

Contents

Weekly Volume 23 Number 6 February 14, 2017

AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 1375 experts in gastroenterology and hepatology from 68 countries.

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

INDEXING/ABSTRACTING

World Journal of Gastroenterology (*WJG*) is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. The 2015 edition of Journal Citation Reports® released by Thomson Reuters (ISI) cites the 2015 impact factor for *WJG* as 2.787 (5-year impact factor: 2.848), ranking *WJG* as 38 among 78 journals in gastroenterology and hepatology (quartile in category Q2).

NAME OF JOURNAL
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

EDITORIAL OFFICE
Jin-Lei Wang, Director
Yuan Qi, Vice Director
Ze-Mao Gong, Vice Director
World Journal of Gastroenterology
Baishideng Publishing Group Inc
8226 Regency Drive,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Inc
8226 Regency Drive,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLICATION DATE
February 14, 2017

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/bpg/gerinfo/204

ONLINE SUBMISSION
http://www.wjgnet.com/esps/

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v23.i6.931

World J Gastroenterol 2017 February 14; 23(6): 931-934
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Prophylactic stenting for esophageal stricture prevention after endoscopic submucosal dissection

Ke-Da Shi, Feng Ji

Ke-Da Shi, Feng Ji, Department of Gastroenterology, First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310000, Zhejiang Province, China

Author contributions: Shi KD and Ji F equally contributed to the analysis of the literature and writing of the manuscript.

Conflict-of-interest statement: Both authors declare no conflict of interest related to this publication.

Manuscript source: Invited manuscript

Correspondence to: Feng Ji, MD, PhD, Deputy Director, Department of Gastroenterology, First Affiliated Hospital, School of Medicine, Zhejiang University, No. 79 Qingchun Rd., Hangzhou 310000, Zhejiang Province, China. jifeng1126@sina.com
Telephone: +86-571-87236568
Fax: +86-571-87236568

Received: August 28, 2016
Peer-review started: September 1, 2016
First decision: September 20, 2016
Revised: October 6, 2016
Accepted: November 12, 2016
Article in press: November 13, 2016
Published online: February 14, 2017

Abstract

Endoscopic submucosal dissection (ESD) of superficial esophageal cancer has been increasingly used as an alternative to surgery because it is minimally invasive and has a high rate of en bloc resection. However, a high rate of esophageal stricture is observed after ESD for large lesions, which can dramatically decrease the patient's quality of life. Stricture prevention is necessary to allow for endoscopic therapy to expand. We, herein, review the most recent evidence and discuss the role of the metallic self-expandable stent and the biodegradable stent in esophageal stricture prevention. Limited studies suggested that prophylactic stenting could reduce the stricture rate without increasing the number of complications. In addition, the number of bougie dilation procedures was significantly lower with stent placement. Esophageal stenting is a promising option for post-ESD stricture prevention. However, current evidence is too preliminary to formulate practice standards. Future studies are needed to further validate the efficacy and safety of prophylactic stenting and determine the best strategy for stricture prevention. Stent migration is the most common complication. A new stent that has advantages of a low migration rate and minimal tissue reaction will need to be developed. Therefore, randomized controlled trials with long-term follow-up periods are required before prophylactic stenting could be considered a valid option to prevent post-ESD stricture.

Key words: Biodegradable stent; Stricture prevention; Esophageal stricture; Metallic self-expandable stent; Endoscopic submucosal dissection

Core tip: Esophageal stenting is a promising option for esophageal stricture prevention. Current evidence is too preliminary to formulate practice standards. Randomized controlled trials with long-term follow-up periods and cost-effective studies are required before prophylactic stenting could be considered a valid option to prevent post-endoscopic submucosal dissection stricture.

Shi KD, Ji F. Prophylactic stenting for esophageal stricture prevention after endoscopic submucosal dissection. *World J Gastroenterol* 2017; 23(6): 931-934 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i6/931.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i6.931

INTRODUCTION

Endoscopic submucosal dissection (ESD) of superficial esophageal cancer has been increasingly used as an alternative to surgery because it is minimally invasive and has a high rate of en bloc resection. Despite these advantages, a high rate of esophageal stricture is observed after ESD for large lesions, which can dramatically decrease the patient's quality of life[1-3]. The rate of strictures after endoscopic resection for circumferential or near-circumferential lesions can be as high as 88%[4]. Although oral steroids were administered in one study, 45% of patients still suffered from stricture[5]. Therefore, post-ESD esophageal stricture prevention is needed to allow for endoscopic therapy to expand. In this editorial, we discuss the use of prophylactic stenting in the prevention of post-ESD stricture and include a discussion of our future vision related to this topic. PubMed and Web of Science were searched using the following search strategy: (ESD OR endoscopic resection) AND esophageal stricture AND stent, until 31 August 2016. Studies investigating the role of stent placement after esophageal ESD were included. We searched the reference lists of the relevant studies to identify other studies on the same topic. Currently, no conclusive recommendation is evident in the literature related to the type of stent to use in stricture prevention. According to the current studies, the self-expandable metallic stent and the biodegradable stent are two potential options.

SELF-EXPANDABLE METALLIC STENTS

Temporary placement of a self-expandable metallic stent is increasingly used in practice to prevent post-ESD stricture. Ye and colleagues reported on their application of fully-covered stent placement after circumferential endoscopic resection of esophageal lesions[6]. More information about the stent used can be found in Table 1. A total of 23 patients were included in this prospective study. During the follow-up period, 4 patients had stent migration, and 3 of those 4 patients developed esophageal strictures. The stricture rate in this study was 17.3% (4/23), which was significantly lower than the stricture rate reported in previous studies. However, only one endoscopist from a high-volume center performed the study, which raises concerns about potential bias. Because the post-ESD stricture rate could be affected by the experience and operative skills of the endoscopist, the results of this study may not be generalizable. Another limitation of this study was the absence of sham control to further validate the advantage of stent placement. The only randomized controlled trial (RCT) to date to investigate this question was conducted by Wen and his colleagues[7]. In this study, patients who had mucosal defects that exceeded 75% of the circumference of the esophagus after ESD treatment were randomized in a 1:1 fashion to either a group that received treatment with a fully-covered esophageal stent or an observation group. The study showed a significantly lower rate of stricture in the group that received stent placement compared to the observational group (18.2% *vs* 72.7%, respectively, $P < 0.05$). Moreover, the number of bougie dilation procedures was significantly lower in the stent placement group compared to the observational group (mean 0.45, range 0-3 *vs* mean 3.9, range 0-17, respectively, $P < 0.05$). In addition, complications were comparable between the two groups. Interestingly, the two patients who developed stricture in the stent group both had stent migration. The stent migration rate could be affected by the design of the stents. Different types of stents have their own characteristics. A small portion of exposed bare metal at both ends of the partially covered metallic stent helps to prevent migration. However, the granulation tissue ingrowth at the ends of the stent could lead to stent-induced strictures. In contrast, fully-covered stents do not have any exposed bare metal, but they are more prone to migration. A new stent that has advantages of both a low migration rate and minimal tissue reaction will need to be developed. Is successful stent placement without migration enough for stricture prevention? The answer may be "no". Bhat and colleagues reported a 28% stricture rate after stent removal or migration[8]. The circumferential lesion left at the ends of the stent after stent removal is prone to forming strictures. In addition, the potential pro-inflammatory action of the implanted stent makes it unlikely to be the single best choice for esophageal stricture prevention. Although the results in previously mentioned studies are inspiring, the studies involved a small sample size and a short-term follow-up period; therefore, the results need to be confirmed in future larger trials. A recent meta-analysis conducted by Oliveira and his colleagues demonstrated that the use of preventive therapy after extensive ESD reduces the risk of stricture without increasing the number of complications[9]. Moreover, the placement of a fully-covered stent and the use of local corticosteroid injections have the most promising results in reducing post-ESD stricture among different types of strategies. It is worth mentioning that these results were based on three small RCTs and several retrospective studies. The high degree of heterogeneity across the included studies raises additional concerns about the potential bias of this analysis. Therefore, RCTs with larger sample sizes comparing different treatment strategies are still needed to clarify this issue. In endoscopy, the

Table 1 Studies using esophageal stenting to prevent post-endoscopic submucosal dissection stricture

Ref.	Type of study	Population	Type of stent	Time of removal	Stricture rate	Stent migration rate
Ye *et al*[6], 2016	Cohort	Circumferential	Fully-covered self-expandable metallic stents (CZES stent; Sigma, China)	12 wk	17.4%	17.4%
Wen *et al*[7], 2014	RCT	Mucosal defect > 3/4	Fully-covered self-expandable metallic stents (CZES stent; Sigma, China)	8 wk	18.2%	18.2%
Saito *et al*[11], 2008	Case report	Mucosal defect > 3/4	PLLA esophageal stent (Tanaka-Marui stent; Marui Textile Machinery Co., Japan)	Self-degradable	0	0
Saito *et al*[10], 2007	Case report	Mucosal defect > 3/4	PLLA esophageal stent (Tanaka-Marui stent; Marui Textile Machinery Co., Japan)	Self-degradable	0	77.0%

RCT: Randomized controlled trial; PLLA: Poly-L-lactic acid.

advancement of a new concept could be affected by the availability and cost-effectiveness of technology. Since stents are associated with a high cost, evidence on cost-effectiveness is required in future studies.

BIODEGRADABLE STENTS

Saito *et al*[10] reported the successful application of biodegradable stents to post-ESD esophageal stricture prevention. Despite having a high rate of early stent migration, the efficacy of stricture prevention reached 100%, but caution should be taken as the data are based on short-term follow-up case reports[10]. This group also reported 2 cases of successful application of biodegradable stents in the prevention of re-stricture formation after balloon dilatation of post-ESD strictures[11]. Experience in using these types of stents is learned from animal model-based experiments[12,13]. Biodegradable stents have the potential to mitigate stent-related complications and do not require removal. The main limitations of biodegradable stents are a hyperplastic tissue reaction and stent migration. The poly-L-lactic acid (PLLA) stent and the polydioxanone stent are two types of biodegradable stents that are currently available. The PLLA-biodegradable stent has a high rate of early stent migration, which makes it unlikely to be the best choice for stricture prevention[10,14]. Meanwhile, polydioxanone stents have a migration rate of 20%, but they could induce a severe hyperplastic tissue reaction[15,16]. A hyperplastic tissue reaction delays the healing of the mucosa and poses a challenge when removing the stent. Moreover, it might prevent adequate surveillance for local recurrence. Although the ideal stent has not yet been developed, possible options to minimize tissue hyperplasia after biodegradable stent placement should be considered in future studies. These options include steroid injection, consumption of a drug-eluting layer, and coverage with biodegradable membranes or extracellular matrix scaffolds.

CONCLUSION

All patients with extensive esophageal ESD should receive some type of preventive treatment. Esophageal stenting is a promising option for esophageal stricture prevention, especially when corticosteroid treatment is contraindicated in certain patients. Current evidence is too preliminary to formulate practice standards. RCTs with long-term follow-up periods are required before prophylactic esophageal stenting could be considered as a valid option to prevent post-ESD stricture.

REFERENCES

1 **Katada C**, Muto M, Manabe T, Boku N, Ohtsu A, Yoshida S. Esophageal stenosis after endoscopic mucosal resection of superficial esophageal lesions. *Gastrointest Endosc* 2003; **57**: 165-169 [PMID: 12556777 DOI: 10.1067/mge.2003.73]

2 **Ono S**, Fujishiro M, Niimi K, Goto O, Kodashima S, Yamamichi N, Omata M. Predictors of postoperative stricture after esophageal endoscopic submucosal dissection for superficial squamous cell neoplasms. *Endoscopy* 2009; **41**: 661-665 [PMID: 19565442 DOI: 10.1055/s-0029-1214867]

3 **Ono S**, Fujishiro M, Niimi K, Goto O, Kodashima S, Yamamichi N, Omata M. Long-term outcomes of endoscopic submucosal dissection for superficial esophageal squamous cell neoplasms. *Gastrointest Endosc* 2009; **70**: 860-866 [PMID: 19577748 DOI: 10.1016/j.gie.2009.04.044]

4 **van Vilsteren FG**, Pouw RE, Seewald S, Alvarez Herrero L, Sondermeijer CM, Visser M, Ten Kate FJ, Yu Kim Teng KC, Soehendra N, Rösch T, Weusten BL, Bergman JJ. Stepwise radical endoscopic resection versus radiofrequency ablation for Barrett's oesophagus with high-grade dysplasia or early cancer: a multicentre randomised trial. *Gut* 2011; **60**: 765-773 [PMID: 21209124 DOI: 10.1136/gut.2010.229310]

5 **Tang B**, Bai JY, Zhao XY, Fan CQ, Yang X, Deng L, Yang SM, Yu J. Endoscopic submucosal dissection for superficial esophageal cancer with near-circumferential lesions: our experience with 40 patients. *Surg Endosc* 2015; **29**: 2141-2148 [PMID: 25303920 DOI: 10.1007/s00464-014-3909-8]

6 **Ye LP**, Zheng HH, Mao XL, Zhang Y, Zhou XB, Zhu LH. Complete circular endoscopic resection using submucosal tunnel technique combined with esophageal stent placement for circumferential superficial esophageal lesions. *Surg Endosc* 2016; **30**: 1078-1085 [PMID: 26092023 DOI: 10.1007/s00464-015-4301-z]

7 **Wen J**, Lu Z, Yang Y, Liu Q, Yang J, Wang S, Wang X, Du H, Meng J, Wang H, Linghu E. Preventing stricture formation by covered esophageal stent placement after endoscopic submucosal dissection for early esophageal cancer. *Dig Dis Sci* 2014; **59**: 658-663 [PMID: 24323178 DOI: 10.1007/s10620-013-2958-5]

8 **Bhat YM**, Kane SD, Shah JN, Hamerski CM, Binmoeller KF. Single-session circumferential EMR and metal stent placement for the treatment of long-segment Barrett's esophagus with high-grade intraepithelial neoplasia. *Gastrointest Endosc* 2014; **80**: 331 [PMID: 25034839 DOI: 10.1016/j.gie.2014.04.058]

9 **Oliveira JF**, Moura EG, Bernardo WM, Ide E, Cheng S, Sulbaran M, Santos CM, Sakai P. Prevention of esophageal stricture after

endoscopic submucosal dissection: a systematic review and meta-analysis. *Surg Endosc* 2016; **30**: 2779-2791 [PMID: 26487197 DOI: 10.1007/s00464-015-4551-9]

10 **Saito Y**, Tanaka T, Andoh A, Minematsu H, Hata K, Tsujikawa T, Nitta N, Murata K, Fujiyama Y. Usefulness of biodegradable stents constructed of poly-l-lactic acid monofilaments in patients with benign esophageal stenosis. *World J Gastroenterol* 2007; **13**: 3977-3980 [PMID: 17663513 DOI:10.3748/wjg.v13.i29.3977]

11 **Saito Y**, Tanaka T, Andoh A, Minematsu H, Hata K, Tsujikawa T, Nitta N, Murata K, Fujiyama Y. Novel biodegradable stents for benign esophageal strictures following endoscopic submucosal dissection. *Dig Dis Sci* 2008; **53**: 330-333 [PMID: 17713855 DOI: 10.1007/s10620-007-9873-6]

12 **Barret M**, Pratico CA, Camus M, Beuvon F, Jarraya M, Nicco C, Mangialavori L, Chaussade S, Batteux F, Prat F. Amniotic membrane grafts for the prevention of esophageal stricture after circumferential endoscopic submucosal dissection. *PLoS One* 2014; **9**: e100236 [PMID: 24992335 DOI: 10.1371/journal.pone.0100236]

13 **Pauli EM**, Schomisch SJ, Furlan JP, Marks AS, Chak A, Lash RH, Ponsky JL, Marks JM. Biodegradable esophageal stent placement does not prevent high-grade stricture formation after circumferential mucosal resection in a porcine model. *Surg Endosc* 2012; **26**: 3500-3508 [PMID: 22684976 DOI: 10.1007/s00464-012-2373-6]

14 **Tanaka T**, Takahashi M, Nitta N, Furukawa A, Andoh A, Saito Y, Fujiyama Y, Murata K. Newly developed biodegradable stents for benign gastrointestinal tract stenoses: a preliminary clinical trial. *Digestion* 2006; **74**: 199-205 [PMID: 17341853 DOI: 10.1159/000100504]

15 **Hair CS**, Devonshire DA. Severe hyperplastic tissue stenosis of a novel biodegradable esophageal stent and subsequent successful management with high-pressure balloon dilation. *Endoscopy* 2010; **42** Suppl 2: E132-E133 [PMID: 20405380 DOI: 10.1055/s-0029-1244011]

16 **Orive-Calzada A**, Alvarez-Rubio M, Romero-Izquierdo S, Cobo Martin M, Juanmartiñena JF, Ogueta-Fernández M, Molina-Alvarez E, Eraña-Ledesma L. Severe epithelial hyperplasia as a complication of a novel biodegradable stent. *Endoscopy* 2009; **41** Suppl 2: E137-E138 [PMID: 19544266 DOI: 10.1055/s-0029-1214634]

P- Reviewer: Iizuka T, Kim GH, Tsuji Y, Wang HP
S- Editor: Gong ZM **L- Editor**: A **E- Editor**: Liu WX

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v23.i6.935

World J Gastroenterol 2017 February 14; 23(6): 935-948
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Familial pancreatic cancer: Concept, management and issues

Hiroyuki Matsubayashi, Kyoichi Takaori, Chigusa Morizane, Hiroyuki Maguchi, Masamichi Mizuma, Hideaki Takahashi, Keita Wada, Hiroko Hosoi, Shinichi Yachida, Masami Suzuki, Risa Usui, Toru Furukawa, Junji Furuse, Takamitsu Sato, Makoto Ueno, Yoshimi Kiyozumi, Susumu Hijioka, Nobumasa Mizuno, Takeshi Terashima, Masaki Mizumoto, Yuzo Kodama, Masako Torishima, Takahisa Kawaguchi, Reiko Ashida, Masayuki Kitano, Keiji Hanada, Masayuki Furukawa, Ken Kawabe, Yoshiyuki Majima, Toru Shimosegawa

Hiroyuki Matsubayashi, Division of Endoscopy, Shizuoka Cancer Center, Shizuoka 411-8777, Japan

Hiroyuki Matsubayashi, Yoshimi Kiyozumi, Clinic of Genetic Medicine, Shizuoka Cancer Center, Shizuoka 411-8777, Japan

Kyoichi Takaori, Department of Surgery, Kyoto University Graduate School of Medicine, Sakyo, Kyoto 606-8397, Japan

Chigusa Morizane, Hiroko Hosoi, Division of Hepatobiliary and Pancreatic Oncology, National Cancer Center Hospital, Chuoku, Tokyo 104-0045, Japan

Hiroyuki Maguchi, Center for Gastroenterology, Teine-Keijinkai Hospital, Sapporo, Hokkaido 006-0811, Japan

Masamichi Mizuma, Department of Surgery, Tohoku University Graduate School of Medicine, Sendai, Miyagi 980-8574, Japan

Hideaki Takahashi, Department of Hepatobiliary and Pancreatic Oncology, National Cancer Center Hospital East, Kashiwa, Chiba 277-8577, Japan

Keita Wada, Department of Surgery, Teikyo University School of Medicine, Itabashi, Tokyo 173-8606, Japan

Shinichi Yachida, Masami Suzuki, Risa Usui, Division of Cancer Genomics, National Cancer Center Research Institute, Chuoku, Tokyo 104-0045, Japan

Toru Furukawa, Institute for Integrated Medical Sciences, Tokyo Women's Medical University, Shinjuku, Tokyo 162-8666, Japan

Junji Furuse, Department of Internal Medicine, Kyorin University School of Medicine, Mitaka, Tokyo 181-8611, Japan

Takamitsu Sato, Division of Gastroenterology, Yokohama City University Graduate School of Medicine, Yokohama, Kanagawa 236-0004, Japan

Makoto Ueno, Division of Hepatobiliary and Pancreatic Medical Oncology, Kanagawa Cancer Center, Yokohama, Kanagawa 241-8515, Japan

Susumu Hijioka, Nobumasa Mizuno, Department of Gastroenterology, Aichi Cancer Center Hospital, Nagoya, Aichi 464-8681, Japan

Takeshi Terashima, Department of Gastroenterology, Kanazawa University Graduate School of Medicine, Kanazawa, Ishikawa 920-8641, Japan

Masaki Mizumoto, Department of Surgery, Japan Bapist Hospital, Kyotoshi, Kyoto 606-8273, Japan

Yuzo Kodama, Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Sakyo, Kyoto 606-8397, Japan

Masako Torishima, Clinical Genetics Unit, Kyoto University Graduate School of Medicine, Sakyo, Kyoto 606-8397, Japan

Takahisa Kawaguchi, Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Sakyo, Kyoto 606-8397, Japan

Reiko Ashida, Osaka Medical Center for Cancer and Cardiovascular Diseases, Higashinari, Osaka 537-8511, Japan

Masayuki Kitano, Second Department of Internal Medicine, Wakayama Medical University, Wakayamashi, Wakayama 641-8509, Japan

Keiji Hanada, Department of Gastroenterology, Onomichi General Hospital, Onomichi, Hiroshima 722-8508, Japan

Masayuki Furukawa, Department of Gastroenterology, National

Kyushu Cancer Center, Fukuokashi, Fukuoka 811-1395, Japan

Ken Kawabe, Department of Medicine and Bioregulatory Sciences, Kyushu University Graduate School of Medical Science, Fukuokashi, Fukuoka 812-8582, Japan

Yoshiyuki Majima, Pancreatic Cancer Action Network, Chiyoda, Tokyo 102-0071, Japan

Toru Shimosegawa, Department of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Miyagi 980-8574, Japan

Author contributions: All authors are core members of Japanese Familial Pancreatic Cancer Study Group; Matsubayashi H drafted and completed the manuscript, and all other authors reviewed and provided beneficial comments and discussions.

Conflict-of-interest statement: The authors have no conflict of interest relevant to this review.

Manuscript source: Invited manuscript

Correspondence to: Hiroyuki Matsubayashi, MD, PhD, Division of Endoscopy, Shizuoka Cancer Center, 1007, Shimonagakubo, Nagaizumi, Suntogun, Shizuoka 411-8777, Japan. h.matsubayashi@scchr.jp
Telephone: +81-55-9895222
Fax: +81-55-9895692

Received: August 7, 2016
Peer-review started: August 10, 2016
First decision: September 5, 2016
Revised: September 7, 2016
Accepted: January 18, 2017
Article in press: January 18, 2017
Published online: February 14, 2017

Abstract

Familial pancreatic cancer (FPC) is broadly defined as two first-degree-relatives with pancreatic cancer (PC) and accounts for 4%-10% of PC. Several genetic syndromes, including Peutz-Jeghers syndrome, hereditary pancreatitis, hereditary breast-ovarian cancer syndrome (HBOC), Lynch syndrome, and familial adenomatous polyposis (FAP), also have increased risks of PC, but the narrowest definition of FPC excludes these known syndromes. When compared with other familial tumors, proven genetic alterations are limited to a small proportion (< 20%) and the familial aggregation is usually modest. However, an ethnic deviation (Ashkenazi Jewish > Caucasian) and a younger onset are common also in FPC. In European countries, "anticipation" is reported in FPC families, as with other hereditary syndromes; a trend toward younger age and worse prognosis is recognized in the late years. The resected pancreases of FPC kindred often show multiple pancreatic intraepithelial neoplasia (PanIN) foci, with various K-*ras* mutations, similar to colorectal polyposis seen in the FAP patients. As with HBOC patients, a patient who is a *BRCA* mutation carrier with unresectable pancreatic cancer (accounting for 0%-19% of FPC patients) demonstrated better outcome following platinum and Poly (ADP-ribose) polymerase inhibitor treatment. Western countries have established FPC registries since the 1990s and several surveillance projects for high-risk individuals are now ongoing to detect early PCs. Improvement in lifestyle habits, including non-smoking, is recommended for individuals at risk. In Japan, the FPC study group was initiated in 2013 and the Japanese FPC registry was established in 2014 by the Japan Pancreas Society.

Key words: Familial pancreatic cancer; Registry; High risk; Genetic; Surveillance

Core tip: The incidence of pancreatic cancer increases with the number of family members with pancreatic cancer (PC). Familial pancreatic cancer (FPC) is defined as at least two first-degree relatives with PC that does not meet the criteria of other hereditary cancer syndromes. FPC has some epidemiological, pathological, and therapeutic characteristics. Since the 1990s, FPC registries have been established for use in studies to follow up high-risk individuals with family history of PC and hereditary cancer syndromes. Japan initiated a nationwide FPC registry in 2014, and several projects are expected at both the clinical and basic levels.

Matsubayashi H, Takaori K, Morizane C, Maguchi H, Mizuma M, Takahashi H, Wada K, Hosoi H, Yachida S, Suzuki M, Usui R, Furukawa T, Furuse J, Sato T, Ueno M, Kiyozumi Y, Hijioka S, Mizuno N, Terashima T, Mizumoto M, Kodama Y, Torishima M, Kawaguchi T, Ashida R, Kitano M, Hanada K, Furukawa M, Kawabe K, Majima Y, Shimosegawa T. Familial pancreatic cancer: Concept, management and issues. *World J Gasteroenterol* 2017; 23(6): 935-948 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i6/935.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i6.935

INTRODUCTION

Today, in both Japan and Unites States, the number of patients with pancreatic cancer (PC) is gradually increasing[1,2]. The nationwide cancer deaths due to PC is now total over 30000, so that PC ranks fourth among all human cancers (http://ganjoho.jp/reg_stat/

Table 1 Relative risk of pancreatic cancer in hereditary cancer syndromes

Inherited syndrome	Relative risk	Cumulative risk of PC	Responsible gene
Peutz-Jeghers syndrome[27]	132	11%-36%	*STK11*
Hereditary pancreatitis[28-31]	53-87	40%-55%	*PRSS1*
Familial atypical multiple mole melanoma[32,33]	13-22	17%	*CDKN2A*
Hereditary breast-ovarian cancer syndrome[34-37,73]	4-13	2%-7%	*BRCA1, BRCA2*
Lynch syndrome[38,39]	5-9	4%	*MLH1, MSH2, MSH6, PMS2*
Familial adenomatous polyposis[40]	5	-	*APC, MUTYH*

PC: Pancreatic cancer.

statistics/dl/index.html#mortality)[1]. A survey by the Japanese Pancreas Society (2012) indicated an overall 5 year survival for PC patients of only 13.0%. However, when treated when the tumor size is ⩽ 10 mm or within UICC-Stage 0, the 5 year survival increases to 80.4% and 85.8%, respectively[2]. The best strategy for curing this deadly cancer is currently thought to be early detection by following high-risk individuals and resection at a suitable time.

The risk factors for PC include image-detectable pancreatic diseases and lifestyle factors. The former includes pancreatic cysts[3,4], pancreatic duct dilation[3], intraductal papillary mucinous neoplasm (IPMN)[5], and chronic pancreatitis[6,7], while the latter includes smoking[8-10], diabetes mellitus[10-12], obesity[13,14], and low vitamin intake[15], among others. A family history of PC is another known risk, and one that cannot be modified by individual effort or by medicine.

Various human cancers show family history as a risk of the same cancer developing in related family members[16-18]. Several case-control studies and cohort studies have demonstrated an increased risk of PC in those who have a first degree relative (FDR) who is a PC patient [2.1[19]-5.3[20] of odds ratio (OR) and 1.5[21]-1.7[22] of relative risk (RR)[23]]. The incidence of PC increases with the number of family members with PC, so that persons with one FDR with PC have a 4.5 fold increased risk of PC, those with two FDRs have a 6.4 fold increased risk, and those with three or more FDRs have up to a 32 fold risk[24]. The presence of two or more pancreatic cancer patients within FDRs, and without association with known hereditary genetic syndromes, is defined as familial pancreatic cancer (FPC).

The incidence of FPC among total cases of PC is 4%-10%. However, highly affected families are rare (*i.e.*, families with three or more PC cases within FDRs account for only 0.5% of all PC cases in Japan)[10], and their inherited risk is not as high as that of other human malignancies (*e.g.*, melanoma, prostate cancer, ovarian cancer, and breast cancer) as confirmed by a study of a large number of twins in Nordic countries[25]. Several environmental factors (tobacco smoke, asbestos, radon)[10,26] have been reported in cases of FPC, and we must bear in mind that "familial PC" is not a synonym for "inherited PC". With the mentioned criteria, pathogenic germline mutation has been proven in less than 20% of FPC cases, and this is far lower than is observed in other familial cancers associated with the pancreatic neoplasms, such as multiple endocrine neoplasia type 1 (MEN1) and von Hippel-Lindau disease.

Higher risks of PC are also associated with some inherited syndromes, such as Peutz-Jeghers syndrome (PJS)[27], hereditary pancreatitis (HP)[28-31], familial atypical multiple mole melanoma (FAMMM)[32,33], hereditary breast-ovarian cancer (HBOC)[34-37], hereditary nonpolyposis colorectal cancer [HNPCC, Lynch syndrome (LS)][38,39], familial adenomatous polyposis (FAP)[40], and Werner syndrome[41] (Table 1). However, these syndromes are excluded from the definition of FPC in its narrowest meaning. In western countries, high risk individuals (HRI) with a family history of PC and hereditary cancer syndromes have been participating in nationwide or institutional FPC registries[42], and clinical surveillance and basic research have been performed to detect PC in its early stage. This review has focused on the concept and the current outcomes of surveillance of HRI.

CHARACTERISTICS OF FPC

Epidemiology

FPC has several epidemiological features that distinguish it from ordinary PC. Similar to other familial cancers, FPC shows a trend toward a younger onset [FPC: age 58[43]-68[44], compared to sporadic PC (SPC): age 61[43]-74[44]] and an ethnic deviation (Ashkenazi Jewish > Caucasian)[34]. The lifetime risk of PC also increases with decreasing age of onset of PC in family members[44,45]. Meanwhile, similar to the sporadic cases, smoking (especially current smoking)[10,26] and diabetes (recent onset of diabetes)[10] are also risks for FPC.

A pedigree of FPC also incurs an increased risk of developing cancer or cancer death from diseases other than PC, such as in melanoma (OR = 16.8, $P < 0.0001$), endometrial cancer (OR = 5.26, $P = 0.034$), breast cancer [weighted standardized mortality ratio (wSMR): 1.7], ovarian cancer (wSMR: 2.1), and bile duct cancer (wSMR: 3.0)[46]. Several studies have also demonstrated an unexplained worse prognosis in familial cases than in sporadic cases[26,47], albeit some showed no difference[48]. Surprisingly, two European registries (EUROPAC[30] and FaPaCa[49,50]) that analyzed 106 FPC families (264 affected individuals) through three

Figure 1 Worldwide mapping of the (familial) pancreatic cancer registries and genetic research institutions (1-24). 1: Memorial Sloan-Kettering Cancer Center, Familial Pancreatic Cancer Family Registry; 2: Johns Hopkins University, National Familial Pancreas Tumor Registry; 3: Indiana University, Familial Pancreatic Cancer Registry; 4: NorthShore University, Pancreatic Cancer Family Registry; 5: Northwestern University, Pancreatic Cancer Family Registry; 6: University of Nebraska Medical Center and Creighton University, Pancreatic Cancer Family Registry; 7: Huntsman Cancer Institute and University of Utah, Familial Pancreatic Cancer Registry; 8: University of Washington, Familial Pancreatic Cancer Registry; 9: Columbia University, Pancreatic Cancer Registry; 10: Thomas Jefferson University, Jefferson Pancreas Tumor Registry; 11: University of Oklahoma, National Pancreatic Cancer Registry; 12: Oregon Health & Science University, Oregon Pancreas Tumor Registry; 13: Dana-Farber Cancer Institute, Pancreatic Cancer Genes Study; 14: University of Pittsburgh, Pancreatic Adenocarcinoma Gene-Environment Risk Study and Registry; 15: Karmanos Cancer Center and Wayne State University, Pancreatic Cancer Genetic Study; 16: Mayo Clinic, Pancreatic Cancer Genetic Study; 17: University of Texas and MD Anderson Cancer Center, Pancreatic Cancer Genetic Study; 18: Mount Sinai Hospital, Toronto, Familial Gastrointestinal Cancer Registry; 19: Philipps University of Marburg, German National Case Collection Familial Pancreatic Cancer; 20: University of Liverpool, European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer; 21: National Registry for Familial Pancreatic Cancer in Italy; 22: Ramon y Cajal University Hospital, Madrid, Spanish Registry of Hereditary Pancreatic Cancer; 23: The Kinghorn Cancer Center, Australian Pancreatic Cancer Genome Initiative; 24: Kyoto University, Japanese Familial Pancreatic Cancer Registry.

generations [dates of birth: 1900-1919, 1920-1939, 1940-1969] observed "anticipation" in the affected kindred of FPC patients[51]; that is, a trend existed toward younger age and worse prognosis in the latest generation.

Pathology and molecular biology

As is found with colorectal polyposis in numerous FAP patients, the pancreatic histology of FPC kindred often demonstrates multiple precancerous lesions[48] or pancreatic intraepithelial neoplasias (PanINs)[52,53]. PanINs with various mutations of *KRAS* codon 12 are frequently recognized in the vicinity of ordinary PC[54]; however, they are 2.75-fold more frequent in the FPC than in the SPC pancreas[55]. These precursor lesions sometimes appear in the clinical image as small cystic lesions[52,56] and are more often recognized in the pancreases of FPC families than in those of *CDKN2A/p16* mutation carriers (By contrast, PC is 10 times more frequent in the latter group)[57]. These lesions in FPC kindred are associated with lobular parenchymal atrophy and chronic pancreatitis-like changes observable by endoscopic ultrasonography (EUS)[53].

Despite the difference in the numbers of precursor lesions[48,53], a blind review of histological observation of 519 FPCs and 561 SPCs by expert pathologists did not show any significant difference in terms of tumor size, location, neural invasion, angiolymphatic invasion, lymph nodal metastasis, and pathological stage[58]. The genome-wide allelic status[59,60], and genetic and epigenetic alterations[61] are also similar between SPC and FPC.

GENETICS AND CLINICAL MANAGEMENT OF FPC

Familial pancreatic cancer registry

Figure 1 shows a global map of the institutional and nationwide pancreatic cancer registries, including FPC registries. The National Familial Pancreas Tumor Registry (NFPTR) (http://pathology.jhu.edu/pancreas/nfptr/history.php) was founded in 1994 at Johns

Hopkins University (Baltimore, United States)[62]. This was followed by the European Registry of Hereditary Pancreatitis and Familial Pancreas Cancer (EUROPAC: http://www.europac-org.eu/)[30] (1997) at Liverpool University (Liverpool, United Kingdom) and the German National Case Collection for Familial Pancreatic Carcinoma (FaPaCa: http://www.fapaca.de/)[49,50] (1999) at Phillips University (Marburg, Germany). The NFPTR had enrolled 4322 families as of 2012; of these, 1376 families had one or more cases of PC in their FDRs. The FaPaCa had 452 registered FPC families as of 2009[49]. National FPC registries have also been established in Italy (2007)[63] and in Spain (2009)[64]. In Japan, a kickoff meeting was held at Kyoto among international experts in October 2012[65]. A committee was assembled in 2013 and the nationwide registry of FPC (Japanese Familial Pancreatic Cancer Registry: JFPCR: http://jfpcr.com) was officially established in 2014 by the Japan Pancreas Society.

Consortiums and symposiums have also been organized among several high volume centers and/or FPC registries in North America [Pancreatic Cancer Genetic Epidemiology Consortium (PACGENE) in 2002, funded from the National Cancer Institute][62] and across the globe [International Symposium on Inherited Diseases of the Pancreas[66] initiated in 1997, Pancreatic Cancer Cohort Consortium (PanScan) in 2006[8,67], and International Cancer of the Pancreas Screening Consortium (CAPS) in 2011[68]]. The aim has been to gather information on patients and families of PC and to study the cause of PC, with the ultimate goal of improving the clinical practice of counseling and screening of the HRIs, and to devise new early detection methods for PC and better treatments. To date, a large number of clinical studies have been conducted under the FPC registries, mostly concerning risk assessment and screening of family members of FPC patients, in parallel with basic research on pancreatic carcinogenesis[69].

Genetics associated with familial pancreatic cancer

The establishment of FPC registries was followed by a long period of basic research on FPC, as well as pursuit of its causative genes[62]. As already mentioned, several hereditary cancer syndromes have increased risks for the development of PC (Table 1)[23,70]. Genes responsible for FPC have included *ATM* (mutation rate: 2.4%)[71], *BRCA1* (0-1%)[72,73], *BRCA2* (8%-19%)[35,74,75], *CHEK2* (2.9%)[76], and *PALB2* (3.1%-3.7%)[77,78]. However, the known germline mutations account for less than 20% of FPC cases. These genes all function in the homologous recombination of the double strand DNA repair system, or the so-called Fanconi anemia (FA) pathway[79,80], and their germline mutations have also been reported in familial breast cancers[81].

BRCA1/2 mutation carriers have a mild to moderate level of risk for PC (relative risks: 2-8, lifetime risks: 2%-17%), but some specific mutation types may have further increased risks. For instance, *BRCA2* 6174delT, which is a Jewish founder mutation, was detected in 13% (3/23) of Jewish PC cases and the odds for having PC was 12.8[82]. Similarly, the *BRCA2* K3326X mutation was detected in 5.6% (5/144) of American FPC cases[83]. A murine model confirmed that a germline *BRCA2* mutation suffices to promote carcinogenesis by the *KRAS* mutation[84], which is recognized in nearly 90% of PC cases[54]. This may also explain the function of *BRCA2* mutation in FPC. Other genes working in conjunction with FA complementation groups, such as *FANCA*[85], *FANCC*[86], and *FANCG*[86], have been reported to show very low incidences of mutation in FPC (0%-0.5%).

Most recently, the PACGENE study group, which included six American and Canadian institutions, used custom genotyping arrays (iSelect Collaborative Oncological Gene-Environment Study array: iCOGS array) to analyze a single nucleotide polymorphism of 985 PC cases [906 cases with a family history of PC and 79 cases with early-onset ($\leqslant$ 50 years old)]. This group discovered evidence supporting an association of two genetic loci with PC: 7p21.1 (*HDAC9*) and 21q22.3 (*COL6A2*)[87].

SURVEILLANCE OF HIGH RISK INDIVIDUALS

Surveillance conditions

Screening the high-risk population is thought to be an effective strategy for early diagnosis of PC; however, several issues concerning screening have been raised[68]. These include the nature of the pathological lesion that represents the best target for surgical resection, the degree of risk expected for the screening, the best modality or combination of multiple modalities, the best age for initiating screening, the optimal screening interval, and the cost benefit and mental burden for the subjects.

Targeted pathological lesions: The CAPS consortium summit held in Baltimore (2011) concluded that the success of a screening program for HRIs is defined as the detection and treatment of high-grade precursors (PanIN[52] and IPMN[88]) - UICC-stage Ⅰ A PC (T1N0M0; limited to the pancreas and no more than 2 cm in size)[68]. Today, the overall survival of UICC-stage Ⅰ A cancer is unsatisfactory (5-year survival: 68.7%). The ideal for a targeted lesion is thought as high-grade precursors - UICC-stage 0 PC (5-year survival: 85.8%)[2].

Screening candidates and lifestyle guidance at surveillance: A high predictive value can be obtained by surveillance if the conditions of the high-risk group enrolled in a screening protocol are well examined. This is important from the viewpoint of the advantage-

Table 2 Screening candidates with high risks[1]

Individuals with ⩾ 3 affected relatives, with ⩾ 1 affected FDR
Individuals with ⩾ 2 affected FDRs with PC, with ⩾ 1 affected FDR, reaching a certain age
Individuals with ⩾ 2 affected relatives with PC, with ⩾ 1 affected FDR
Peutz-Jeghers syndrome patients, regardless of family history of PC
CDKN2A mutation carriers with one affected FDR
BRCA2 mutation carriers with one affected FDR
BRCA2 mutation carriers with two affected family member pf PC
PALB2 mutation carriers with one affected FDR
Mismatch repair gene mutation carrier (lynch syndrome) with one affected FDR

[1]Quoted from the reference[69]. FDR: First-degree relative; PC: Pancreatic cancer.

Table 3 Non-genetic risk factors of pancreatic cancer

Factors	Risk level
Smoking[8-10]	OR = 1.5-2.2
Diabetes[11,12]	RR = 1.8-1.9
Obesity[13,14]	RR = 1.1-1.4
Chronic pancreatitis[6,7]	SIR = 13-14
Intraductal papillary mucinous neoplasm[5]	SIR = 16
Dilated main pancreatic duct[3]	HR = 6.4
Pancreatic cyst[3,4]	HR = 6.2; OR = 10.3

SIR: Standardized incidence ratio.

disadvantage balance, especially concerning the economic and mental burden placed on the individuals who undergo this surveillance.

The risk level of the candidate individual is assessed based on the numbers of affected family members[24] and hereditary syndromes (Table 1). "PancPro"[89,90] is free software for estimating PC risk (based mainly on hereditary risk) that uses prospective data obtained from 961 families enrolled in the NFPTR; this software is actually applied to the screening programs in Italy[90]. The international consortiums recommended that an individual who had a 5[68,91] to 10[66,92-95] fold risk undergo PC screening. However, we must bear in mind that a complete view of the genetic susceptibility of PC is still unavailable and huge amounts of data from whole genome sequencing are needed for accurate assessment. At present, the CAPS consortium has proposed nine conditions for candidate HRIs (Table 2), within a setting of greater than a 5-fold risk or a 5% of lifetime risk of PC[68].

A screening strategy should also evaluate the risk factors of lifestyle and pancreatic diseases, such as smoking[8,10,26,66,96], obesity[13,14,66], physical inactivity[14], diabetes[10-12,66], chronic pancreatitis[6,7,66], IPMN[88], pancreatic cyst[3,4], pancreatic duct ectasia[3], *etc.* (Table 3). For instance, a patient with diabetes mellitus and a smoking history and a patient with one FDR with PC each showed a 10-fold risk when compared with negative controls[10]. The initial counseling should be used to present modifiable risks related to the lifestyle to HRIs and their improvement should be recommended; *i.e.*, smoking cessation, a healthy diet high in fruits and vegetables, higher intakes of vitamin D (> 600 IU)[15], and regular exercise to control weight (body mass index: < 25 kg/m^2)[66].

Modalities of screening: Consensus could not be reached at the international consortium regarding the modality that is the most suitable for screening[66]. Many institutions currently use EUS as their standard modality[70], based on its ability to detect small pancreatic lesions (< 1 cm)[97-100]. Kamata *et al*[100] prospectively compared the sensitivity of detecting a PC using EUS, enhanced computed tomography (CT), or magnetic resonance imaging (MRI) during the screening of 167 consecutive cases of IPMN; these authors concluded that EUS had the best sensitivity. EUS is also superior at detecting risk findings frequently seen in HRIs, such as duct ectasia, cysts[3], and subtle parenchymal findings of the pancreas[53,97,100-102]. However, agreement is poor in terms of these characteristic findings, even among expert endosonographers[103]. First, visualization by EUS largely depends on the operator's skill[104]. The choice of EUS scopes is also contentious[105], as convex and radial types each have their own different peculiarities[106]. Other drawbacks of EUS include the necessity for a relatively long-time fasting period and conscious sedation, with a limited observation area in cases with a reconstructed upper gastrointestinal tract. In this sense, abdominal ultrasonography is a handy tool that may substitute for EUS if visualization of the pancreas is good without any blind spots[3], for the subjects with slim abdominal trunk.

MRI or magnetic resonance cholangiopancreatography (MRCP) is good at visualization of the pancreatic ductal systems. Dilation of the pancreatic duct and cyst formation are risk factors for PC[3,4] and are actually frequently recognized in HRIs (cyst in 38.9% and duct ectasia in 2.3%)[102], making MRCP a promising tool for assessing the risk level of HRIs. CT scans have a high spatial resolution; however, the healthy examiners are exposed to radiation. Long-term screening for breast cancer with low-dose radiation may possibly increase the incidence of cancer in *BRCA* mutation carriers (*BRCA1*: < 2%, *BRCA2*: < 4%)[107]. This risk is especially high when radiation exposure occurs at age 20 or younger (OR = 2.0, 95%CI: 1.3-3.1) or is repeated five or more times (OR = 1.8, 95%CI: 1.1-3.0)[108]. Excessive use of CT should be avoided in a *BRCA* mutant cohort. Endoscopic retrograde cholangiopancreatography (ERCP) is too invasive for routine screening and carries its own risk of procedure-associated pancreatitis; nevertheless, it is used for further investigation as it has the advantage of obtaining pathological samples[42,109-111]. Repeated pancreatic juice cytology with placement of endoscopic nasopancreatic duct drainage is effective for detecting early pancreatic carcinoma or carcinoma in situ spreading within the pancreatic duct[109]. EUS-guided fine needle

aspiration (EUS-FNA) can target small invasive carcinoma, although only limited tissues can be obtained from carcinoma in situ, and dissemination is a risk[112].

In summary, EUS and MRI are considered the most accurate image tools[100-102,113] with high agreement among the consortium experts (agreement, EUS: 83.7% and MRI/MRCP: 73.5%)[68]. EUS-FNA and ERCP are applicable when abnormal findings or their changes are observed in other images[42,97]. In addition to image analysis, serum tumor markers, including elevated carcinoembryonic antigen and CA19-9, should be checked each time[42,49,50,68].

When to start screening: Screening in many institutions is started at 40 years of age[64,97] or 10 years younger than the age of the youngest relative with PC[42,49]. As PC develops in cases of PJS at a young age (40.8 years)[27], screening is started at 30 years old[97]. However, detection of pancreatic lesions increases after age 50-60[102,114]. No consensus has been reached regarding the age to initiate screening and more than half (51%) of the experts in CAPS consortium voted the initial screening at age 50[68].

Screening interval: Many institutions opt for yearly screening[42,50,95,97,114] if the latest EUS and/or CT is normal (73.5% of agreement by CAPS consortium)[68]. Once an abnormal finding is observed, subsequent screening is done every 3-6 mo[50,97] or 3-12 mo[42,68]. The endorsed screening interval for a non-suspicious cyst is 6-12 mo (agree: 83.7%), 3 mo for a newly detected solid lesion if surgery is not imminent (agree: 85.7%), and 3 mo for an indeterminate main pancreatic duct stricture (agree: 95.9%)[68]. The natural history and progression of FPC still require study to determine the appropriate duration for screening intervals in relation to the risk level.

Surgical indications and procedures: As already mentioned, the characteristics of pancreatic histology in FPC kindred are multifocal PanINs or IPMNs[55] associated with duct ectasia and parenchymal atrophy[53]. The surgical indication for IPMN lesions can be determined according to established Fukuoka guidelines[88]. However, detection of PanIN3 (carcinoma *in situ*) or minimally invasive cancer is difficult, as these cancers are tiny and do not form a solid mass or a nodule.

The extent of resection is controversial, depending on the therapeutic concept. The choices are to remove all precancerous lesions[42] or to resect only a targeted area that includes nodular or cystic lesions[97,115]. In cases of HBOC with the *BRCA* mutation, risk-reducing salpingo-oophorectomy is affordable and has an acceptable level of complications[116]. However, for the pancreas, total pancreatectomy (TP) has severe complications, including a considerable level of postsurgical in-hospital mortality (cf. nationwide: 23%, high-volume hospital: 5%, in Germany)[117,118] and subsequent serious glycemic control failure (mortality: 4%-8% per year)[119]. A secondary pancreatectomy for the remnant pancreas can be conducted without increasing morbidity and mortality[120], so resection of the target area, rather than TP, has been preferable thus far.

For many years, TP with pancreatic transplantation has been conducted in patients with type 1 diabetes[119] and TP combined with islet autotransplantation has been performed on chronic pancreatitis patients with intractable pain[121]. However, most recently, due to the improvements in post-surgical quality of life, these treatment procedures have been considered and actually indicated for FPC kindred with premalignant lesions[119,122,123]. Further improvements are expected in the future.

Present outcomes of surveillance of high risk individuals

Several surveillance results have been reported from single or collaborated FPC registries in western countries; their protocol conditions and outcomes are summarized in Table 4[42,50,91,92,94,95,97,101,114,124-127]. Some of the cases from the same registry may appear in more than one report; therefore, interpretation of cumulative data needs caution. About 5%-20% of the screened HRIs underwent surgery for suspected lesions. Roughly one third of the resected cases were benign lesions that underwent unnecessary treatment, and only less than one fifth were borderline precursors and carcinoma *in situ*, or definitive targets of the surveillance (Table 4). A small proportion of PC was resected at an early phase (T1N0M0)[94], but some PC cases were detected at the advanced unresectable stage. These outcomes testified to the difficulty of providing an accurate diagnosis of PCs at the curative stage.

Psychological and economical aspects of surveillance

Screening participants who are FPC kindred commonly express grief from the experience of family death due to PC[128-130], and are distressed by the high mortality and uncertainty related to prevention and early detection[128]. Their motivation for participating in surveillance is "possible early detection of (a precursor stage of) PC" (95%-100%)[131], and they want to control their cancer risk by seeking information and resources to prevent PC[128]. Research conducted by the Mayo Clinic indicated that 67% (238/361) of FPC kindred felt they had a higher lifetime risk of PC when compared to people of the same age, race, and gender, and 95% were likely to undergo blood test surveillance and 75% were likely to undergo EUS surveillance[130]. A study at the University of Toronto revealed that the perception of PC risk was higher in FPC kindred than in *BRCA2* mutation carriers (42% *vs* 15%)[129]. Most participants had anxiety and worry at the beginning, although only occasionally or sometimes[128,130]; however, this gradually decreased as surveillance progressed (over

Table 4 Outcomes of pancreatic cancer surveillance of high risk individual

Ref.	Year	Country/registry	Entry period	Subjects conditions	Age (range), yr	*n*	Duration (mo)	Modality (surveillance → examination)	Ratio of surgical cases (*n*)	Pathology of the pancreatic lesion: *n*			Ratio of unresectable advanced PC (*n*)
										Benign[1]	Border/CIS[2]	PC	
Brentnall *et al*[42]	1999	United States	NA	FPC kindred	41 (28-65)	14	15	EUS, CT → ERCP	50.0% (7)	0	7[5]	0	0% (0)
Canto *et al*[101]	2004	United States	1998-2001	FPC kindred, PJS	58 (NA)	38	22	EUS → CT, EUS-FNA, ERCP	18.4% (7)	4	2	1	0% (0)
Canto *et al*[97]	2006	United States	2001-2004	FPC kindred, PJS	52 (32-77)	78	12	EUS, CT → EUS-FNA, ERCP	9.0% (7)	4	3	0	1.3% (1)
Langer *et al*[50]	2009	FaPaCa	1999-2007	FPC kindred, BRCA2 (+)[4], CDKN2A (+) FAMMM family	60 (35-85)	76	NA	EUS, MRI → EUS-FNA	9.2% (7)[3]	6	0	0	0% (0)
Poley *et al*[95]	2009	Netherlands	2005-2007	FPC kindred, HP, PJS, FAMMM, BRCA1/2 (+), TP53 (+)	50 (32-75)	44	Initial[6]	EUS → CT, MRI	6.8% (3)	0	0	3	0% (0)
Verna *et al*[124]	2010	United States	2005-2008	FPC kindred, BRCA1/2 (+), LS, FAMMM	52 (29-77)	51	Initial	EUS, MRI → EUS-FNA, ERCP	9.8% (5)	4	0	1	2.0% (1)
Ludwig *et al*[114]	2011	United States	2002-2009	FPC kindred, BRCA1/2(+)	54 (33-86)	109	Initial	MRI → EUS, EUS-FNA	5.5% (6)	3	2	1	0% (0)
Vasen *et al*[125]	2011	Netherlands	2000-2010	CDKN2A-Leiden (+)	56 (39-72)	79	48	MRI	6.3% (5)	0	0	5	2.5% (2)
Zubarik *et al*[126]	2011	United States	2006-2009	FDR of PC with sCA19-9↑	59 (NA)	26	NA	EUS → EUS-FNA	11.5% (3)	2	0	1	0% (0)
Al-Sukhni *et al*[127]	2012	Canada	2003-2011	FPC kindred, PJS, HP, CDKN2A (+), BRCA1/2 (+), STK11 (+)	54 (22-89)	262	50	MRI → MRI, EUS, EUS-FNA, ERCP	1.5% (4)	3	0	1	0.8% (2)
Sud *et al*[91]	2014	United States	2008-2011	FPC kindred, HP, CDKN2A (+), BRCA1/2 (+), PJS, LS	51 (20-75)	16	NA	EUS → EUS-FNA	18.8% (3)	1	0	2	0% (0)
Del Chiaro *et al*[92]	2015	Sweden	2010-2013	FPC kindred, individuals with increased genetic risk	50 (23-76)	40	13	MRI → EUS, EUS-FNA	12.5% (5)	2	0	3	0% (0)
Vasen *et al*[94]	2016	FaPaCa	2000-2015	FPC kindred, CDKN2A (+), BRCA1/2 (+), PALB2 (+)	46-56 (25-81)	411	16-53	MRI ± EUS → EUS, CT → EUS-FNA	7.3% (30)	15	4	11	1.0% (4)

[1]Benign lesions included low-moderate grade of intraductal papillary mucinous neoplasm (IPMN), grade 1-2 of pancreatic intraepithelial neoplasm (PanIN), serous cystadenoma, and neuroendocrine tumor; [2]High-grade precursors and PanIN3; [3]No lesion detected in one case of resected pancreas; [4](+): mutation carrier; [5]Wide spread dysplasia; [6]Evaluated only by the initial surveillance, one resectable pancreatic cancer case (T1N0M0) not resected because of metastatic melanoma. PC: Pancreatic cancer; FPC: Familial pancreatic cancer; PJS: Peutz-Jeghers syndrome; HP: Hereditary pancreatitis; FAMMM: Familial atypical multiple mole melanoma; LS: Lynch syndrome; FDR: First degree relative; FaPaCa: German national case collection for familial pancreatic cancer; NA: Not available, EUS: Endoscopic ultrasonography; EUS-FNA: EUS-guided fine needle aspiration; CT: Computed tomography; ERCP: Endoscopic retrograde cholangiopancreatography; MRI: Magnetic resonance imaging.

a 3-year period of follow-up)[129,131]. This trend was significant in younger participants[132]. The German FaPaCa registry showed that only 39% (80/205) of HRIs participated in the recommended surveillance. The psychological status of these non-participants is still unknown.

Several studies have analyzed the cost-effectiveness of the PC surveillance of HRIs; however, they are not consistent in terms of the applied modality and the target group. For example, Rulyak *et al*[133] evaluated a one-time screening by EUS and ERCP and reported an incremental cost-effectiveness ratio of $16885/life-year saved. They concluded that surveillance remained cost-effective if the prevalence of dysplasia was at least 16% or if the sensitivity of EUS was at least 84%. Bruenderman *et al*[134] estimated costs per year of life of MRI/MRCP surveillance for *CDKN2A* (*p16*)-*Leiden* mutation carriers at $4545, and concluded it to be affordable. By contrast, Latchford *et al*[135] estimated a life-saved cost of over $350000 for total surveillance of PJS patients that followed the American Gastroenterology Association guidelines and recommended its performance only on a research basis. Rubenstein *et al*[136] applied a Markov model to FPC kindred in a setting of 45-year-old-males with positive EUS findings of chronic pancreatitis and compared four different strategies: doing nothing, prophylactic TP, annual EUS surveillance, and annual EUS-FNA surveillance.

The "doing nothing" strategy provided the lowest cost, the greatest remaining years of life, and the best quality-adjusted life years, when compared to the smallest benefit in these aspects obtained with prophylactic TP.

CHEMOTHERAPY FOR FAMILIAL PANCREATIC CANCER WITH *BRCA* MUTATION

For unresectable PC, on the basis of current evidence, FOLFIRINOX (fluorouracil, folic acid, irinotecan, and oxaliplatin) and gemcitabine-based regimens are standard choices of chemotherapy (median survival: 11 mo and 6-9 mo, respectively)[70]. However, in agreement with the response observed in HBOC patients[137-139], PC patients with *BRCA1/2* mutation carriers respond well to platinum-based chemotherapy[140] and poly (ADP-ribose) polymerase (PARP) inhibitors[138,141], as determined in several studies. For example, Golan *et al*[140] compared overall survival (OS) of 43 patients with stage Ⅲ-Ⅳ PC with *BRCA* mutation carriers in terms of their chemotherapy regimen-either platinum or non-platinum. Superior OS was observed for patients treated with platinum chemotherapy (n = 22) than with non-platinum (n = 21) (22 mo *vs* 9 mo, P = 0.039). A similar effect was confirmed in an experiment using xenografts by Lohse *et al*[142], who reported that PC xenografts harvested from *BRCA* mutation carriers and implanted into nude mice showed sensitivity to both gemcitabine and cisplatin. By contrast, xenografts from *BRCA* wild cases showed sensitivity only to gemcitabine. A joint study by Johns Hopkins University and the MD Anderson Cancer Center[143] analyzed effectiveness of platinum-based chemotherapy in metastatic PC patients (n = 549) by familial cancer history, although germline *BRCA* status was not described, and demonstrated a superior OS in patients with family history of either breast, ovarian, or pancreatic cancer (HR = 0.49, P = 0.003). Survival was strongly associated with the number of relatives with *BRCA*-related malignancy (P = 0.009).

Kaufman *et al*[138] reported that a PARP inhibitor (PARPi) treatment induced a 22% response ratio with 4.6 mo of progression-free survival in *BRCA*-mutant PC patients who had already showed progression resistant to the gemcitabine treatment. PARPi is effective for PC cases with deficiency in the homologous recombination pathway; *i.e.*, in cases with either mutation of *ATM*, *BRCA1*, *BRCA2*, or *CHEK2*. This outcome is explained by a synthetic lethal theory, where apoptosis is induced by blocking both the single- and double-strand DNA break repair system[139]. Currently, data are lacking with respect to PARPi use against FPC in causative mutation carriers. Future outcomes are expected.

CONCLUSION

In addition to classical risk factors, hereditary factors including family history of pancreatic cancer and some genetic syndromes must be taken into account when screening to detect early pancreatic cancer. Since the 1990s, basic and clinical research has accumulated much scientific data on FPC. However, to date, screening of HRIs has had unsatisfactory outcomes. In 2014, the JFPCR was established in Japan, and projects have just begun for early detection and better outcomes of PC. Success in this venture will depend on improvement of all aspects, including genetic medicine, screening and treatment methods, and better understanding of what determines a HRI.

REFERENCES

1 **American Cancer Society**. Cancer Facts and Figures 2016. Available from: URL: http://www.cancerorg/research/cancerfactsstatistics/2016

2 **Egawa S**, Toma H, Ohigashi H, Okusaka T, Nakao A, Hatori T, Maguchi H, Yanagisawa A, Tanaka M. Japan Pancreatic Cancer Registry; 30th year anniversary: Japan Pancreas Society. *Pancreas* 2012; **41**: 985-992 [PMID: 22750974 DOI: 10.1097/MPA.0b013e318258055c]

3 **Tanaka S**, Nakao M, Ioka T, Takakura R, Takano Y, Tsukuma H, Uehara H, Suzuki R, Fukuda J. Slight dilatation of the main pancreatic duct and presence of pancreatic cysts as predictive signs of pancreatic cancer: a prospective study. *Radiology* 2010; **254**: 965-972 [PMID: 20177107 DOI: 10.1148/radiol.09090992]

4 **Matsubara S**, Tada M, Akahane M, Yagioka H, Kogure H, Sasaki T, Arizumi T, Togawa O, Nakai Y, Sasahira N, Hirano K, Tsujino T, Isayama H, Toda N, Kawabe T, Ohtomo K, Omata M. Incidental pancreatic cysts found by magnetic resonance imaging and their relationship with pancreatic cancer. *Pancreas* 2012; **41**: 1241-1246 [PMID: 22699201 DOI: 10.1097/MPA.0b013e31824f5970]

5 **Tanno S**, Nakano Y, Koizumi K, Sugiyama Y, Nakamura K, Sasajima J, Nishikawa T, Mizukami Y, Yanagawa N, Fujii T, Okumura T, Obara T, Kohgo Y. Pancreatic ductal adenocarcinomas in long-term follow-up patients with branch duct intraductal papillary mucinous neoplasms. *Pancreas* 2010; **39**: 36-40 [PMID: 19745777 DOI: 10.1097/MPA.0b013e3181b91cd0]

6 **Lowenfels AB**, Maisonneuve P, Cavallini G, Ammann RW, Lankisch PG, Andersen JR, Dimagno EP, Andrén-Sandberg A, Domellöf L. Pancreatitis and the risk of pancreatic cancer. International Pancreatitis Study Group. *N Engl J Med* 1993; **328**: 1433-1437 [PMID: 8479461]

7 **Talamini G**, Falconi M, Bassi C, Sartori N, Salvia R, Caldiron E, Frulloni L, Di Francesco V, Vaona B, Bovo P, Vantini I, Pederzoli P, Cavallini G. Incidence of cancer in the course of chronic pancreatitis. *Am J Gastroenterol* 1999; **94**: 1253-1260 [PMID: 10235203]

8 **Lynch SM**, Vrieling A, Lubin JH, Kraft P, Mendelsohn JB, Hartge P, Canzian F, Steplowski E, Arslan AA, Gross M, Helzlsouer K, Jacobs EJ, LaCroix A, Petersen G, Zheng W, Albanes D, Amundadottir L, Bingham SA, Boffetta P, Boutron-Ruault MC, Chanock SJ, Clipp S, Hoover RN, Jacobs K, Johnson KC, Kooperberg C, Luo J, Messina C, Palli D, Patel AV, Riboli E, Shu XO, Rodriguez Suarez L, Thomas G, Tjønneland A, Tobias GS, Tong E, Trichopoulos D, Virtamo J, Ye W, Yu K, Zeleniuch-Jacquette A, Bueno-de-Mesquita HB, Stolzenberg-Solomon RZ. Cigarette smoking and pancreatic cancer: a pooled analysis from the pancreatic cancer cohort consortium. *Am J Epidemiol* 2009; **170**: 403-413 [PMID: 19561064 DOI: 10.1093/aje/kwp134]

9 **Bosetti C**, Lucenteforte E, Silverman DT, Petersen G, Bracci PM, Ji BT, Negri E, Li D, Risch HA, Olson SH, Gallinger S, Miller

AB, Bueno-de-Mesquita HB, Talamini R, Polesel J, Ghadirian P, Baghurst PA, Zatonski W, Fontham E, Bamlet WR, Holly EA, Bertuccio P, Gao YT, Hassan M, Yu H, Kurtz RC, Cotterchio M, Su J, Maisonneuve P, Duell EJ, Boffetta P, La Vecchia C. Cigarette smoking and pancreatic cancer: an analysis from the International Pancreatic Cancer Case-Control Consortium (Panc4). *Ann Oncol* 2012; **23**: 1880-1888 [PMID: 22104574 DOI: 10.1093/annonc/mdr541]

10 **Matsubayashi H**, Maeda A, Kanemoto H, Uesaka K, Yamazaki K, Hironaka S, Miyagi Y, Ikehara H, Ono H, Klein A, Goggins M. Risk factors of familial pancreatic cancer in Japan: current smoking and recent onset of diabetes. *Pancreas* 2011; **40**: 974-978 [PMID: 21487321 DOI: 10.1097/MPA.0b013e3182156e1b]

11 **Huxley R**, Ansary-Moghaddam A, Berrington de González A, Barzi F, Woodward M. Type-II diabetes and pancreatic cancer: a meta-analysis of 36 studies. *Br J Cancer* 2005; **92**: 2076-2083 [PMID: 15886696 DOI: 10.1038/sj.bjc.6602619]

12 **Ben Q**, Xu M, Ning X, Liu J, Hong S, Huang W, Zhang H, Li Z. Diabetes mellitus and risk of pancreatic cancer: A meta-analysis of cohort studies. *Eur J Cancer* 2011; **47**: 1928-1937 [PMID: 21458985 DOI: 10.1016/j.ejca.2011.03.003]

13 **Larsson SC**, Orsini N, Wolk A. Body mass index and pancreatic cancer risk: A meta-analysis of prospective studies. *Int J Cancer* 2007; **120**: 1993-1998 [PMID: 17266034]

14 **Stolzenberg-Solomon RZ**, Adams K, Leitzmann M, Schairer C, Michaud DS, Hollenbeck A, Schatzkin A, Silverman DT. Adiposity, physical activity, and pancreatic cancer in the National Institutes of Health-AARP Diet and Health Cohort. *Am J Epidemiol* 2008; **167**: 586-597 [PMID: 18270373 DOI: 10.1093/aje/kwm361]

15 **Skinner HG**. Vitamin D for the treatment and prevention of pancreatic cancer. *Cancer Biol Ther* 2008; **7**: 437-439 [PMID: 18421252]

16 **Ait Ouakrim D**, Lockett T, Boussioutas A, Hopper JL, Jenkins MA. Screening participation for people at increased risk of colorectal cancer due to family history: a systematic review and meta-analysis. *Fam Cancer* 2013; **12**: 459-472 [PMID: 23700069 DOI: 10.1007/s10689-013-9658-3]

17 **Turati F**, Edefonti V, Talamini R, Ferraroni M, Malvezzi M, Bravi F, Franceschi S, Montella M, Polesel J, Zucchetto A, La Vecchia C, Negri E, Decarli A. Family history of liver cancer and hepatocellular carcinoma. *Hepatology* 2012; **55**: 1416-1425 [PMID: 22095619 DOI: 10.1002/hep.24794]

18 **Win AK**, Reece JC, Ryan S. Family history and risk of endometrial cancer: a systematic review and meta-analysis. *Obstet Gynecol* 2015; **125**: 89-98 [PMID: 25560109 DOI: 10.1097/AOG.0000000000000563]

19 **Inoue M**, Tajima K, Takezaki T, Hamajima N, Hirose K, Ito H, Tominaga S. Epidemiology of pancreatic cancer in Japan: a nested case-control study from the Hospital-based Epidemiologic Research Program at Aichi Cancer Center (HERPACC). *Int J Epidemiol* 2003; **32**: 257-262 [PMID: 12714546]

20 **Falk RT**, Pickle LW, Fontham ET, Correa P, Fraumeni JF. Life-style risk factors for pancreatic cancer in Louisiana: a case-control study. *Am J Epidemiol* 1988; **128**: 324-336 [PMID: 3394699]

21 **Coughlin SS**, Calle EE, Patel AV, Thun MJ. Predictors of pancreatic cancer mortality among a large cohort of United States adults. *Cancer Causes Control* 2000; **11**: 915-923 [PMID: 11142526]

22 **Hemminki K**, Li X. Familial and second primary pancreatic cancers: a nationwide epidemiologic study from Sweden. *Int J Cancer* 2003; **103**: 525-530 [PMID: 12478670 DOI: 10.1002/ijc.10863]

23 **Matsubayashi H**. Familial pancreatic cancer and hereditary syndromes: screening strategy for high-risk individuals. *J Gastroenterol* 2011; **46**: 1249-1259 [PMID: 21847571 DOI: 10.1007/s00535-011-0457-z]

24 **Klein AP**, Brune KA, Petersen GM, Goggins M, Tersmette AC, Offerhaus GJ, Griffin C, Cameron JL, Yeo CJ, Kern S, Hruban RH. Prospective risk of pancreatic cancer in familial pancreatic cancer kindreds. *Cancer Res* 2004; **64**: 2634-2638 [PMID: 15059921]

25 **Mucci LA**, Hjelmborg JB, Harris JR, Czene K, Havelick DJ, Scheike T, Graff RE, Holst K, Möller S, Unger RH, McIntosh C, Nuttall E, Brandt I, Penney KL, Hartman M, Kraft P, Parmigiani G, Christensen K, Koskenvuo M, Holm NV, Heikkilä K, Pukkala E, Skytthe A, Adami HO, Kaprio J. Familial Risk and Heritability of Cancer Among Twins in Nordic Countries. *JAMA* 2016; **315**: 68-76 [PMID: 26746459 DOI: 10.1001/jama.2015.17703]

26 **Yeo TP**, Hruban RH, Brody J, Brune K, Fitzgerald S, Yeo CJ. Assessment of "gene-environment" interaction in cases of familial and sporadic pancreatic cancer. *J Gastrointest Surg* 2009; **13**: 1487-1494 [PMID: 19459017]

27 **Giardiello FM**, Brensinger JD, Tersmette AC, Goodman SN, Petersen GM, Booker SV, Cruz-Correa M, Offerhaus JA. Very high risk of cancer in familial Peutz-Jeghers syndrome. *Gastroenterology* 2000; **119**: 1447-1453 [PMID: 11113065]

28 **Whitcomb DC**, Applebaum S, Martin SP. Hereditary pancreatitis and pancreatic carcinoma. *Ann N Y Acad Sci* 1999; **880**: 201-209 [PMID: 10415865]

29 **Rebours V**, Boutron-Ruault MC, Schnee M, Férec C, Maire F, Hammel P, Ruszniewski P, Lévy P. Risk of pancreatic adenocarcinoma in patients with hereditary pancreatitis: a national exhaustive series. *Am J Gastroenterol* 2008; **103**: 111-119 [PMID: 18184119]

30 **Howes N**, Lerch MM, Greenhalf W, Stocken DD, Ellis I, Simon P, Truninger K, Ammann R, Cavallini G, Charnley RM, Uomo G, Delhaye M, Spicak J, Drumm B, Jansen J, Mountford R, Whitcomb DC, Neoptolemos JP. Clinical and genetic characteristics of hereditary pancreatitis in Europe. *Clin Gastroenterol Hepatol* 2004; **2**: 252-261 [PMID: 15017610]

31 **Lowenfels AB**, Maisonneuve P, DiMagno EP, Elitsur Y, Gates LK, Perrault J, Whitcomb DC. Hereditary pancreatitis and the risk of pancreatic cancer. International Hereditary Pancreatitis Study Group. *J Natl Cancer Inst* 1997; **89**: 442-446 [PMID: 9091646]

32 **Lynch HT**, Fusaro RM, Lynch JF, Brand R. Pancreatic cancer and the FAMMM syndrome. *Fam Cancer* 2008; **7**: 103-112 [PMID: 17992582 DOI: 10.1007/s10689-007-9166-4]

33 **Vasen HF**, Gruis NA, Frants RR, van Der Velden PA, Hille ET, Bergman W. Risk of developing pancreatic cancer in families with familial atypical multiple mole melanoma associated with a specific 19 deletion of p16 (p16-Leiden). *Int J Cancer* 2000; **87**: 809-811 [PMID: 10956390]

34 **Lynch HT**, Deters CA, Lynch JF, Brand RE. Familial pancreatic carcinoma in Jews. *Fam Cancer* 2004; **3**: 233-240 [PMID: 15516847 DOI: 10.1007/s10689-004-9549-8]

35 **Murphy KM**, Brune KA, Griffin C, Sollenberger JE, Petersen GM, Bansal R, Hruban RH, Kern SE. Evaluation of candidate genes MAP2K4, MADH4, ACVR1B, and BRCA2 in familial pancreatic cancer: deleterious BRCA2 mutations in 17%. *Cancer Res* 2002; **62**: 3789-3793 [PMID: 12097290]

36 **Breast Cancer Linkage Consortium**. Cancer risks in BRCA2 mutation carriers. *J Natl Cancer Inst* 1999; **91**: 1310-1316 [PMID: 10433620]

37 **Brose MS**, Rebbeck TR, Calzone KA, Stopfer JE, Nathanson KL, Weber BL. Cancer risk estimates for BRCA1 mutation carriers identified in a risk evaluation program. *J Natl Cancer Inst* 2002; **94**: 1365-1372 [PMID: 12237282]

38 **Kastrinos F**, Mukherjee B, Tayob N, Wang F, Sparr J, Raymond VM, Bandipalliam P, Stoffel EM, Gruber SB, Syngal S. Risk of pancreatic cancer in families with Lynch syndrome. *JAMA* 2009; **302**: 1790-1795 [PMID: 19861671 DOI: 10.1001/jama.2009.1529]

39 **Aarnio M**, Sankila R, Pukkala E, Salovaara R, Aaltonen LA, de la Chapelle A, Peltomäki P, Mecklin JP, Järvinen HJ. Cancer risk in mutation carriers of DNA-mismatch-repair genes. *Int J Cancer* 1999; **81**: 214-218 [PMID: 10188721]

40 **Giardiello FM**, Offerhaus GJ, Lee DH, Krush AJ, Tersmette AC, Booker SV, Kelley NC, Hamilton SR. Increased risk of thyroid and pancreatic carcinoma in familial adenomatous polyposis. *Gut* 1993; **34**: 1394-1396 [PMID: 8244108]

41 **Chun SG**, Yee NS. Werner syndrome as a hereditary risk factor for exocrine pancreatic cancer: potential role of WRN in pancreatic tumorigenesis and patient-tailored therapy. *Cancer Biol Ther* 2010; **10**: 430-437 [PMID: 20657174 DOI: 10.4161/cbt.10.5.12763]

42 **Brentnall TA**, Bronner MP, Byrd DR, Haggitt RC, Kimmey MB.

Early diagnosis and treatment of pancreatic dysplasia in patients with a family history of pancreatic cancer. *Ann Intern Med* 1999; **131**: 247-255 [PMID: 10454945]

43 **James TA**, Sheldon DG, Rajput A, Kuvshinoff BW, Javle MM, Nava HR, Smith JL, Gibbs JF. Risk factors associated with earlier age of onset in familial pancreatic carcinoma. *Cancer* 2004; **101**: 2722-2726 [PMID: 15534880]

44 **Brune KA**, Lau B, Palmisano E, Canto M, Goggins MG, Hruban RH, Klein AP. Importance of age of onset in pancreatic cancer kindreds. *J Natl Cancer Inst* 2010; **102**: 119-126 [PMID: 20068195]

45 **Del Chiaro M**, Zerbi A, Falconi M, Bertacca L, Polese M, Sartori N, Boggi U, Casari G, Longoni BM, Salvia R, Caligo MA, Di Carlo V, Pederzoli P, Presciuttini S, Mosca F. Cancer risk among the relatives of patients with pancreatic ductal adenocarcinoma. *Pancreatology* 2007; **7**: 459-469 [PMID: 17912010]

46 **Wang L**, Brune KA, Visvanathan K, Laheru D, Herman J, Wolfgang C, Schulick R, Cameron JL, Goggins M, Hruban RH, Klein AP. Elevated cancer mortality in the relatives of patients with pancreatic cancer. *Cancer Epidemiol Biomarkers Prev* 2009; **18**: 2829-2834 [PMID: 19843679 DOI: 10.1158/1055-9965.EPI-09-0557]

47 **Ji J**, Forsti A, Sundquist J, Lenner P, Hemminki K. Survival in familial pancreatic cancer. *Pancreatology* 2008; **8**: 252-256 [PMID: 18497537 DOI: 10.1159/000134272]

48 **Humphris JL**, Johns AL, Simpson SH, Cowley MJ, Pajic M, Chang DK, Nagrial AM, Chin VT, Chantrill LA, Pinese M, Mead RS, Gill AJ, Samra JS, Kench JG, Musgrove EA, Tucker KM, Spigelman AD, Waddell N, Grimmond SM, Biankin AV. Clinical and pathologic features of familial pancreatic cancer. *Cancer* 2014; **120**: 3669-3675 [PMID: 25313458 DOI: 10.1002/cncr.28863]

49 **Schneider R**, Slater EP, Sina M, Habbe N, Fendrich V, Matthäi E, Langer P, Bartsch DK. German national case collection for familial pancreatic cancer (FaPaCa): ten years experience. *Fam Cancer* 2011; **10**: 323-330 [PMID: 21207249 DOI: 10.1007/s10689-010-9414-x]

50 **Langer P**, Kann PH, Fendrich V, Habbe N, Schneider M, Sina M, Slater EP, Heverhagen JT, Gress TM, Rothmund M, Bartsch DK. Five years of prospective screening of high-risk individuals from families with familial pancreatic cancer. *Gut* 2009; **58**: 1410-1418 [PMID: 19470496]

51 **McFaul CD**, Greenhalf W, Earl J, Howes N, Neoptolemos JP, Kress R, Sina-Frey M, Rieder H, Hahn S, Bartsch DK. Anticipation in familial pancreatic cancer. *Gut* 2006; **55**: 252-258 [PMID: 15972300]

52 **Takaori K**, Hruban RH, Maitra A, Tanigawa N. Pancreatic intraepithelial neoplasia. *Pancreas* 2004; **28**: 257-262 [PMID: 15084967]

53 **Brune K**, Abe T, Canto M, O'Malley L, Klein AP, Maitra A, Volkan Adsay N, Fishman EK, Cameron JL, Yeo CJ, Kern SE, Goggins M, Hruban RH. Multifocal neoplastic precursor lesions associated with lobular atrophy of the pancreas in patients having a strong family history of pancreatic cancer. *Am J Surg Pathol* 2006; **30**: 1067-1076 [PMID: 16931950]

54 **Matsubayashi H**, Watanabe H, Yamaguchi T, Ajioka Y, Nishikura K, Iwafuchi M, Yamano M, Kijima H, Saito T. Multiple K-ras mutations in hyperplasia and carcinoma in cases of human pancreatic carcinoma. *Jpn J Cancer Res* 1999; **90**: 841-848 [PMID: 10543256]

55 **Shi C**, Klein AP, Goggins M, Maitra A, Canto M, Ali S, Schulick R, Palmisano E, Hruban RH. Increased Prevalence of Precursor Lesions in Familial Pancreatic Cancer Patients. *Clin Cancer Res* 2009; **15**: 7737-7743 [PMID: 19996207 DOI: 10.1158/1078-0432.CCR-09-0004]

56 **Kimura W**, Nagai H, Kuroda A, Muto T, Esaki Y. Analysis of small cystic lesions of the pancreas. *Int J Pancreatol* 1995; **18**: 197-206 [PMID: 8708390 DOI: 10.1007/BF02784942]

57 **Potjer TP**, Schot I, Langer P, Heverhagen JT, Wasser MN, Slater EP, Klöppel G, Morreau HM, Bonsing BA, de Vos Tot Nederveen Cappel WH, Bargello M, Gress TM, Vasen HF, Bartsch DK. Variation in precursor lesions of pancreatic cancer among high-risk groups. *Clin Cancer Res* 2013; **19**: 442-449 [PMID: 23172884 DOI: 10.1158/1078-0432.CCR- 12-2730]

58 **Singhi AD**, Ishida H, Ali SZ, Goggins M, Canto M, Wolfgang CL, Meriden Z, Roberts N, Klein AP, Hruban RH. A histomorphologic comparison of familial and sporadic pancreatic cancers. *Pancreatology* 2015; **15**: 387-391 [PMID: 25959245 DOI: 10.1016/j.pan.2015.04.003]

59 **Abe T**, Fukushima N, Brune K, Boehm C, Sato N, Matsubayashi H, Canto M, Petersen GM, Hruban RH, Goggins M. Genome-wide allelotypes of familial pancreatic adenocarcinomas and familial and sporadic intraductal papillary mucinous neoplasms. *Clin Cancer Res* 2007; **13**: 6019-6025 [PMID: 17947463]

60 **Norris AL**, Roberts NJ, Jones S, Wheelan SJ, Papadopoulos N, Vogelstein B, Kinzler KW, Hruban RH, Klein AP, Eshleman JR. Familial and sporadic pancreatic cancer share the same molecular pathogenesis. *Fam Cancer* 2015; **14**: 95-103 [PMID: 25240578 DOI: 10.1007/s10689-014-9755-y]

61 **Brune K**, Hong SM, Li A, Yachida S, Abe T, Griffith M, Yang D, Omura N, Eshleman J, Canto M, Schulick R, Klein AP, Hruban RH, Iacobuzio-Donohue C, Goggins M. Genetic and epigenetic alterations of familial pancreatic cancers. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 3536-3542 [PMID: 19064568]

62 **Petersen GM**, de Andrade M, Goggins M, Hruban RH, Bondy M, Korczak JF, Gallinger S, Lynch HT, Syngal S, Rabe KG, Seminara D, Klein AP. Pancreatic cancer genetic epidemiology consortium. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 704-710 [PMID: 16614112 DOI: 10.1158/1055-9965.EPI-05-0734]

63 **Del Chiaro M**, Zerbi A, Capurso G, Zamboni G, Maisonneuve P, Presciuttini S, Arcidiacono PG, Calculli L, Falconi M; Italian Registry for Familial Pancreatic Cancer. Familial pancreatic cancer in Italy. Risk assessment, screening programs and clinical approach: a position paper from the Italian Registry. *Dig Liver Dis* 2010; **42**: 597-605 [PMID: 20627831 DOI: 10.1016/j.dld.2010.04.016]

64 **Mocci E**, Guillen-Ponce C, Earl J, Marquez M, Solera J, Salazar-López MT, Calcedo-Arnáiz C, Vázquez-Sequeiros E, Montans J, Muñoz-Beltrán M, Vicente-Bártulos A, González-Gordaliza C, Sanjuanbenito A, Guerrero C, Mendía E, Lisa E, Lobo E, Martínez JC, Real FX, Malats N, Carrato A. PanGen-Fam: Spanish registry of hereditary pancreatic cancer. *Eur J Cancer* 2015; **51**: 1911-1917 [PMID: 26212471 DOI: 10.1016/j.ejca.2015.07.004]

65 **Wada K**, Takaori K, Traverso LW, Hruban RH, Furukawa T, Brentnall TA, Hatori T, Sano K, Takada T, Majima Y, Shimosegawa T. Clinical importance of Familial Pancreatic Cancer Registry in Japan: a report from kick-off meeting at International Symposium on Pancreas Cancer 2012. *J Hepatobiliary Pancreat Sci* 2013; **20**: 557-566 [PMID: 23604538 DOI: 10.1007/s00534-013-0611-5]

66 **Brand RE**, Lerch MM, Rubinstein WS, Neoptolemos JP, Whitcomb DC, Hruban RH, Brentnall TA, Lynch HT, Canto MI. Advances in counselling and surveillance of patients at risk for pancreatic cancer. *Gut* 2007; **56**: 1460-1469 [PMID: 17872573 DOI: 10.1136/gut.2006.108456]

67 **Jacobs EJ**, Chanock SJ, Fuchs CS, Lacroix A, McWilliams RR, Steplowski E, Stolzenberg-Solomon RZ, Arslan AA, Bueno-de-Mesquita HB, Gross M, Helzlsouer K, Petersen G, Zheng W, Agalliu I, Allen NE, Amundadottir L, Boutron-Ruault MC, Buring JE, Canzian F, Clipp S, Dorronsoro M, Gaziano JM, Giovannucci EL, Hankinson SE, Hartge P, Hoover RN, Hunter DJ, Jacobs KB, Jenab M, Kraft P, Kooperberg C, Lynch SM, Sund M, Mendelsohn JB, Mouw T, Newton CC, Overvad K, Palli D, Peeters PH, Rajkovic A, Shu XO, Thomas G, Tobias GS, Trichopoulos D, Virtamo J, Wactawski-Wende J, Wolpin BM, Yu K, Zeleniuch-Jacquotte A. Family history of cancer and risk of pancreatic cancer: a pooled analysis from the Pancreatic Cancer Cohort Consortium (PanScan). *Int J Cancer* 2010; **127**: 1421-1428 [PMID: 20049842 DOI: 10.1002/ijc.25148]

68 **Canto MI**, Harinck F, Hruban RH, Offerhaus GJ, Poley JW, Kamel I, Nio Y, Schulick RS, Bassi C, Kluijt I, Levy MJ, Chak A, Fockens P, Goggins M, Bruno M; International Cancer of Pancreas Screening (CAPS) Consortium. International Cancer of the Pancreas Screening (CAPS) Consortium summit on the management of patients with increased risk for familial pancreatic cancer. *Gut* 2013; **62**: 339-347 [PMID: 23135763 DOI: 10.1136/gutjnl-2012-303108]

69 **Hruban RH**, Canto MI, Goggins M, Schulick R, Klein AP. Update on familial pancreatic cancer. *Adv Surg* 2010; **44**: 293-311 [PMID: 20919528]

70 **Kamisawa T**, Wood LD, Itoi T, Takaori K. Pancreatic cancer. *Lancet* 2016; **388**: 73-85 [PMID: 26830752 DOI: 10.1016/S0140-6736(16)00141-0]

71 **Roberts NJ**, Jiao Y, Yu J, Kopelovich L, Petersen GM, Bondy ML, Gallinger S, Schwartz AG, Syngal S, Cote ML, Axilbund J, Schulick R, Ali SZ, Eshleman JR, Velculescu VE, Goggins M, Vogelstein B, Papadopoulos N, Hruban RH, Kinzler KW, Klein AP. ATM mutations in patients with hereditary pancreatic cancer. *Cancer Discov* 2012; **2**: 41-46 [PMID: 22585167 DOI: 10.1158/2159-8290.CD-11-0194]

72 **Axilbund JE**, Argani P, Kamiyama M, Palmisano E, Raben M, Borges M, Brune KA, Goggins M, Hruban RH, Klein AP. Absence of germline BRCA1 mutations in familial pancreatic cancer patients. *Cancer Biol Ther* 2009; **8**: 131-135 [PMID: 19029836]

73 **Lynch HT**, Deters CA, Snyder CL, Lynch JF, Villeneuve P, Silberstein J, Martin H, Narod SA, Brand RE. BRCA1 and pancreatic cancer: pedigree findings and their causal relationships. *Cancer Genet Cytogenet* 2005; **158**: 119-125 [PMID: 15796958 DOI: 10.1016/j.cancergencyto.2004.01.032]

74 **Goggins M**, Schutte M, Lu J, Moskaluk CA, Weinstein CL, Petersen GM, Yeo CJ, Jackson CE, Lynch HT, Hruban RH, Kern SE. Germline BRCA2 gene mutations in patients with apparently sporadic pancreatic carcinomas. *Cancer Res* 1996; **56**: 5360-5364 [PMID: 8968085]

75 **Hahn SA**, Greenhalf B, Ellis I, Sina-Frey M, Rieder H, Korte B, Gerdes B, Kress R, Ziegler A, Raeburn JA, Campra D, Grützmann R, Rehder H, Rothmund M, Schmiegel W, Neoptolemos JP, Bartsch DK. BRCA2 germline mutations in familial pancreatic carcinoma. *J Natl Cancer Inst* 2003; **95**: 214-221 [PMID: 12569143]

76 **Bartsch DK**, Krysewski K, Sina-Frey M, Fendrich V, Rieder H, Langer P, Kress R, Schneider M, Hahn SA, Slater EP. Low frequency of CHEK2 mutations in familial pancreatic cancer. *Fam Cancer* 2006; **5**: 305-308 [PMID: 16858628 DOI: 10.1007/s10689-006-7850-4]

77 **Jones S**, Hruban RH, Kamiyama M, Borges M, Zhang X, Parsons DW, Lin JC, Palmisano E, Brune K, Jaffee EM, Iacobuzio-Donahue CA, Maitra A, Parmigiani G, Kern SE, Velculescu VE, Kinzler KW, Vogelstein B, Eshleman JR, Goggins M, Klein AP. Exomic sequencing identifies PALB2 as a pancreatic cancer susceptibility gene. *Science* 2009; **324**: 217 [PMID: 19264984]

78 **Slater EP**, Langer P, Niemczyk E, Strauch K, Butler J, Habbe N, Neoptolemos JP, Greenhalf W, Bartsch DK. PALB2 mutations in European familial pancreatic cancer families. *Clin Genet* 2010; **78**: 490-494 [PMID: 20412113]

79 **Michl J**, Zimmer J, Tarsounas M. Interplay between Fanconi anemia and homologous recombination pathways in genome integrity. *EMBO J* 2016; **35**: 909-923 [PMID: 27037238 DOI: 10.15252/embj.201693860]

80 **Renwick A**, Thompson D, Seal S, Kelly P, Chagtai T, Ahmed M, North B, Jayatilake H, Barfoot R, Spanova K, McGuffog L, Evans DG, Eccles D, Easton DF, Stratton MR, Rahman N. ATM mutations that cause ataxia-telangiectasia are breast cancer susceptibility alleles. *Nat Genet* 2006; **38**: 873-875 [PMID: 16832357 DOI: 10.1038/ng1837]

81 **Mathew CG**. Fanconi anaemia genes and susceptibility to cancer. *Oncogene* 2006; **25**: 5875-5884 [PMID: 16998502 DOI: 10.1038/sj.onc.1209878]

82 **Figer A**, Irmin L, Geva R, Flex D, Sulkes J, Sulkes A, Friedman E. The rate of the 6174delT founder Jewish mutation in BRCA2 in patients with non-colonic gastrointestinal tract tumours in Israel. *Br J Cancer* 2001; **84**: 478-481 [PMID: 11207041]

83 **Martin ST**, Matsubayashi H, Rogers CD, Philips J, Couch FJ, Brune K, Yeo CJ, Kern SE, Hruban RH, Goggins M. Increased prevalence of the BRCA2 polymorphic stop codon K3326X among individuals with familial pancreatic cancer. *Oncogene* 2005; **24**: 3652-3656 [PMID: 15806175]

84 **Skoulidis F**, Cassidy LD, Pisupati V, Jonasson JG, Bjarnason H, Eyfjord JE, Karreth FA, Lim M, Barber LM, Clatworthy SA, Davies SE, Olive KP, Tuveson DA, Venkitaraman AR. Germline Brca2 heterozygosity promotes Kras(G12D) -driven carcinogenesis in a murine model of familial pancreatic cancer. *Cancer Cell* 2010; **18**: 499-509 [PMID: 21056012]

85 **Rogers CD**, Couch FJ, Brune K, Martin ST, Philips J, Murphy KM, Petersen G, Yeo CJ, Hruban RH, Goggins M. Genetics of the FANCA gene in familial pancreatic cancer. *J Med Genet* 2004; **41**: e126 [PMID: 15591268 DOI: 10.1136/jmg.2004.024851]

86 **Rogers CD**, van der Heijden MS, Brune K, Yeo CJ, Hruban RH, Kern SE, Goggins M. The genetics of FANCC and FANCG in familial pancreatic cancer. *Cancer Biol Ther* 2004; **3**: 167-169 [PMID: 14726700]

87 **Childs EJ**, Chaffee KG, Gallinger S, Syngal S, Schwartz AG, Cote ML, Bondy ML, Hruban RH, Chanock SJ, Hoover RN, Fuchs CS, Rider DN, Amundadottir LT, Stolzenberg-Solomon R, Wolpin BM, Risch HA, Goggins MG, Petersen GM, Klein AP. Association of Common Susceptibility Variants of Pancreatic Cancer in Higher-Risk Patients: A PACGENE Study. *Cancer Epidemiol Biomarkers Prev* 2016; **25**: 1185-1191 [PMID: 27197284 DOI: 10.1158/1055-9965.EPI-15-1217]

88 **Tanaka M**, Fernández-del Castillo C, Adsay V, Chari S, Falconi M, Jang JY, Kimura W, Levy P, Pitman MB, Schmidt CM, Shimizu M, Wolfgang CL, Yamaguchi K, Yamao K. International consensus guidelines 2012 for the management of IPMN and MCN of the pancreas. *Pancreatology* 2012; **12**: 183-197 [PMID: 22687371 DOI: 10.1016/j.pan.2012.04.004]

89 **Wang W,** Chen S, Brune KA, Hruban RH, Parmigiani G, Klein AP. PancPRO: risk assessment for individuals with a family history of pancreatic cancer. *J Clin Oncol* 2007; **25**: 1417-1422 [PMID: 17416862 DOI: 10.1200/JCO.2006.09.2452]

90 **Leonardi G**, Marchi S, Falconi M, Zerbi A, Ussia V, de Bortoli N, Mosca F, Presciuttini S, Del Chiaro M. "PancPro" as a tool for selecting families eligible for pancreatic cancer screening: an Italian study of incident cases. *Dig Liver Dis* 2012; **44**: 585-588 [PMID: 22281375 DOI: 10.1016/j.dld.2011.12.019]

91 **Sud A**, Wham D, Catalano M, Guda NM. Promising outcomes of screening for pancreatic cancer by genetic testing and endoscopic ultrasound. *Pancreas* 2014; **43**: 458-461 [PMID: 24622079 DOI: 10.1097/MPA.0000000000000052]

92 **Del Chiaro M**, Verbeke CS, Kartalis N, Pozzi Mucelli R, Gustafsson P, Hansson J, Haas SL, Segersvärd R, Andren-Sandberg Å, Löhr JM. Short-term Results of a Magnetic Resonance Imaging-Based Swedish Screening Program for Individuals at Risk for Pancreatic Cancer. *JAMA Surg* 2015; **150**: 512-518 [PMID: 25853369 DOI: 10.1001/jamasurg.2014.3852]

93 **Harinck F**, Konings IC, Kluijt I, Poley JW, van Hooft JE, van Dullemen HM, Nio CY, Krak NC, Hermans JJ, Aalfs CM, Wagner A, Sijmons RH, Biermann K, van Eijck CH, Gouma DJ, Dijkgraaf MG, Fockens P, Bruno MJ. A multicentre comparative prospective blinded analysis of EUS and MRI for screening of pancreatic cancer in high-risk individuals. *Gut* 2016; **65**: 1505-1513 [PMID: 25986944 DOI: 10.1136/gutjnl-2014-308008]

94 **Vasen H**, Ibrahim I, Ponce CG, Slater EP, Matthäi E, Carrato A, Earl J, Robbers K, van Mil AM, Potjer T, Bonsing BA, de Vos Tot Nederveen Cappel WH, Bergman W, Wasser M, Morreau H, Klöppel G, Schicker C, Steinkamp M, Figiel J, Esposito I, Mocci E, Vazquez-Sequeiros E, Sanjuanbenito A, Muñoz-Beltran M, Montans J, Langer P, Fendrich V, Bartsch DK. Benefit of Surveillance for Pancreatic Cancer in High-Risk Individuals: Outcome of Long-Term Prospective Follow-Up Studies From Three European Expert Centers. *J Clin Oncol* 2016; **34**: 2010-2019 [PMID: 27114589 DOI: 10.1200/JCO.2015.64.0730]

95 **Poley JW**, Kluijt I, Gouma DJ, Harinck F, Wagner A, Aalfs C, van Eijck CH, Cats A, Kuipers EJ, Nio Y, Fockens P, Bruno MJ. The yield of first-time endoscopic ultrasonography in screening individuals at a high risk of developing pancreatic cancer. *Am J Gastroenterol* 2009; **104**: 2175-2181 [PMID: 19491823 DOI: 10.1038/ajg.2009.276]

96 **Lowenfels AB**, Maisonneuve P, Whitcomb DC, Lerch MM,

DiMagno EP. Cigarette smoking as a risk factor for pancreatic cancer in patients with hereditary pancreatitis. *JAMA* 2001; **286**: 169-170 [PMID: 11448279]
97 **Canto MI**, Goggins M, Hruban RH, Petersen GM, Giardiello FM, Yeo C, Fishman EK, Brune K, Axilbund J, Griffin C, Ali S, Richman J, Jagannath S, Kantsevoy SV, Kalloo AN. Screening for early pancreatic neoplasia in high-risk individuals: a prospective controlled study. *Clin Gastroenterol Hepatol* 2006; **4**: 766-781; quiz 665 [PMID: 16682259]
98 **Greenhalf W**, Neoptolemos JP. Increasing survival rates of patients with pancreatic cancer by earlier identification. *Nat Clin Pract Oncol* 2006; **3**: 346-347 [PMID: 16826198 DOI: 10.1038/ncponc0483]
99 **Yasuda I**, Iwashita T, Doi S, Nakashima M, Moriwaki H. Role of EUS in the early detection of small pancreatic cancer. *Dig Endosc* 2011; **23** Suppl 1: 22-25 [PMID: 21535195 DOI: 10.1111/j.1443-1661.2011.01113.x]
100 **Kamata K**, Kitano M, Kudo M, Sakamoto H, Kadosaka K, Miyata T, Imai H, Maekawa K, Chikugo T, Kumano M, Hyodo T, Murakami T, Chiba Y, Takeyama Y. Value of EUS in early detection of pancreatic ductal adenocarcinomas in patients with intraductal papillary mucinous neoplasms. *Endoscopy* 2014; **46**: 22-29 [PMID: 24218310 DOI: 10.1055/s-0033-1353603]
101 **Canto MI**, Goggins M, Yeo CJ, Griffin C, Axilbund JE, Brune K, Ali SZ, Jagannath S, Petersen GM, Fishman EK, Piantadosi S, Giardiello FM, Hruban RH. Screening for pancreatic neoplasia in high-risk individuals: an EUS-based approach. *Clin Gastroenterol Hepatol* 2004; **2**: 606-621 [PMID: 15224285]
102 **Canto MI**, Hruban RH, Fishman EK, Kamel IR, Schulick R, Zhang Z, Topazian M, Takahashi N, Fletcher J, Petersen G, Klein AP, Axilbund J, Griffin C, Syngal S, Saltzman JR, Mortele KJ, Lee J, Tamm E, Vikram R, Bhosale P, Margolis D, Farrell J, Goggins M. Frequent detection of pancreatic lesions in asymptomatic high-risk individuals. *Gastroenterology* 2012; **142**: 796-804; quiz e14-15 [PMID: 22245846 DOI: 10.1053/j.gastro.2012.01.005]
103 **Topazian M**, Enders F, Kimmey M, Brand R, Chak A, Clain J, Cunningham J, Eloubeidi M, Gerdes H, Gress F, Jagannath S, Kantsevoy S, LeBlanc JK, Levy M, Lightdale C, Romagnuolo J, Saltzman JR, Savides T, Wiersema M, Woodward T, Petersen G, Canto M. Interobserver agreement for EUS findings in familial pancreatic-cancer kindreds. *Gastrointest Endosc* 2007; **66**: 62-67 [PMID: 17382940 DOI: 10.1016/j.gie.2006.09.018]
104 **Eisen GM**, Dominitz JA, Faigel DO, Goldstein JA, Petersen BT, Raddawi HM, Ryan ME, Vargo JJ, Young HS, Wheeler-Harbaugh J, Hawes RH, Brugge WR, Carrougher JG, Chak A, Faigel DO, Kochman ML, Savides TJ, Wallace MB, Wiersema MJ, Erickson RA. Guidelines for credentialing and granting privileges for endoscopic ultrasound. *Gastrointest Endosc* 2001; **54**: 811-814 [PMID: 11726873]
105 **Kanazawa K**, Imazu H, Mori N, Ikeda K, Kakutani H, Sumiyama K, Hino S, Ang TL, Omar S, Tajiri H. A comparison of electronic radial and curvilinear endoscopic ultrasonography in the detection of pancreatic malignant tumor. *Scand J Gastroenterol* 2012; **47**: 1313-1320 [PMID: 22943477 DOI: 10.3109/00365521.2012.719930]
106 **Katanuma A**, Maguchi H, Osanai M, Takahashi K. The difference in the capability of delineation between convex and radial arrayed echoendoscope for pancreas and biliary tract; case reports from the standpoint of both convex and radial arrayed echoendoscope. *Dig Endosc* 2011; **23** Suppl 1: 2-8 [PMID: 21535191 DOI: 10.1111/j.1443-1661.2011.01131.x]
107 **Lowry KP**, Lee JM, Kong CY, McMahon PM, Gilmore ME, Cott Chubiz JE, Pisano ED, Gatsonis C, Ryan PD, Ozanne EM, Gazelle GS. Annual screening strategies in BRCA1 and BRCA2 gene mutation carriers: a comparative effectiveness analysis. *Cancer* 2012; **118**: 2021-2030 [PMID: 21935911 DOI: 10.1002/cncr.26424]
108 **Jansen-van der Weide MC**, Greuter MJ, Jansen L, Oosterwijk JC, Pijnappel RM, de Bock GH. Exposure to low-dose radiation and the risk of breast cancer among women with a familial or genetic predisposition: a meta-analysis. *Eur Radiol* 2010; **20**: 2547-2556 [PMID: 20582702 DOI: 10.1007/s00330-010-1839-y]
109 **Hanada K**, Okazaki A, Hirano N, Izumi Y, Teraoka Y, Ikemoto J, Kanemitsu K, Hino F, Fukuda T, Yonehara S. Diagnostic strategies for early pancreatic cancer. *J Gastroenterol* 2015; **50**: 147-154 [PMID: 25501287 DOI: 10.1007/s00535-014-1026-z]
110 **Matsubayashi H**, Sasaki K, Nagata K, Kanemoto H, Kiuchi R, Ono H. Pancreatic carcinoma mimicking diffuse-type autoimmune pancreatitis: important diagnostic role of pancreatic juice cytology using endoscopic naso-pancreatic drainage. *J Dig Dis* 2012; **13**: 287-290 [PMID: 22500792 DOI: 10.1111/j.1751-2980.2012.00584.x]
111 **Ohtsuka T**, Ideno N, Aso T, Nagayoshi Y, Kono H, Mori Y, Takahata S, Oda Y, Aishima S, Igarashi H, Ito T, Ishigami K, Nakamura M, Mizumoto K, Tanaka M. Role of endoscopic retrograde pancreatography for early detection of pancreatic ductal adenocarcinoma concomitant with intraductal papillary mucinous neoplasm of the pancreas. *J Hepatobiliary Pancreat Sci* 2013; **20**: 356-361 [PMID: 22878836 DOI: 10.1007/s00534-012-0541-7]
112 **Maguchi H**, Takahashi K, Osanai M, Katanuma A. Small pancreatic lesions: is there need for EUS-FNA preoperatively? What to do with the incidental lesions? *Endoscopy* 2006; **38** Suppl 1: S53-S56 [PMID: 16802225 DOI: 10.1055/s-2006-946653]
113 **Kitano M**, Kudo M, Yamao K, Takagi T, Sakamoto H, Komaki T, Kamata K, Imai H, Chiba Y, Okada M, Murakami T, Takeyama Y. Characterization of small solid tumors in the pancreas: the value of contrast-enhanced harmonic endoscopic ultrasonography. *Am J Gastroenterol* 2012; **107**: 303-310 [PMID: 22008892 DOI: 10.1038/ajg.2011.354]
114 **Ludwig E**, Olson SH, Bayuga S, Simon J, Schattner MA, Gerdes H, Allen PJ, Jarnagin WR, Kurtz RC. Feasibility and yield of screening in relatives from familial pancreatic cancer families. *Am J Gastroenterol* 2011; **106**: 946-954 [PMID: 21468009 DOI: 10.1038/ajg.2011.65]
115 **Davis B**, Lowy AM. Surgical management of hereditary pancreatic cancer. *Med Clin North Am* 2000; **84**: 749-759 [PMID: 10872430]
116 **Kauff ND**, Barakat RR. Risk-reducing salpingo-oophorectomy in patients with germline mutations in BRCA1 or BRCA2. *J Clin Oncol* 2007; **25**: 2921-2927 [PMID: 17617523 DOI: 10.1200/JCO.2007.11.3449]
117 **Nimptsch U**, Krautz C, Weber GF, Mansky T, Grützmann R. Nationwide In-hospital Mortality Following Pancreatic Surgery in Germany is Higher than Anticipated. *Ann Surg* 2016; **264**: 1082-1090 [PMID: 26978570 DOI: 10.1097/SLA.0000000000001693]
118 **Müller MW**, Friess H, Kleeff J, Dahmen R, Wagner M, Hinz U, Breisch-Girbig D, Ceyhan GO, Büchler MW. Is there still a role for total pancreatectomy? *Ann Surg* 2007; **246**: 966-974; discussion 974-975 [PMID: 18043098]
119 **Mehrabi A**, Golriz M, Adili-Aghdam F, Hafezi M, Ashrafi M, Morath C, Zeier M, Hackert T, Schemmer P. Expanding the indications of pancreas transplantation alone. *Pancreas* 2014; **43**: 1190-1193 [PMID: 25333402 DOI: 10.1097/MPA.0000000000000181]
120 **Miyazaki M**, Yoshitomi H, Shimizu H, Ohtsuka M, Yoshidome H, Furukawa K, Takayasiki T, Kuboki S, Okamura D, Suzuki D, Nakajima M. Repeat pancreatectomy for pancreatic ductal cancer recurrence in the remnant pancreas after initial pancreatectomy: is it worthwhile? *Surgery* 2014; **155**: 58-66 [PMID: 24238124 DOI: 10.1016/j.surg.2013.06.050]
121 **Bellin MD**, Gelrud A, Arreaza-Rubin G, Dunn TB, Humar A, Morgan KA, Naziruddin B, Rastellini C, Rickels MR, Schwarzenberg SJ, Andersen DK. Total pancreatectomy with islet autotransplantation: summary of a National Institute of Diabetes and Digestive and Kidney diseases workshop. *Pancreas* 2014; **43**: 1163-1171 [PMID: 25333399 DOI: 10.1097/MPA.0000000000000236]
122 **Heidt DG**, Burant C, Simeone DM. Total pancreatectomy: indications, operative technique, and postoperative sequelae. *J Gastrointest Surg* 2007; **11**: 209-216 [PMID: 17390175 DOI: 10.1007/s11605-006-0025-7]
123 **Wu W**, Dodson R, Makary MA, Weiss MJ, Hirose K, Cameron

JL, Ahuja N, Pawlik TM, Wolfgang CL, He J. A Contemporary Evaluation of the Cause of Death and Long-Term Quality of Life After Total Pancreatectomy. *World J Surg* 2016; **40**: 2513-2518 [PMID: 27177647 DOI: 10.1007/s00268-016-3552-8]

124 **Verna EC**, Hwang C, Stevens PD, Rotterdam H, Stavropoulos SN, Sy CD, Prince MA, Chung WK, Fine RL, Chabot JA, Frucht H. Pancreatic cancer screening in a prospective cohort of high-risk patients: a comprehensive strategy of imaging and genetics. *Clin Cancer Res* 2010; **16**: 5028-5037 [PMID: 20876795 DOI: 10.1158/1078-0432.CCR-09-3209]

125 **Vasen HF**, Wasser M, van Mil A, Tollenaar RA, Konstantinovski M, Gruis NA, Bergman W, Hes FJ, Hommes DW, Offerhaus GJ, Morreau H, Bonsing BA, de Vos tot Nederveen Cappel WH. Magnetic resonance imaging surveillance detects early-stage pancreatic cancer in carriers of a p16-Leiden mutation. *Gastroenterology* 2011; **140**: 850-856 [PMID: 21129377 DOI: 10.1053/j.gastro.2010.11.048]

126 **Zubarik R**, Gordon SR, Lidofsky SD, Anderson SR, Pipas JM, Badger G, Ganguly E, Vecchio J. Screening for pancreatic cancer in a high-risk population with serum CA 19-9 and targeted EUS: a feasibility study. *Gastrointest Endosc* 2011; **74**: 87-95 [PMID: 21704809 DOI: 10.1016/j.gie.2011.03.1235]

127 **Al-Sukhni W**, Borgida A, Rothenmund H, Holter S, Semotiuk K, Grant R, Wilson S, Moore M, Narod S, Jhaveri K, Haider MA, Gallinger S. Screening for pancreatic cancer in a high-risk cohort: an eight-year experience. *J Gastrointest Surg* 2012; **16**: 771-783 [PMID: 22127781 DOI: 10.1007/s11605-011-1781-6]

128 **Underhill M**, Berry D, Dalton E, Schienda J, Syngal S. Patient experiences living with pancreatic cancer risk. *Hered Cancer Clin Pract* 2015; **13**: 13 [PMID: 26029287 DOI: 10.1186/s13053-015-0034-1]

129 **Maheu C**, Vodermaier A, Rothenmund H, Gallinger S, Ardiles P, Semotiuk K, Holter S, Thayalan S, Esplen MJ. Pancreatic cancer risk counselling and screening: impact on perceived risk and psychological functioning. *Fam Cancer* 2010; **9**: 617-624 [PMID: 20623197 DOI: 10.1007/s10689-010-9354-5]

130 **Breitkopf CR**, Sinicrope PS, Rabe KG, Brockman TA, Patten CA, McWilliams RR, Ehlers S, Petersen GM. Factors influencing receptivity to future screening options for pancreatic cancer in those with and without pancreatic cancer family history. *Hered Cancer Clin Pract* 2012; **10**: 8 [PMID: 22738386 DOI: 10.1186/1897-4287-10-8]

131 **Konings IC**, Sidharta GN, Harinck F, Aalfs CM, Poley JW, Kieffer JM, Kuenen MA, Smets EM, Wagner A, van Hooft JE, van Rens A, Fockens P, Bruno MJ, Bleiker EM. Repeated participation in pancreatic cancer surveillance by high-risk individuals imposes low psychological burden. *Psychooncology* 2016; **25**: 971-978 [PMID: 26632416 DOI: 10.1002/pon.4047]

132 **Hart SL**, Torbit LA, Crangle CJ, Esplen MJ, Holter S, Semotiuk K, Borgida A, Ardiles P, Rothenmund H, Gallinger S. Moderators of cancer-related distress and worry after a pancreatic cancer genetic counseling and screening intervention. *Psychooncology* 2012; **21**: 1324-1330 [PMID: 21774034 DOI: 10.1002/pon.2026]

133 **Rulyak SJ**, Kimmey MB, Veenstra DL, Brentnall TA. Cost-effectiveness of pancreatic cancer screening in familial pancreatic cancer kindreds. *Gastrointest Endosc* 2003; **57**: 23-29 [PMID: 12518126]

134 **Bruenderman E**, Martin RC. A cost analysis of a pancreatic cancer screening protocol in high-risk populations. *Am J Surg* 2015; **210**: 409-416 [PMID: 26003200 DOI: 10.1016/j.amjsurg.2014.11.017]

135 **Latchford A**, Greenhalf W, Vitone LJ, Neoptolemos JP, Lancaster GA, Phillips RK. Peutz-Jeghers syndrome and screening for pancreatic cancer. *Br J Surg* 2006; **93**: 1446-1455 [PMID: 17115408]

136 **Rubenstein JH**, Scheiman JM, Anderson MA. A clinical and economic evaluation of endoscopic ultrasound for patients at risk for familial pancreatic adenocarcinoma. *Pancreatology* 2007; **7**: 514-525 [PMID: 17912015 DOI: 10.1159/000108969]

137 **Alsop K**, Fereday S, Meldrum C, deFazio A, Emmanuel C, George J, Dobrovic A, Birrer MJ, Webb PM, Stewart C, Friedlander M, Fox S, Bowtell D, Mitchell G. BRCA mutation frequency and patterns of treatment response in BRCA mutation-positive women with ovarian cancer: a report from the Australian Ovarian Cancer Study Group. *J Clin Oncol* 2012; **30**: 2654-2663 [PMID: 22711857 DOI: 10.1200/JCO.2011.39.8545]

138 **Kaufman B**, Shapira-Frommer R, Schmutzler RK, Audeh MW, Friedlander M, Balmaña J, Mitchell G, Fried G, Stemmer SM, Hubert A, Rosengarten O, Steiner M, Loman N, Bowen K, Fielding A, Domchek SM. Olaparib monotherapy in patients with advanced cancer and a germline BRCA1/2 mutation. *J Clin Oncol* 2015; **33**: 244-250 [PMID: 25366685 DOI: 10.1200/JCO.2014.56.2728]

139 **Ashworth A**. A synthetic lethal therapeutic approach: poly(ADP) ribose polymerase inhibitors for the treatment of cancers deficient in DNA double-strand break repair. *J Clin Oncol* 2008; **26**: 3785-3790 [PMID: 18591545 DOI: 10.1200/JCO.2008.16.0812]

140 **Golan T**, Kanji ZS, Epelbaum R, Devaud N, Dagan E, Holter S, Aderka D, Paluch-Shimon S, Kaufman B, Gershoni-Baruch R, Hedley D, Moore MJ, Friedman E, Gallinger S. Overall survival and clinical characteristics of pancreatic cancer in BRCA mutation carriers. *Br J Cancer* 2014; **111**: 1132-1138 [PMID: 25072261 DOI: 10.1038/bjc.2014.418]

141 **Oza AM**, Cibula D, Benzaquen AO, Poole C, Mathijssen RH, Sonke GS, Colombo N, Špaček J, Vuylsteke P, Hirte H, Mahner S, Plante M, Schmalfeldt B, Mackay H, Rowbottom J, Lowe ES, Dougherty B, Barrett JC, Friedlander M. Olaparib combined with chemotherapy for recurrent platinum-sensitive ovarian cancer: a randomised phase 2 trial. *Lancet Oncol* 2015; **16**: 87-97 [PMID: 25481791 DOI: 10.1016/S1470-2045(14)71135-0]

142 **Lohse I**, Borgida A, Cao P, Cheung M, Pintilie M, Bianco T, Holter S, Ibrahimov E, Kumareswaran R, Bristow RG, Tsao MS, Gallinger S, Hedley DW. BRCA1 and BRCA2 mutations sensitize to chemotherapy in patient-derived pancreatic cancer xenografts. *Br J Cancer* 2015; **113**: 425-432 [PMID: 26180923 DOI: 10.1038/bjc.2015.220]

143 **Fogelman D**, Sugar EA, Oliver G, Shah N, Klein A, Alewine C, Wang H, Javle M, Shroff R, Wolff RA, Abbruzzese JL, Laheru D, Diaz LA. Family history as a marker of platinum sensitivity in pancreatic adenocarcinoma. *Cancer Chemother Pharmacol* 2015; **76**: 489-498 [PMID: 26126726 DOI: 10.1007/s00280-015-2788-6]

P- Reviewer: Kamisawa T, Zhang ZM **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Liu WX

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v23.i6.949

World J Gastroenterol 2017 February 14; 23(6): 949-956
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

MINIREVIEWS

Lymphocytic esophagitis: Still an enigma a decade later

Carol Rouphael, Ilyssa O Gordon, Prashanthi N Thota

Carol Rouphael, Department of General Internal Medicine, Cleveland Clinic Foundation, Cleveland, OH 44195, United States

Ilyssa O Gordon, Department of Pathology, Cleveland Clinic Foundation, Cleveland, OH 44195, United States

Prashanthi N Thota, Department of Gastroenterology and Hepatology, Digestive Disease and Surgery Institute, Cleveland Clinic Foundation, Cleveland, OH 44195, United States

Author contributions: All authors contributed to this paper with conception and design of the study, literature review and analysis, drafting, revision and final approval of the manuscript.

Conflict-of-interest statement: All authors have no conflict of interest.

Manuscript source: Invited manuscript

Correspondence to: Prashanthi N Thota, MD, FACG, Director, Esophageal Center, Department of Gastroenterology and Hepatology, Digestive Disease and Surgery Institute, Cleveland Clinic Foundation, Cleveland, OH 44195, United States. thotap@ccf.org
Telephone: +1-216-4440780
Fax: +1-216-4454222

Received: October 24, 2016
Peer-review started: October 27, 2016
First decision: December 2, 2016
Revised: December 14, 2016
Accepted: January 18, 2017
Article in press: January 18, 2017
Published online: February 14, 2017

Abstract

Lymphocytic esophagitis (LE) is a clinicopathologic entity first described by Rubio *et al* in 2006. It is defined as peripapillary intraepithelial lymphocytosis with spongiosis and few or no granulocytes on esophageal biopsy. This definition is not widely accepted and the number of lymphocytes needed to make the diagnosis varied in different studies. Multiple studies have described potential clinical associations and risk factors for LE, such as old age, female gender and smoking history. This entity was reported in inflammatory bowel disease in the pediatric population but not in adults. Other associations include gastroesophageal reflux disease and primary esophageal motility disorders. The most common symptom is dysphagia, with a normal appearing esophagus on endoscopy, though esophageal rings, webs, nodularities, furrows and strictures have been described. Multiple treatment modalities have been used such as proton pump inhibitors and topical steroids. Esophageal dilation seems to be therapeutic when dysphagia is present along with esophageal narrowing secondary to webs, rings or strictures. The natural history of the disease remains unclear and needs to be better delineated. Overall, lymphocytic esophagitis seems to have a chronic and benign course, except for two cases of esophageal perforation in the literature, thought to be secondary to this entity.

Key words: Lymphocytic esophagitis; Intraepithelial lymphocytes; Spongiosis; Gastroesophageal reflux disease; CD4 T-cells; Dysphagia; Inflammatory bowel disease; Esophageal rings; Proton pump inhibitors; Esophageal dilation

Core tip: Lymphocytic esophagitis has recently been described in 2006 and subsequently, multiple groups have attempted to describe its clinical associations and risk factors with minimal information available on

treatment. We performed a PubMed search of all case reports and retrospective studies published in English about lymphocytic esophagitis. The objective of this paper is to present a scientific review of all aspects of this emerging clinical entity known to date.

Rouphael C, Gordon IO, Thota PN. Lymphocytic esophagitis: Still an enigma a decade later. *World J Gastroenterol* 2017; 23(6): 949-956 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i6/949.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i6.949

INTRODUCTION

Lymphocytic esophagitis (LE) is a new clinicopathologic entity that was first described by Rubio *et al*[1] in 2006 and subsequently, more frequently diagnosed and reported by clinicians and pathologists over the last decade. It consists of peripapillary intraepithelial lymphocytes (IELs) with no or rare granulocytes on esophageal biopsies. Despite LE being an emerging entity, esophageal IELs have been described years ago and several studies, as early as the 1970s, suggested a possible association between gastroesophageal reflux disease (GERD) and an increased number of IELs[2]. The co-occurrence of lymphocytes and granulocytes in reflux esophagitis has also been reported[2-4]. IELs with irregular nuclear contours were described on the esophageal biopsy specimens of patients with reflux esophagitis[4]. In the late 1990s, Wang *et al*[5] found that IELs were not an independent marker of reflux esophagitis as no correlation was noted between the number of neutrophils and T lymphocytes, despite the presence of a correlation between the number of T lymphocytes and eosinophils. Around that time, in a Swedish study looking for Menetrier's disease and varioliform lymphocytic gastritis in baboons, one esophageal biopsy specimen was noted to be infiltrated by lymphocytes with round irregular contours and a lack of granulocytes[6]. The latter study was the basis for Rubio's group to start looking for human biopsy specimens with similar characteristics and describe LE for the first time. They characterized this condition by heavy peripapillary intraepithelial lymphocytic infiltration with no or rare granulocytes[1]. Consequently, multiple studies done on LE noted intercellular edema known as spongiosis[7,8] on pathology slides, a criterion that was later added to Rubio *et al* definition. The widely accepted definition for lymphocytic esophagitis is increased peripapillary IELs by more than 20 IELs per high power field with little or no granulocytes, along with spongiosis (Figure 1).

HISTOPATHOLOGY

The presence of neutrophils, eosinophils or increased lymphocytes on esophageal biopsy indicates an inflammatory process. A normal esophageal mucosa has a small number of dispersed lymphocytes, mostly with irregular nuclear contours, and mainly in the peripapillary epithelium[9]. An acceptable count of IELs in a normal esophagus was reported to be 10 IELs/HPF[10]. The lymphocytic population in the esophagus is part of the gut-associated lymphoid tissue: Cytotoxic CD8+ T cells are normally seen in the squamous epithelium along with Langerhans cells. Helper CD4+ T cells and B lymphocytes reside in the lamina propria[11].

Given that LE is defined as an increased number of IELs, we would expect a peripapillary predominance of cytotoxic CD8+ T cells in the esophageal squamous epithelium. This has been inconsistent however, and a recent study demonstrated a CD4+ T-cell-predominant intraepithelial lymphocytosis in patients with primary esophageal motility disorder (PEMD) raising the possibility of a new clinicopathologic entity that they labeled "dysmotility-associated LE"[12]. In contrast, in cases of LE in patients with normal esophageal motility, the number of CD4+ and CD8+ T lymphocytes varied. Indeed, one study found a CD8+ T-cell predominance when LE was associated with GERD in the absence of PEMD[13]. A better immunophenotypic characterization of LE is hence needed for a superior definition. Another aspect of deficiency in the definition of LE is reflected in the various studies performed with different cut-offs used to demarcate an increased IEL count, ranging between $\geqslant$ 10 IEL/HPF[14] and $\geqslant$ 50 IEL/HPF[15,16] with a cut off of $\geqslant$ 20 IEL/HPF being commonly used[17,18]. Moreover, the acceptable number of a "few granulocytes" needs to be better defined, as granulocytic inflammation is typically seen in GERD.

CLINICAL FEATURES OF LE

The incidence of LE has been on the rise in the United States over the past few years. It is unclear whether this is true increase in incidence or simply secondary to the condition being better recognized by pathologists and clinicians. In one study of 81 subjects with LE, 81.5% were diagnosed between 2006 and 2009 as compared to 6.2% diagnosed between 1998 and 2001[18]. It has been published that LE is being detected at a rate of 1 in a 1000 on endoscopies and biopsies performed in the outpatient setting[7]. It is also unclear whether LE is more commonly seen in the western world or developing countries. Despite the fact that a Swedish group first characterized it, most published case reports and studies looking for clinical associations and potential risk factors took place in the United States. To the best of our knowledge, only three case reports in the English literature described cases of LE outside the United States, including Japan[19], Portugal[20] and Australia[21]. In contrast to the findings of Rubio *et al*[1], it is becoming evident that LE seems to affect older women to a larger extent, in their sixth decade[12,14,17,18], in contrast to eosinophilic esophagitis (EoE) seen in younger men. Smoking was also found

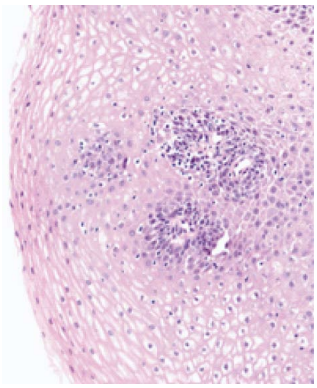

Figure 1 Histopathologic findings in lymphocytic esophagitis: increased peripapillary intraepithelial lymphocytes with spongiosis (hematoxylin and eosin stain; × 20 magnification).

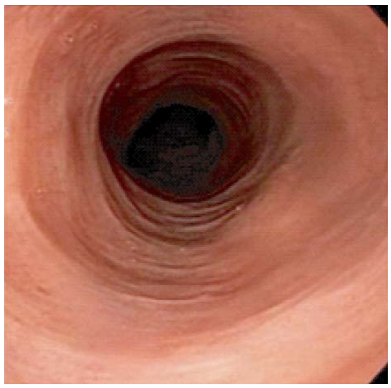

Figure 2 Endoscopy showing narrowed esophagus with subtle strictures.

to be associated with LE in multiple retrospective studies[14,17]. Patients tend to present with dysphagia as the most common symptom, though reflux/heartburn, chest pain, nausea and abdominal pain have been reported as well[8,12,14,18].

ENDOSCOPIC FINDINGS

Endoscopic findings vary from normal mucosa in up to one-third of the cases (19.8%-35%)[12,18] to esophageal rings, strictures, furrows and webs (Figure 2). For instance, Cohen *et al*[18] demonstrated that the esophagus looked normal in approximately 30% of their subjects, with the most common lesions in others being esophageal rings (19.6%), esophagitis (13%) and strictures (8.6%). Erythema, nodularities, furrows and webs were observed to a lesser extent. Purdy *et al*[8] noted a significant difference in endoscopic findings between LE subjects and controls. Similar to Cohen *et al*[18] findings, a normal appearance of the esophagus was the most common finding in both groups. It is noteworthy however that when Putra *et al*[22] compared patients with PEMD to their controls (GERD patients with no dysmotility disorder), patients with LE were more likely to have a normal upper endoscopy than patients with "dysmotility negative-GERD" (P = 0.004). No significant difference was noted however between both groups when looking at rings, furrows, esophagitis, possible Barrett's esophagus, ulcer or stricture, findings that were much less encountered in both groups than a normal esophageal mucosa. The majority of the retrospective studies conducted to look for clinical associations and risk factors for LE, found that LE most commonly presented with a normal esophagus. This observation was in contrast to Pasricha *et al*[14] findings, who noted that 82% (23/27) of their sample had abnormal endoscopic findings in the esophagus. In addition, all of the case reports published to date, report abnormal esophageal findings. For instance, Zhang *et al*[23] reported the case of a 66-year-old female with history of dysphagia, who was found to have multiple concentric rings in her mid and lower esophagus with biopsies consistent with LE. Those endoscopic findings are similar to EoE given the lesions and the location. It is hence important to realize that a feline esophagus is not specific for EoE. In addition, the most common presentation of LE seems to be dysphagia with a normal esophageal mucosa on endoscopy, hence the need for a biopsy, not only to rule out EoE, but also to look for LE. The esophageal abnormalities associated with LE have been reported in multiple locations of the esophagus. In a patient with systemic lupus erythematosus and Behçet's disease, multiple rings were noted in the upper third of the esophagus exclusively, with small outpouchings developing in the cervical esophagus years later and requiring dilation[20]. In another patient with lymphoma and esophagitis, presenting with dysphagia and food impaction, endoscopy revealed mid-esophageal rings as well as a distal esophageal stricture[24]. Lesions seem to develop in all 3 parts of the esophagus with the mid esophagus being mostly co-involved with the proximal or distal parts. As a matter of fact, in their study, Xue *et al*[12] specified that most biopsies were obtained from the mid-esophagus and had a higher IEL count compared to other locations in the esophagus.

CLINICAL ASSOCIATIONS AND RISK FACTORS FOR LE

Since Rubio *et al*[1] first described LE, multiple groups throughout the United States attempted to better characterize this entity through retrospective studies trying to identify this emerging entity's clinical features. Numerous potential clinical associations were looked at, including inflammatory bowel disease (IBD)[15,16,25], esophageal dysmotility disorders[22], hypersensitivity and mucosal insults[8,14,17], celiac sprue, common variable immunodeficiency disorder[26] and GERD, as presented below (Table 1). Other groups tried to establish LE as an independent and distinct clinical entity, many of them failing to find any potential correlates[8,12,14]. To

Table 1 Summary of retrospective studies on lymphocytic esophagitis

Ref.	Number of Patients with Lymphocytic Esophagitis	Gender Distribution *n* (%)	Mean age[1] of patients with LE (yr)	Associated conditions	Treatment
Rubio *et al*[1], 2006	20	Female: 10; Male: 10	31.3 Range: (2-28)	Crohn's disease, asthma, liver cirrhosis, Sclerosing cholangitis	Not available
Purdy *et al*[8], 2008	42	Not Available	44 Range: (2-81)	Allergies (drug and non-drug), *Helicobacter pylori* gastritis, Crohn's disease	Not available
Xue *et al*[12], 2015	45 (21 LE-NG) (24 LE-FG)	LE-NG: Female: 15; Male: 7 LE-FG: Female: 15; Male: 9	LE-NG: 59 ± 12 LE-FG: 65 ± 13	Motility abnormalities	Not available
Putra *et al*[22], 2016	10/22 with nutcracker esophagus 7/33 with ineffective motility 5/14 with diffuse esophageal spasm	Nutcracker esophagus: Female: 11; Male: 11 Ineffective motility: Female: 20; Male: 13 Ineffective motility: Female: 8; Male: 6	Nutcracker Esophagus: 54 ± 11 Ineffective motility: 56 ± 14 Diffuse spasm: 62 ± 11	Nutcracker esophagus, ineffective motility, diffuse esophageal spasm	Diltiazem Proton pump inhibitors Botulinum toxin Peppermint Nitroglycerin sublingual
Kissiedu *et al*[17], 2016	33	Female: 6; Male: 27	67 ± 10	Smoking, hyperlipidemia, cryotherapy, radio-frequency ablation, endoscopic mucosal resection	Not available
Pasricha *et al*[14], 2016	27	Female: 17; Male: 10	56 ± 16	IBD, GERD, Barrett's esophagus, achalasia, drug allergy, asthma, eczema, IBS, cancer, tobacco, alcohol, drug use	Proton pump inhibitors Fluticasone GI cocktail (maalox, donnatal, lidocaine) Prednisone taper
Cohen *et al*[18], 2012	81	Female: 44; Male: 37	51 Range: (19-84)	GERD, achalasia, allergies, asthma, eczema, IBD, hypothyroidism, alcohol use, tobacco use	Proton pump inhibitors Anti-tumor necrosis factor agent
Basseri *et al*[25], 2013	4/47 patients with IBD	Female: 23; Male: 24	39.3 ± 14.6	GERD, asthma, IBD, diabetes mellitus, celiac disease, smoking, alcohol use	Not available
Sutton *et al*[16], 2014	31	Female: 15; Male: 16	8.9	Crohn's disease, GERD, Infectious/inflammatory disorders, Polyps/neoplasms, Immune disorders, mechanical disorders, functional abdominal pain	Not available
Ebach *et al*[15], 2011	Crohn's and LymphocyticEsophagitis: 17 out of 60 patients with Crohn's	Crohn's disease patients: Female: 17; Male: 43	Crohn's disease patients: 13.3 Range: (4.7-20.7)	IBD	Not available
Haque *et al*[7], 2012	119	Female: 72; Male: 47	63 (Median)	*Helicobacter pylori* gastritis, celiac disease, duodenal lymphocytosis, Crohn's disease	Not available

[1]Age expressed as mean ± SD, unless otherwise stated. LE-NG: Lymphocytic esophagitis-no granulocytes; LE-FG: Lymphocytic esophagitis-few granulocytes; IBS: Inflammatory bowel syndrome; IBD: Inflammatory bowel disease; GERD: Gastroesophageal reflux disease.

date, LE remains a "disease" with no established definition or clinical associations.

IBD

Esophageal Crohn's disease (CD) is well recognized and is known to occur in approximately 6.5% of the affected juvenile patients as compared to 0.3%-2% in adults with CD[27]. In severe forms, it is characterized by sharply demarcated ulcers or erosions, and the mucosa surrounding those lesions is typically normal[28]. Of 20 esophageal biopsy cases with histologic LE, 8 cases of CD (including 7 pediatric cases) were identified by Rubio *et al*[1], suggesting a potential correlation with CD in the juvenile popu-

lation. Subsequently, Purdy *et al*[8] confirmed a potential association between CD and LE in the pediatric population. In their study comprising 42 patients with LE from a mixed adult and pediatric database, 38% of the pediatric subset with LE had CD (3 of 8 children). Similarly, Ebach *et al*[15] studied an exclusively pediatric cohort and found that LE was present in 28% of 60 patients with CD as compared to 4.4% of 68 children without CD. A comparable association was found in the study by Sutton *et al*[16], where LE was present in 12.2% of 49 patients with CD as compared to 5% of 496 patients without CD. These results were not reproducible in the adult population however, as shown by the work of Basseri *et al*[25], where only one out of 47 patients with LE had CD. Similarly, results were consistent in the studies conducted by Haque *et al*[7] and Xue *et al*[12], where none of the 119 and 45 adult patients with LE respectively, had CD. LE might hence be a manifestation of CD or, possibly, an indicator of IBD activity in the pediatric population, but remains nonspecific and most likely not associated with IBD in adults.

Esophageal motility disorders

An interesting association of LE with PEMD was noted by Xue *et al*[12]. In their initial work, they compared the clinical and histological characteristics of 3 groups: LE-No Granulocytes (LE-NG), LE-few granulocytes (LE-FG) and their control group which consisted of patients with "reflux esophagitis with increased IELs" (REIL). Out of the 21 subjects in the LE-NG group, 11 were tested for motility abnormality, which was confirmed in 10 subjects. PEMD were also found in 6/10 patients tested in the LE-FG group (24 patients) and in 6 of 11 tested in the REIL group (28 patients). Interestingly, the prevalence of PEMD was significantly higher in patients with CD4+ T-cell predominant IELs as compared to CD8+ T-cell predominant esophagitis, a finding that suggests a potential association between PEMD and CD4+ T-cell predominant esophagitis. It might hence be necessary to characterize the T-cell subpopulation in patients with LE for diagnostic purposes in some cases.

Esophageal mucosal injuries

Reechoing the definition of LE as peripapillary intraepithelial lymphocytosis with spongiosis, one would think of LE as an entity with histopathologic similarities to acute spongiotic dermatitis raising the hypothesis of a possible irritant to the esophageal mucosa resulting in lymphocytosis and epithelial edema. Purdy *et al*[8] refuted this hypothesis in their work and demonstrated no associations between LE and allergic disorders, asthma or celiac disease. They also looked at GERD as a potential cause of mucosal irritation and injury and found no correlation between reflux and LE, which brings us to a controversy in the present literature: Is GERD associated with LE? A recent study, published by Olson *et al*[13] in the form of an abstract, concluded that LE could be associated with GERD with CD8+ T-cell predominant IELs. Another study by Kissiedu *et al*[17] looked at post-ablation surveillance biopsies in patients with Barrett's esophagus. A significantly higher prevalence of LE was noted on surveillance biopsies as compared to pre-ablation specimens, suggesting mucosal injury to be a potential trigger to the development of this condition. Yet, it is noteworthy that all those patients had GERD. A similar mechanism of mucosal injury could hypothetically explain the association noted between LE and smoking.

Other potential associations investigated

In addition to the above-mentioned clinical associations, authors have investigated other hypothetical correlations. Pasricha *et al*[14] excluded patients with lichen planus from their study: according to the authors, lichen planus and LE are two different entities histologically as lichen planus is characterized by lichenoid lymphocytic infiltration, which is absent in LE. The potential link between LE and connective tissue disorders where analyzed in a case report from Portugal[20] where dysphagia secondary to LE was not associated with systemic lupus erythematosus and Behçet's disease flare-ups, as dysphagia and flare-ups would occur at different points in time. LE was also reported in a patient with common variable immune deficiency receiving intravenous immunoglobulin (IVIG) infusions[26]. No other reports in the literature described LE in patients with immune deficiency disorders or in patients receiving IVIG. It is hence unclear whether LE is linked to common variable immune deficiency, IVIG or is simply an independent entity in this specific patient. Some studies also looked at a potential co-occurrence of lymphocytosis in the esophagus and other parts of the gut. One would wonder whether LE would co-occur with lymphocytic colitis for instance. In their study, Purdy *et al*[8] looked at biopsies from other gastrointestinal sites obtained at the time of esophageal biopsies: Out of the 30 patients with LE, 23 had stomach biopsies which showed normal mucosa (4/23), Helicobacter pylori gastritis (4/23), focally enhanced gastritis (3/23), inactive chronic gastritis (6/23) or other random findings. Small bowel biopsies where obtained in 15 patients and revealed normal mucosa in 8 specimens, CD in one, autoimmune enteropathy in one, and epithelial lymphocytosis in two patients. The other 2 specimens showed nonspecific changes. Six patients had concurrent colon biopsies performed. One was normal, 2 had CD, 2 showed hyperplastic polyps and one had autoimmune enteropathy. The authors comment however that no pattern was noted and that interestingly, these GI tract findings corresponded to known chronic conditions of the patients. In a case report with multiple gastric biopsies taken concurrently with the esophageal biopsies that revealed LE, gastric biopsies were negative for lymphocytosis or

Table 2 Summary of case reports on lymphocytic esophagitis

Ref.	Age (yr)	Gender	Associated conditions	Treatment
Figueiredo *et al*[20], 2014	30	Male	Behçet's disease Systemic lupus erythematosus	Endoscopic dilation Swallowed fluticasone
Mandaliya *et al*[24], 2012	74	Male	Lymphoma Esophagitis	Endoscopic dilation Botox injections
Zhang *et al*[23], 2016	66	Female	Opioid overdose	Omeprazole 40 mg twice daily
Maejima *et al*[19], 2015	68	Male	Food impaction	Endoscopic dilation
Niewiarowski *et al*[31], 2016	82	Female	Acute food impaction	Not available
Vangimalla *et al*[26], 2016	67	Male	Common variable immune deficiency	Acid suppression Endoscopic dilation
Hendy *et al*[21], 2013	35	Female	Not available	Topical steroids (fluticasone)

eosinophilic gastritis[23]. Haque *et al*[7] also looked at the co-occurrence of celiac disease and duodenal lymphocytosis with LE and EoE. There was no significant difference in duodenal lymphocytosis co-occurrence between the LE and the EoE groups. On the other hand, celiac disease was more commonly noted in patients with LE as compared to EoE, although this was not statistically significant given the small number of patients (Table 2).

TREATMENT

Multiple treatments have been tried including proton pump inhibitors (PPIs), topical steroids, oral prednisone, botox injections and esophageal dilations. Few retrospective studies actually addressed treatment. Cohen *et al*[18] conducted a study in two stages: they initially performed a retrospective chart review of patients with LE and investigated potential clinical associations with this condition. The second part of the study aimed at exploring the natural history of LE by sending out surveys to their 81 patients with LE with a 3.3-year median follow-up. Out of the 29 patients who completed the survey, approximately 60% reported improvement in their GI symptoms that they thought was as a result of therapy, either with PPIs or after starting an anti-tumor necrosis factor agent for patients with IBD. Esophageal dilation contributed to symptomatic improvement as well. Pasricha *et al*[14] also collected information on treatment changes after establishing a diagnosis of LE. Out of their 27 patients, one-third had a change in treatment that consisted of either a PPI (6), inhaled fluticasone (1), gastrointestinal cocktail (1) or prednisone taper (1) with improvement noted in patients treated with PPIs or inhaled fluticasone. It is unclear how PPIs result in symptom improvement. Despite the fact that PPIs have actually been associated with lymphocytic and collagenous colitis[29], they actually seem to be therapeutic in LE, most likely secondary to their anti-inflammatory effect. Clinicians have been prescribing PPIs for LE, as LE is thought to be potentially associated with GERD, and given that improvement is being reported. For instance, Zhang *et al*[23] treated their patient with omeprazole 40 mg twice daily with symptomatic improvement within days. As already mentioned, topical steroids have also been suggested with symptom resolution, assuming LE and EoE belong to the same family. Kasirye *et al*[30] opted to treat their patient with 220 μg/puff, two puffs three times daily, as their patient was already treated with PPIs and Histamine 2-receptor antagonists with incomplete resolution of their symptoms. Additionally, therapeutic esophageal dilations have been performed in patients presenting with dysphagia (with or without food impaction). Based on the few case reports published, dilation can be repeated as needed[26,31].

NATURAL HISTORY, PROGNOSIS AND FUTURE DIRECTIONS

Extensive study of the natural history of LE is lacking. We identified one study looking at the clinical course of LE *via* a survey sent out to patients, which found that 70 of 81 patients with LE (87%) were alive after a 3.3-year median follow up[18]. Of the 29 subjects who completed the survey, 96.5% continued to have GI symptoms, but reported improvement in their symptoms with medical management, which included PPIs or anti-tumor necrosis factor agents (for patients with IBD); 66% were satisfied with their current gastrointestinal health, 22 had repeat endoscopies of which only 2 patients had normal biopsy, 9 had persistent LE and the rest had other forms of esophagitis or CD[18]. Given that 9 out of 22 patients had persistent LE on repeat biopsy, one would hypothesize that LE might be a form of chronic esophagitis independent of other diseases. As a matter of fact, Mandaliya *et al*[24] described the case of a patient who presented with a 3-year history of dysphagia leading to the diagnosis of LE. Five endoscopies performed four years later, showed persistent LE endoscopically and histologically, requiring serial dilations[24], which supports the possible chronic nature of this entity. It is noteworthy that there are two cases of esophageal perforation in the setting of LE published in the literature[21,24]. In one case, perforation occurred following endoscopic removal of acutely impacted meat. Repeat endoscopy, after the acute episode resolved, showed tight rings and a stricture of the esophagus with pathology con-

sistent with LE[24]. The other case was of a previously healthy 35 year-old female who presented with fever, chest pain and shock, with CT chest showing diffuse thickening of the esophagus and bilateral pleural effusions, exudative in nature with > 60% neutrophils. She was hence resuscitated and started on antibiotics for microperforation. A week later, an upper endoscopy was performed and biopsies from the mid and distal esophagus showed LE, thought to be the cause behind her initial presentation[21].

Overall, LE appears to be a benign entity except for two cases of esophageal perforation. Furthermore, it seems to have a chronic course[20,24], either because appropriate treatment is still not found or because more research is needed to further characterize its mysterious nature. According to the literature published to date, it remains unclear whether LE is associated with any of the clinical entities discussed. Although multiple studies are exploring this entity and trying to attribute it to a disease or investigating its clinical associations, LE might end up being a diagnosis of exclusion. It might also end up being a phenotypic expression of different pathologic processes rather than an actual disease. Prospective studies are needed to depict appropriate treatments of this condition and its possible subsets: CD4+ *vs* CD8+ T- cell-predominant LE, as well as to follow the clinical course of patients with LE to be able to better characterize the behavior of this new entity with time.

CONCLUSION

As Haque *et al*[7] perfectly described it, LE remains an entity "in search of a disease". Increasing in prevalence since it was defined in 2006, it seems to occur more commonly in older females and is associated with smoking. Multiple groups attempted to better characterize this entity and study potential associations with clinical diseases such as IBD, motility disorders, GERD, mucosal injuries and hypersensitivity reactions with inconclusive results and sometimes, conflicting conclusions. PPIs, topical steroids and esophageal dilations are used as effective treatment modalities with good short-term results but unclear long-term outcomes. Prospective studies are needed to define the disease, delineate the disease course, treatment options and long-term outcomes.

REFERENCES

1 **Rubio CA**, Sjödahl K, Lagergren J. Lymphocytic esophagitis: a histologic subset of chronic esophagitis. *Am J Clin Pathol* 2006; **125**: 432-437 [PMID: 16613348 DOI: 10.1309/7LAB -LGY0 -8UEM-3H26]

2 **Ismail-Beigi F**, Horton PF, Pope CE. Histological consequences of gastroesophageal reflux in man. *Gastroenterology* 1970; **58**: 163-174 [PMID: 5413015 DOI: 10.1016/S0016-5085(70)80004-X]

3 **Butt AM**, Murch SH, Ng CL, Kitching P, Montgomery SM, Phillips AD, Walker-Smith JA, Thomson MA. Upregulated eotaxin expression and T cell infiltration in the basal and papillary epithelium in cows' milk associated reflux oesophagitis. *Arch Dis Child* 2002; **87**: 124-130 [PMID: 12138061 DOI: 10.1136/adc.87.2.124]

4 **Cucchiara S**, D'Armiento F, Alfieri E, Insabato L, Minella R, De Magistris TM, Scoppa A. Intraepithelial cells with irregular nuclear contours as a marker of esophagitis in children with gastroesophageal reflux disease. *Dig Dis Sci* 1995; **40**: 2305-2311 [PMID: 7587806 DOI: 10.1007/BF02063229]

5 **Wang HH**, Mangano MM, Antonioli DA. Evaluation of T-lymphocytes in esophageal mucosal biopsies. *Mod Pathol* 1994; **7**: 55-58 [PMID: 8159653]

6 **Rubio CA**, Hubbard G. Hyperplastic foveolar gastropathy and hyperplastic foveolar gastritis in baboons. *In Vivo* 1996; **10**: 507-510 [PMID: 8899430]

7 **Haque S**, Genta RM. Lymphocytic oesophagitis: clinicopathological aspects of an emerging condition. *Gut* 2012; **61**: 1108-1114 [PMID: 22157333 DOI: 10.1136/gutjnl-2011-301014]

8 **Purdy JK**, Appelman HD, Golembeski CP, McKenna BJ. Lymphocytic esophagitis: a chronic or recurring pattern of esophagitis resembling allergic contact dermatitis. *Am J Clin Pathol* 2008; **130**: 508-513 [PMID: 18794041 DOI: 10.1309/ D3PCF6D6YY MQRX9A]

9 **Carmack SW**, Lash RH, Gulizia JM, Genta RM. Lymphocytic disorders of the gastrointestinal tract: a review for the practicing pathologist. *Adv Anat Pathol* 2009; **16**: 290-306 [PMID: 19700939 DOI: 10.1097/PAP.0b013e3181b5073a]

10 **Resnick MB**, Finkelstein Y, Weissler A, Levy J, Yakirevich E. Assessment and diagnostic utility of the cytotoxic T-lymphocyte phenotype using the specific markers granzyme-B and TIA-1 in esophageal mucosal biopsies. *Hum Pathol* 1999; **30**: 397-402 [PMID: 10208460 DOI: 10.1016/S0046-8177(99)90114-4]

11 **Podolsky DK**, Camilleri M, Fitz JG, Kalloo AN, Shanahan F, Wang TC. Yamada's Textbook of Gastroenterology, 2 Volume Set. 6th ed. Wiley, 2015: 49

12 **Xue Y**, Suriawinata A, Liu X, Li Z, Gabbard S, Rothstein R, Lacy B, Lisovsky M. Lymphocytic Esophagitis With CD4 T-cell-predominant Intraepithelial Lymphocytes and Primary Esophageal Motility Abnormalities: A Potential Novel Clinicopathologic Entity. *Am J Surg Pathol* 2015; **39**: 1558-1567 [PMID: 26379147 DOI: 10.1097/PAS.0000000000000493]

13 **Olson N**, Putra J, Liu X, Suriawinata AA, Lisovsky M. Lymphocytic Esophagitis with CD8 T-cell Predominance may be Associated with Gastroesophageal Reflux Disease. Poster, United States and Canadian Academy of Pathology (USCAP) Annual Meeting, March 12-18, 2016, Seattle, WA, United States

14 **Pasricha S**, Gupta A, Reed CC, Speck O, Woosley JT, Dellon ES. Lymphocytic Esophagitis: An Emerging Clinicopathologic Disease Associated with Dysphagia. *Dig Dis Sci* 2016; **61**: 2935-2941 [PMID: 27343035 DOI: 10.1007/s10620-016-4230-2]

15 **Ebach DR**, Vanderheyden AD, Ellison JM, Jensen CS. Lymphocytic esophagitis: a possible manifestation of pediatric upper gastrointestinal Crohn's disease. *Inflamm Bowel Dis* 2011; **17**: 45-49 [PMID: 20848529 DOI: 10.1002/ibd.21347]

16 **Sutton LM**, Heintz DD, Patel AS, Weinberg AG. Lymphocytic esophagitis in children. *Inflamm Bowel Dis* 2014; **20**: 1324-1328 [PMID: 24983984 DOI: 10.1097/MIB.0000000000000100]

17 **Kissiedu J**, Thota PN, Gohel T, Lopez R, Gordon IO. Post-ablation lymphocytic esophagitis in Barrett esophagus with high grade dysplasia or intramucosal carcinoma. *Mod Pathol* 2016; **29**: 599-606 [PMID: 26965580 DOI: 10.1038/modpathol.2016.50]

18 **Cohen S**, Saxena A, Waljee AK, Piraka C, Purdy J, Appelman H, McKenna B, Elmunzer BJ, Singal AG. Lymphocytic esophagitis: a diagnosis of increasing frequency. *J Clin Gastroenterol* 2012; **46**: 828-832 [PMID: 22751335 DOI: 10.1097/MCG. 0b013e31825 00de8]

19 **Maejima R**, Uno K, Iijima K, Fujishima F, Noguchi T, Ara N, Asano N, Koike T, Imatani A, Shimosegawa T. A Japanese case of lymphocytic esophagitis. *Dig Endosc* 2016; **28**: 476-480 [PMID: 26589889 DOI: 10.1111/den.12578]

20 **Figueiredo PC**, Pinto-Marques P, Borralho P, Freitas J. Unusual cause for smoldering dysphagia. Lymphocytic esophagitis.

Dysphagia 2014; **29**: 283-285 [PMID: 23982520 DOI: 10.1007/s00455-013-9489-2]
21 **Hendy PJ**, Wong DS, Florin TH. Spontaneous oesophageal perforation: an unreported complication of lymphocytic oesophagitis. *Gut* 2013; **62**: 1668-1669 [PMID: 23850714 DOI: 10.1136/gutjnl-2013-305455]
22 **Putra J**, Muller KE, Hussain ZH, Parker S, Gabbard S, Brickley EB, Lacy BE, Rothstein R, Lisovsky M. Lymphocytic Esophagitis in Nonachalasia Primary Esophageal Motility Disorders: Improved Criteria, Prevalence, Strength of Association, and Natural History. *Am J Surg Pathol* 2016; **40**: 1679-1685 [PMID: 27526295 DOI: 10.1097/PAS.0000000000000712]
23 **Zhang Z**, Jain D, Brand M. Ringed Esophagus Secondary to Lymphocytic Esophagitis. *Gastroenterol Hepatol* (N Y) 2016; **12**: 237-239 [PMID: 27231454]
24 **Mandaliya R**, Dimarino AJ, Cohen S. Lymphocytic esophagitis mimicking eosinophilic esophagitis. *Ann Gastroenterol* 2012; **25**: 355-357 [PMID: 24714246]
25 **Basseri B**, Vasiliauskas EA, Chan O, Wang HL, Basseri RJ, Pimentel M, Soffer E, Conklin JL. Evaluation of peripapillary lymphocytosis and lymphocytic esophagitis in adult inflammatory bowel disease. *Gastroenterol Hepatol* (N Y) 2013; **9**: 505-511 [PMID: 24719598]
26 **Vangimalla S**, Gordon I, Thota PN. Image of the Month: Lymphocytic Esophagitis in Common Variable Immune Deficiency. *Am J Gastroenterol* 2016; **111**: 170 [PMID: 26882931 DOI: 10.1038/ajg.2015.178]
27 **Feagans J**, Victor D, Joshi V. Crohn disease of the esophagus: a review of the literature. *South Med J* 2008; **101**: 927-930 [PMID: 18708983 DOI: 10.1097/SMJ.0b013e31818047be]
28 **Goldstein NS**, Amin M. Upper Gastrointestinal Tract in Inflammatory Bowel Disease. *Surg Pathol Clin* 2010; **3**: 349-359 [PMID: 26839135 DOI: 10.1016/j.path.2010.05.004]
29 **Masclee GM**, Coloma PM, Kuipers EJ, Sturkenboom MC. Increased risk of microscopic colitis with use of proton pump inhibitors and non-steroidal anti-inflammatory drugs. *Am J Gastroenterol* 2015; **110**: 749-759 [PMID: 25916221 DOI: 10.1038/ajg.2015.119]
30 **Kasirye Y**, John A, Rall C, Resnick J. Lymphocytic esophagitis presenting as chronic dysphagia. *Clin Med Res* 2012; **10**: 83-84 [PMID: 22031480 DOI: 10.3121/cmr.2011.1009]
31 **Niewiarowski TJ**, Stoll LM. Recurrent dysphagia in a patient with chronic lymphocytic esophagitis. *Gastrointest Endosc* 2016; **84**: 1071-1072 [PMID: 26902845 DOI: 10.1016/j.gie.2016.02.016]

P- Reviewer: Blonski W, Smith RC **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Liu WX

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v23.i6.957

World J Gastroenterol 2017 February 14; 23(6): 957-963
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Restoration of energy level in the early phase of acute pediatric pancreatitis

Dóra Mosztbacher, Nelli Farkas, Margit Solymár, Gabriella Pár, Judit Bajor, Ákos Szűcs, József Czimmer, Katalin Márta, Alexandra Mikó, Zoltán Rumbus, Péter Varjú, Péter Hegyi, Andrea Párniczky

Dóra Mosztbacher, First Department of Pediatrics, Semmelweis University, 1083 Budapest, Hungary

Dóra Mosztbacher, Nelli Farkas, Margit Solymár, Katalin Márta, Alexandra Mikó, Zoltán Rumbus, Péter Varjú, Péter Hegyi, Andrea Párniczky, Institute for Translational Medicine, University of Pécs, 7624 Pécs, Hungary

Nelli Farkas, Institute for Bioanalyses, University of Pécs, 7624 Pécs, Hungary

Gabriella Pár, Judit Bajor, József Czimmer, Department of Gastroenterology, First Department of Medicine, University of Pécs, 7624 Pécs, Hungary

Judit Bajor, Péter Hegyi, Department of Translational Medicine, First Department of Medicine, University of Pécs, 7624 Pécs, Hungary

Ákos Szűcs, First Department of Surgery, Semmelweis University, 1082 Budapest, Hungary

Péter Hegyi, Hungarian Academy of Sciences - University of Szeged, Momentum Gastroenterology Multidisciplinary Research Group, 6720 Szeged, Hungary

Andrea Párniczky, Heim Pál Children's Hospital, 1089 Budapest, Hungary

Author contributions: Solymár M, Pár G, Bajor J, Szűcs Á and Czimmer J searched for the articles in Pubmed and screened them using the titles and abstracts; Márta K, Mikó A, Rumbus Z and Varjú P searched for the articles in Embase and screened them using the titles and abstracts; data was collected from the eligible papers by Mosztbacher D and Párniczky A; Farkas N performed the statistical analysis; Mosztbacher D, Hegyi P and Párniczky A drafted the manuscript.

Supported by the Hungarian Scientific Research Fund, No. K116634 to Hegyi P; and the Momentum Grant of the Hungarian Academy of Sciences, No. LP2014-10/2014 to Hegyi P.

Conflict-of-interest statement: All the authors disclaim any form of conflict of interest.

Manuscript source: Invited manuscript

Correspondence to: Andrea Párniczky, MD, Institute for Translational Medicine, University of Pécs, Szigeti út 12., II. em., 7624 Pécs, Hungary. andrea.parniczky@gmail.com
Telephone: +36-72-536246
Fax: +36-72-536247

Received: October 3, 2016
Peer-review started: October 7, 2016
First decision: October 20, 2016
Revised: November 21, 2016
Accepted: January 11, 2017
Article in press: January 11, 2017
Published online: February 14, 2017

Abstract

Acute pancreatitis (AP) is a serious inflammatory disease with rising incidence both in the adult and pediatric populations. It has been shown that mitochondrial injury and energy depletion are the earliest intracellular events in the early phase of AP. Moreover, it has been revealed that restoration of intracellular ATP level restores cellular functions and defends the cells from death. We have recently shown in a systematic review and meta-analysis that early enteral feeding is beneficial in adults; however, no reviews are available concerning the effect of early enteral feeding in pediatric AP. In this minireview, our aim was to systematically analyse the literature on the treatment

of acute pediatric pancreatitis. The preferred reporting items for systematic review (PRISMA-P) were followed, and the question was drafted based on participants, intervention, comparison and outcomes: P: patients under the age of twenty-one suffering from acute pancreatitis; I: early enteral nutrition (per os and nasogastric- or nasojejunal tube started within 48 h); C: nil per os therapy; O: length of hospitalization, need for treatment at an intensive care unit, development of severe AP, lung injury (including lung oedema and pleural effusion), white blood cell count and pain score on admission. Altogether, 632 articles (PubMed: 131; EMBASE: 501) were found. After detailed screening of eligible papers, five of them met inclusion criteria. Only retrospective clinical trials were available. Due to insufficient information from the authors, it was only possible to address length of hospitalization as an outcome of the study. Our mini-meta-analysis showed that early enteral nutrition significantly (SD = 0.806, P = 0.034) decreases length of hospitalization compared with nil per os diet in acute pediatric pancreatitis. In this minireview, we clearly show that early enteral nutrition, started within 24-48 h, is beneficial in acute pediatric pancreatitis. Prospective studies and better presentation of research are crucially needed to achieve a higher level of evidence.

Key words: Pediatric pancreatitis; Enteral nutrition; Nil per os diet; ATP restoration; Length of hospitalization

Core tip: Acute pancreatitis is a serious inflammatory disease with rising incidence both in adult and pediatric medicine. Despite the existing research activities in this field, no specific therapy is available to treat this disease. Results in basic science strongly suggest that early energy restoration could be the first-line treatment for acute pancreatitis. Our minireview suggests that early enteral nutrition should have priority in the treatment of acute pediatric pancreatitis.

Mosztbacher D, Farkas N, Solymár M, Pár G, Bajor J, Szűcs Á, Czimmer J, Márta K, Mikó A, Rumbus Z, Varjú P, Hegyi P, Párniczky A. Restoration of energy level in the early phase of acute pediatric pancreatitis. *World J Gastroenterol* 2017; 23(6): 957-963 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i6/957.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i6.957

INTRODUCTION

Acute pancreatitis (AP) is a serious inflammatory disease with rising incidence both in adult and pediatric populations[1,2]. Common characteristics in both age groups are that no specific therapy is available to treat the disease and that the general supportive treatments at the early phase of the disease are usually volume resuscitation and a nil per os (NPO) diet[3-6]. However, while there is clear evidence in the literature that volume therapy is beneficial, the latter treatment is questionable.

One of the main reasons for the debate is that the pathogenesis of the disease clearly suggests the opposite. Irrespective of the etiological factors, mitochondrial damage and energy depletion are the leading intracellular responses in the early phase of the disease in the exocrine pancreas[7-10]. Bile acids[11-14], ethanol, fatty acids and their non-oxidative metabolites, fatty acid ethyl esthers[8,9,15-18] were shown to elevate the intracellular Ca^{2+} concentration, causing mitochondrial damage and a resultant decrease of intracellular ATP concentration. This will lead to inhibited fluid and bicarbonate secretion and CFTR Cl^- channel dysfunction in the ductal cells and secretory block and intracellular trypsinogen activation in the acinar cells (Figure 1)[9,16,19,20]. Very importantly, restoration of ATP levels both in acinar and ductal cells prevents (at least in part) the toxic effects of the etiological factors[7,21,22] noted above. These data strongly suggest that an energy supply, for example, *via* enteral nutrition, should be beneficial for patients as compared to nil energy.

Notably, early enteral nutrition (EEN) either *via* oral, nasogastric- or nasojejunal tube feeding is beneficial as regards systemic infections, complications, multi-organ failure, need for surgical interventions and mortality[6,23-30]. Enteral nutrition has already been proven to be beneficial in other inflammatory gastrointestinal diseases. The first-line recommendation to induce remission in pediatric Crohn's disease is exclusive enteral nutrition[31]. Enteral nutrition could also be effective in the maintenance of pediatric inflammatory bowel disease remission[32]. With regard to acute pancreatitis, three of the recent and most up-to-date guidelines for acute pancreatitis in adults clearly show the positive effect of enteral nutrition in moderate and severe AP[6,23,24]. Besides the energy supply, enteral nutrition in patients can also have other advantages as a first-line treatment for patients. It is well documented that the gut plays an important role as an immune barrier in the immune system and that EEN facilitates this barrier function. EEN significantly decreases pathogenic bacteria in the stool, alteration of intestinal flora and levels of serum endotoxins. EEN has a favourable effect on immune dysregulation caused by severe acute pancreatitis, which can reduce APACHE II scores, pancreatic sepsis, initial incidences of systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome[33,34].

Recent meta-analyses of adult data showed that EEN is beneficial in all severity groups in AP; however, no systematic review is available concerning the role of EEN in pediatrics[35]. Therefore, the aim was to review

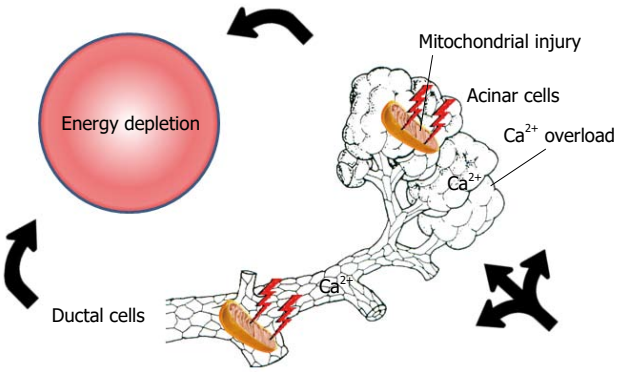

Figure 1 Early events in acute pancreatitis. Bile acids, ethanol, fatty acids or their non-oxidative metabolites, fatty acid ethyl esthers, induce calcium overload, causing mitochondrial damage and a resultant decrease in intracellular ATP concentration both in acinar and ductal cells. This will lead to general energy depletion in the pancreas.

the literature to analyse the effect of EEN *vs* NPO therapy on the outcome of acute pediatric pancreatitis (APP) and aggregate the information in APP leading to a higher statistical power and more robust point estimate than is possible from the individual studies.

The preferred reporting items for systematic review and meta-analysis protocol (PRISMA-P) were followed[36]. Our structured literature search was based on the participants, intervention, comparison and outcomes format: P: patients under the age of twenty-one suffering from acute pancreatitis; I: early enteral nutrition (per os and nasogastric- or nasojejunal tube started within 48 h); C: NPO therapy [per os/nasogastric- or enteral tube after 48 h and total parenteral nutrition (TPN) within or after 48 h]; O: length of hospitalization, need for intensive care unit (ICU), complications, necessity of antibiotics, surgical/non-surgical interventions and mortality.

In February 2016, a literature search was performed on the PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and EMBASE (https://www.embase.com) databases using the following Medical Subject Headings and search terms: "pediatric" OR "paediatric" AND "pancreatitis". The search was limited to human studies, full-text publications with abstracts in English with no time period, resulting in 632 articles altogether (PubMed: 131; EMBASE: 501).

The articles were checked separately. Meta-analyses, reviews, case reports and articles on chronic pancreatitis were excluded and duplicates were removed (Figure 2). Potentially eligible papers were selected, and, finally, five of them with relevant data on EEN or/with NPO therapy in acute pediatric pancreatitis in patients under twenty-one years old were included (Table 1)[37-41]. To reduce the risk of bias, the literature search was independently performed by three researchers following the inclusion criteria noted above.

The details in the collected articles were checked, and only articles where both EEN and NPO were presented separately were used. Two articles met this criterion. The two articles contained three separate data pairs, where EEN was compared to NPO (Figure 3). The following parameters were collected: length of hospitalization (LOH), need for treatment at an ICU, development of severe AP, lung injury (including lung oedema and pleural effusion), white blood cell count and pain score on admission. Only one of the five investigated parameters (LOH) contained a minimum of three items, which were analysed statistically.

Table 1 Studies included in the quantitative synthesis

Ref.	Data	Groups	NO. of patients
Abu-El-Haija *et al*[37], 2016	Yes	EEN	24
		NPO	14
Flores-Calderón *et al*[41], 2009		Only NPO	18
Goh *et al*[40], 2003		Only NPO	12
Raizner *et al*[39], 2013		Only NPO	7
Szabo *et al*[38], 2015	Yes	EEN + IVF lo	55
		NPO + IVF lo	20
	Yes	EEN + IVF hi	96
		NPO + IVF hi	30

EEN: Early enteral nutrition; NPO: Nil per os.

The meta-analytic calculation was made with Comprehensive MetaAnalysis (V3) software using the random effects model (the DerSimonian-Laird method). We calculated a weighted standard difference in means and 95%CI. In the case of one study (Abu-El-Haija *et al*[37], 2016), we converted the median and range values to means and standard deviation using the modified Hozo's formula by Wan *et al*[42]. For a visual inspection, we used a forest plot.

Figure 3 shows the parameters collected from the articles. It was only possible to perform forest plot analyses on LOH. EEN significantly decreased LOH (SD = 0.806, P = 0.034) compared to the standard NPO diet (Figure 3).

DISCUSSION

Several therapeutic recommendations are available in the literature on nutrition in acute pancreatitis. The IAP/APA guideline suggests enteral tube feeding as the first-line therapy in patients requiring nutritional support with predicted severe and severe acute pancreatitis[6]. According to the Japanese guideline, enteral nutrition in the early phase of severe acute pancreatitis can decrease the incidence of complications and elevate the survival rate[24]. Recent meta-analyses of adult studies revealed that EEN decreases mortality, rate of interventions and the incidence of multi-organ failure in severe acute pancreatitis. Moreover, group analyses of 17 parameters including laboratory parameters (such as CRP and white blood cells) and symptoms (such as pain or presence of SIRS) suggested that EEN also has merits in mild acute pancreatitis. Since the incidence of APP has risen in the past twenty years (with 3.6 and 13.2/100000 children affected annually), we

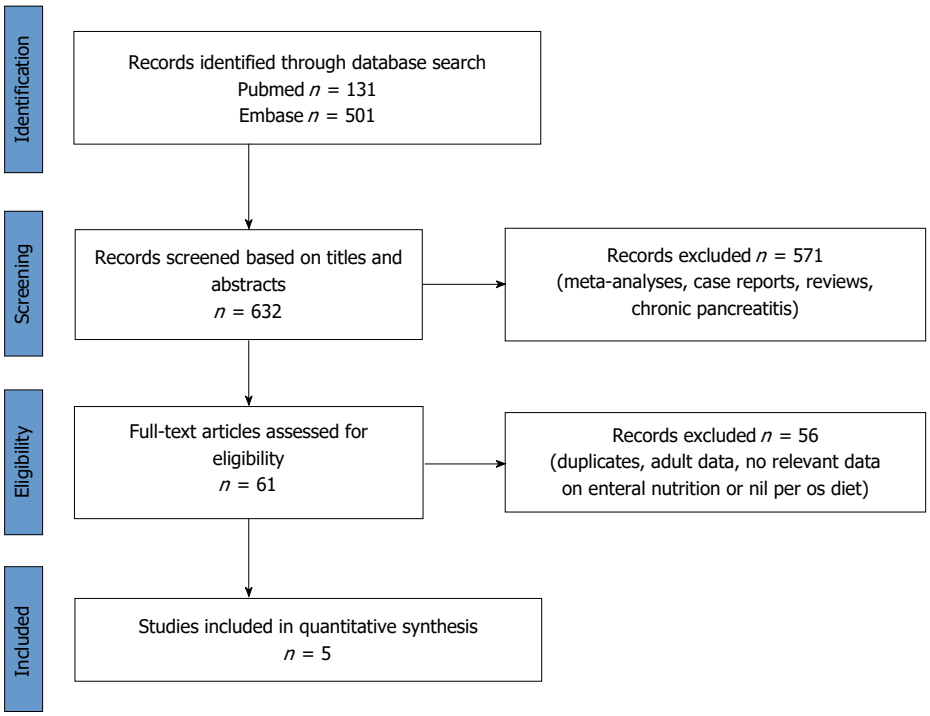

Figure 2 Flow chart on the methods used in the literature search.

Ref.		Therapy	*n*	LOH	Need for ICU	Severe AP	Lung injury	WBC	Pain score
Abu-El-Haija *et al*[37], 2016	1	EEN (< 24 h)	24	81.7 h					
		NPO (< 24 h)	14	94.7 h					
Szabo *et al*[38], 2015	2	NPO (< 48 h) + IVF lo (< 24 h)	20	7.1 d (1.01 SE)	20.0% (8.94 SE)	35.0% (10.7 SE)	11	13.60 (6.44 SD)	4.95 (3.75 SD)
		EEN (< 48 h) + IVF lo (< 24 h)	55	2.8 d (0.24 SE)	1.8% (1.80 SE)	9.1% (3.88 SE)	21	9.89 (3.89 SD)	4.62 (3.50 SD)
	3	NPO (< 48 h) + IVF hi (< 24 h)	30	5.0 d (0.58 SE)	13.0% (6.21 SE)	17.0% (6.80 SE)	14	13.30 (4.76 SD)	6.08 (3.19 SD)
		EEN (< 48 h) + IVF hi (< 24 h)	96	3.2 d (0.22 SE)	1.0% (1.04 SE)	4.2% (2.04 SE)	4	11.30 (5.25 SD)	5.47 (3.57 SD)

Study name	Statistics for each study					
	Std diff in means	Standard error	Lower limit	Upper limit	Z value	*P* value
Abu-El-Haija *et al*[37], 2016	0.089	0.336	-0.570	0.749	0.266	0.790
Szabo *et al*[38], 2015 A	1.554	0.290	0.985	2.122	5.352	0.000
Szabo *et al*[38], 2015 B	0.739	0.214	0.319	1.159	3.447	0.001
	0.806	0.380	0.062	1.551	2.124	0.034

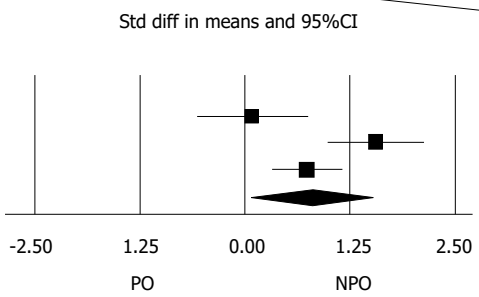

Figure 3 Two articles contained three separate data pairs where early enteral nutrition was compared to nil per os. LOH: Length of hospitalization; EEN: Early enteral nutrition; NPO: Nil per os. ICU: Intensive care unit; AP: Acute pancreatitis; WBC: White blood cell count.

systematically reviewed the literature to understand whether there is any beneficial effect of EEN *vs* NPO in children[43,44].

We faced several difficulties during our review: (1) APP is still underdiagnosed, thus decreasing the possibility of performing clinical trials[45]; (2) the number of studies on the management of these patients is very low, and there is still only a small number of studies focused on understanding the characteristics of the disease[46]; (3) the studies have not focused on the early management of the patients; the groups were therefore not separated; and (4) finally, but very importantly, the methods sections and the quality of data presentation in these articles are very low. Consequently, in many cases, it was impossible to obtain quality analysable data from the manuscripts for a proper broad-spectrum meta-analysis[37-39].

By the end of the search, we identified five articles containing relevant data on nutritional management during the early phase of APP. Raizner *et al*[39] published a retrospective analysis involving seven children with necrotizing pancreatitis. All the children received a

strict NPO diet, five patients received TPN and just one patient was treated with nasojejunal feeding for seven days. All the children required a prolonged hospital stay (with a mean of 20 d) for acute complications, with three of them suffering from late complications[39]. Goh *et al*[40] included twelve patients in their retrospective study. One patient needed a distal pancreatectomy, and eleven patients recovered with conservative management, with none of them receiving EEN. Two patients had acute complications, and two patients had recurrent AP[40]. Flores-Calderon *et al*[41] studied eighteen patients with acute pancreatitis caused by L-asparaginase due to acute lymphoblastic leukemia. All the patients were treated with bowel resting for a mean of 22 d, fourteen of the patients received TPN and four had an elementary diet. Two of the patients required intensive care unit admission, with local complications developing in twelve patients. None of the patients died from complications related to AP. Although these studies point out several disadvantages of that NPO diet, none of them could be enrolled in our meta-analysis.

Finally, it was possible to collect three sets of analysable data pairs where both NPO and EEN were present. Abu-El-Haija *et al*[37] conducted a prospective study of 38 children suffering from mild AP and retrospectively investigated the relationship of nutrition with pain and LOS. EEN feeding meant per os feeding and NPO was identified as oral feeding not being allowed for 24 h. Importantly, EEN, even with high fat intake, did not cause an elevation in pain in children, suggesting that EEN is a well tolerable nutritional possibility in children. The fact that LOS was much shorter in group EEN *vs* NPO points to EEN as a better way of treating APP[37]. The most advanced study was performed by Szabo *et al*[38], where several parameters were collected to understand the effect of EEN on the course of APP. Two hundred and one children suffering from mild AP were enrolled retrospectively. They compared EEN *vs* NPO both with and without aggressive fluid resuscitation. Fluid therapy was administered during the first 24 h, and the type of nutrition was determined during the first 48 h. Besides the beneficial effects of EEN on LOS, they also showed that EEN reduces the severity of the disease. Although our aim was to perform a meta-analysis on several parameters to understand the differences between EEN and NPO, we were only able to perform the statistical analyses on LOS, which clearly showed that EEN is not only a safe method of nutrition but also substantially decreases LOS, resulting in a better and less expensive treatment of mild APP[38].

CONCLUSION

The information collected by basic scientists, retrospective clinical studies and meta-analyses suggests that EEN should have priority in treating APP. However, it is perhaps self-evident that randomized multicenter clinical intervention trials would be crucial to achieving a higher level of evidence.

REFERENCES

1 **Peery AF**, Dellon ES, Lund J, Crockett SD, McGowan CE, Bulsiewicz WJ, Gangarosa LM, Thiny MT, Stizenberg K, Morgan DR, Ringel Y, Kim HP, Dibonaventura MD, Carroll CF, Allen JK, Cook SF, Sandler RS, Kappelman MD, Shaheen NJ. Burden of gastrointestinal disease in the United States: 2012 update. *Gastroenterology* 2012; **143**: 1179-1187.e1-3 [PMID: 22885331 DOI: 10.1053/j.gastro.2012.08.002]

2 **Pant C**, Deshpande A, Olyaee M, Anderson MP, Bitar A, Steele MI, Bass PF, Sferra TJ. Epidemiology of acute pancreatitis in hospitalized children in the United States from 2000-2009. *PLoS One* 2014; **9**: e95552 [PMID: 24805879 DOI: 10.1371/journal.pone.0095552]

3 **Párniczky A**, Czakó L, Dubravcsik Z, Farkas G, Hegyi P, Hritz I, Kelemen D, Morvay Z, Oláh A, Pap Á, Sahin-Tóth M, Szabó F, Szentkereszti Z, Szmola R, Takács T, Tiszlavicz L, Veres G, Szücs Á, Lásztity N. [Pediatric pancreatitis. Evidence based management guidelines of the Hungarian Pancreatic Study Group]. *Orv Hetil* 2015; **156**: 308-325 [PMID: 25662148 DOI: 10.1556/OH.2015.30062]

4 **Hritz I**, Czakó L, Dubravcsik Z, Farkas G, Kelemen D, Lásztity N, Morvay Z, Oláh A, Pap Á, Párniczky A, Sahin-Tóth M, Szentkereszti Z, Szmola R, Szücs Á, Takács T, Tiszlavicz L, Hegyi P. [Acute pancreatitis. Evidence-based practice guidelines, prepared by the Hungarian Pancreatic Study Group]. *Orv Hetil* 2015; **156**: 244-261 [PMID: 25661970 DOI: 10.1556/OH.2015.30059]

5 **Morinville VD**, Husain SZ, Bai H, Barth B, Alhosh R, Durie PR, Freedman SD, Himes R, Lowe ME, Pohl J, Werlin S, Wilschanski M, Uc A. Definitions of pediatric pancreatitis and survey of present clinical practices. *J Pediatr Gastroenterol Nutr* 2012; **55**: 261-265 [PMID: 22357117 DOI: 10.1097/MPG.0b013e31824f1516]

6 **Working Group IAP/APA Acute Pancreatitis Guidelines**. IAP/APA evidence-based guidelines for the management of acute pancreatitis. *Pancreatology* 2013; **13**: e1-15 [PMID: 24054878 DOI: 10.1016/j.pan.2013.07.063]

7 **Maléth J**, Hegyi P. Ca2+ toxicity and mitochondrial damage in acute pancreatitis: translational overview. *Philos Trans R Soc Lond B Biol Sci* 2016; **371**: pii: 20150425 [PMID: 27377719 DOI: 10.1098/rstb.2015.0425]

8 **Maléth J**, Hegyi P, Rakonczay Z, Venglovecz V. Breakdown of bioenergetics evoked by mitochondrial damage in acute pancreatitis: Mechanisms and consequences. *Pancreatology* 2015; **15**: S18-S22 [PMID: 26162756 DOI: 10.1016/j.pan.2015.06.002]

9 **Hegyi P**, Petersen OH. The exocrine pancreas: the acinar-ductal tango in physiology and pathophysiology. *Rev Physiol Biochem Pharmacol* 2013; **165**: 1-30 [PMID: 23881310 DOI: 10.1007/112_2013_14]

10 **Hegyi P**, Pandol S, Venglovecz V, Rakonczay Z. The acinar-ductal tango in the pathogenesis of acute pancreatitis. *Gut* 2011; **60**: 544-552 [PMID: 20876773 DOI: 10.1136/gut.2010.218461]

11 **Venglovecz V**, Rakonczay Z, Ozsvári B, Takács T, Lonovics J, Varró A, Gray MA, Argent BE, Hegyi P. Effects of bile acids on pancreatic ductal bicarbonate secretion in guinea pig. *Gut* 2008; **57**: 1102-1112 [PMID: 18303091 DOI: 10.1136/gut.2007.134361]

12 **Hegyi P**. Bile as a key aetiological factor of acute but not chronic pancreatitis: a possible theory revealed. *J Physiol* 2016; **594**: 6073-6074 [PMID: 27800624 DOI: 10.1113/JP273108]

13 **Venglovecz V**, Hegyi P, Rakonczay Z, Tiszlavicz L, Nardi A, Grunnet M, Gray MA. Pathophysiological relevance of apical large-conductance Ca^{2+}-activated potassium channels in pancreatic duct epithelial cells. *Gut* 2011; **60**: 361-369 [PMID: 20940280 DOI: 10.1136/gut.2010.214213]

14 **Voronina SG**, Gryshchenko OV, Gerasimenko OV, Green AK, Petersen OH, Tepikin AV. Bile acids induce a cationic current, depolarizing pancreatic acinar cells and increasing the intracellular Na+ concentration. *J Biol Chem* 2005; **280**: 1764-1770 [PMID: 15536077 DOI: 10.1074/jbc.M410230200]

15 **Hegyi P**, Rakonczay Z. The role of pancreatic ducts in the pathogenesis of acute pancreatitis. *Pancreatology* 2015; **15**: S13-S17 [PMID: 25921231 DOI: 10.1016/j.pan.2015.03.010]

16 **Maléth J**, Balázs A, Pallagi P, Balla Z, Kui B, Katona M, Judák L, Németh I, Kemény LV, Rakonczay Z, Venglovecz V, Földesi I, Pető Z, Somorácz Á, Borka K, Perdomo D, Lukacs GL, Gray MA, Monterisi S, Zaccolo M, Sendler M, Mayerle J, Kühn JP, Lerch MM, Sahin-Tóth M, Hegyi P. Alcohol disrupts levels and function of the cystic fibrosis transmembrane conductance regulator to promote development of pancreatitis. *Gastroenterology* 2015; **148**: 427-439.e16 [PMID: 25447846 DOI: 10.1053/j.gastro.2014.11.002]

17 **Maléth J**, Hegyi P. Calcium signaling in pancreatic ductal epithelial cells: an old friend and a nasty enemy. *Cell Calcium* 2014; **55**: 337-345 [PMID: 24602604 DOI: 10.1016/j.ceca.2014.02.004]

18 **Criddle DN**. The role of fat and alcohol in acute pancreatitis: A dangerous liaison. *Pancreatology* 2015; **15**: S6-S12 [PMID: 25845855 DOI: 10.1016/j.pan.2015.02.009]

19 **Hegyi P**, Wilschanski M, Muallem S, Lukacs GL, Sahin-Tóth M, Uc A, Gray MA, Rakonczay Z, Maléth J. CFTR: A New Horizon in the Pathomechanism and Treatment of Pancreatitis. *Rev Physiol Biochem Pharmacol* 2016; **170**: 37-66 [PMID: 26856995 DOI: 10.1007/112_2015_5002]

20 **Pallagi P**, Venglovecz V, Rakonczay Z, Borka K, Korompay A, Ozsvári B, Judák L, Sahin-Tóth M, Geisz A, Schnúr A, Maléth J, Takács T, Gray MA, Argent BE, Mayerle J, Lerch MM, Wittmann T, Hegyi P. Trypsin reduces pancreatic ductal bicarbonate secretion by inhibiting CFTR Cl^- channels and luminal anion exchangers. *Gastroenterology* 2011; **141**: 2228-2239.e6 [PMID: 21893120 DOI: 10.1053/j.gastro.2011.08.039]

21 **Judák L**, Hegyi P, Rakonczay Z, Maléth J, Gray MA, Venglovecz V. Ethanol and its non-oxidative metabolites profoundly inhibit CFTR function in pancreatic epithelial cells which is prevented by ATP supplementation. *Pflugers Arch* 2014; **466**: 549-562 [PMID: 23948742 DOI: 10.1007/s00424-013-1333-x]

22 **Criddle DN**, Murphy J, Fistetto G, Barrow S, Tepikin AV, Neoptolemos JP, Sutton R, Petersen OH. Fatty acid ethyl esters cause pancreatic calcium toxicity via inositol trisphosphate receptors and loss of ATP synthesis. *Gastroenterology* 2006; **130**: 781-793 [PMID: 16530519 DOI: 10.1053/j.gastro.2005.12.031]

23 **Tenner S**, Baillie J, DeWitt J, Vege SS. American College of Gastroenterology guideline: management of acute pancreatitis. *Am J Gastroenterol* 2013; **108**: 1400-1415; 1416 [PMID: 23896955 DOI: 10.1038/ajg.2013.218]

24 **Yokoe M**, Takada T, Mayumi T, Yoshida M, Isaji S, Wada K, Itoi T, Sata N, Gabata T, Igarashi H, Kataoka K, Hirota M, Kadoya M, Kitamura N, Kimura Y, Kiriyama S, Shirai K, Hattori T, Takeda K, Takeyama Y, Hirota M, Sekimoto M, Shikata S, Arata S, Hirata K. Japanese guidelines for the management of acute pancreatitis: Japanese Guidelines 2015. *J Hepatobiliary Pancreat Sci* 2015; **22**: 405-432 [PMID: 25973947 DOI: 10.1002/jhbp.259]

25 **Petrov MS**, Whelan K. Comparison of complications attributable to enteral and parenteral nutrition in predicted severe acute pancreatitis: a systematic review and meta-analysis. *Br J Nutr* 2010; **103**: 1287-1295 [PMID: 20370944 DOI: 10.1017/S0007114510000887]

26 **Kalfarentzos F**, Kehagias J, Mead N, Kokkinis K, Gogos CA. Enteral nutrition is superior to parenteral nutrition in severe acute pancreatitis: results of a randomized prospective trial. *Br J Surg* 1997; **84**: 1665-1669 [PMID: 9448611]

27 **Abou-Assi S**, Craig K, O'Keefe SJ. Hypocaloric jejunal feeding is better than total parenteral nutrition in acute pancreatitis: results of a randomized comparative study. *Am J Gastroenterol* 2002; **97**: 2255-2262 [PMID: 12358242 DOI: 10.1111/j.1572-0241.2002.05979.x]

28 **Eckerwall GE**, Tingstedt BB, Bergenzaun PE, Andersson RG. Immediate oral feeding in patients with mild acute pancreatitis is safe and may accelerate recovery--a randomized clinical study. *Clin Nutr* 2007; **26**: 758-763 [PMID: 17719703 DOI: 10.1016/j.clnu.2007.04.007]

29 **Li J**, Xue GJ, Liu YL, Javed MA, Zhao XL, Wan MH, Chen GY, Altaf K, Huang W, Tang WF. Early oral refeeding wisdom in patients with mild acute pancreatitis. *Pancreas* 2013; **42**: 88-91 [PMID: 22836861 DOI: 10.1097/MPA.0b013e3182575fb5]

30 **Petrov MS**, McIlroy K, Grayson L, Phillips AR, Windsor JA. Early nasogastric tube feeding versus nil per os in mild to moderate acute pancreatitis: a randomized controlled trial. *Clin Nutr* 2013; **32**: 697-703 [PMID: 23340042 DOI: 10.1016/j.clnu.2012.12.011]

31 **Ruemmele FM**, Veres G, Kolho KL, Griffiths A, Levine A, Escher JC, Amil Dias J, Barabino A, Braegger CP, Bronsky J, Buderus S, Martín-de-Carpi J, De Ridder L, Fagerberg UL, Hugot JP, Kierkus J, Kolacek S, Koletzko S, Lionetti P, Miele E, Navas López VM, Paerregaard A, Russell RK, Serban DE, Shaoul R, Van Rheenen P, Veereman G, Weiss B, Wilson D, Dignass A, Eliakim A, Winter H, Turner D. Consensus guidelines of ECCO/ESPGHAN on the medical management of pediatric Crohn's disease. *J Crohns Colitis* 2014; **8**: 1179-1207 [PMID: 24909831 DOI: 10.1016/j.crohns.2014.04.005]

32 **Penagini F**, Dilillo D, Borsani B, Cococcioni L, Galli E, Bedogni G, Zuin G, Zuccotti GV. Nutrition in Pediatric Inflammatory Bowel Disease: From Etiology to Treatment. A Systematic Review. *Nutrients* 2016; **8**: pii: E334 [PMID: 27258308 DOI: 10.3390/nu8060334]

33 **Capurso G**, Zerboni G, Signoretti M, Valente R, Stigliano S, Piciucchi M, Delle Fave G. Role of the gut barrier in acute pancreatitis. *J Clin Gastroenterol* 2012; **46** Suppl: S46-S51 [PMID: 22955357 DOI: 10.1097/MCG.0b013e3182652096]

34 **Flint RS**, Windsor JA. The role of the intestine in the pathophysiology and management of severe acute pancreatitis. *HPB* (Oxford) 2003; **5**: 69-85 [PMID: 18332961 DOI: 10.1080/13651820310001108]

35 **Márta K**, Farkas N, Szabó I, Illés A, Vincze Á, Pár G, Sarlós P, Bajor J, Szűcs Á, Czimmer J, Mosztbacher D, Párniczky A, Szemes K, Pécsi D, Hegyi P. Meta-Analysis of Early Nutrition: The Benefits of Enteral Feeding Compared to a Nil Per Os Diet Not Only in Severe, but Also in Mild and Moderate Acute Pancreatitis. *Int J Mol Sci* 2016; **17**: pii: E1691 [PMID: 27775609 DOI: 10.3390/ijms17101691]

36 **Shamseer L**, Moher D, Clarke M, Ghersi D, Liberati A, Petticrew M, Shekelle P, Stewart LA; PRISMA-P Group. Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015: elaboration and explanation. *BMJ* 2015; **349**: g7647 [PMID: 25555855 DOI: 10.1136/bmj.g7647]

37 **Abu-El-Haija M**, Wilhelm R, Heinzman C, Siqueira BN, Zou Y, Fei L, Cole CR. Early Enteral Nutrition in Children With Acute Pancreatitis. *J Pediatr Gastroenterol Nutr* 2016; **62**: 453-456 [PMID: 26488122 DOI: 10.1097/MPG.0000000000001013]

38 **Szabo FK**, Fei L, Cruz LA, Abu-El-Haija M. Early Enteral Nutrition and Aggressive Fluid Resuscitation are Associated with Improved Clinical Outcomes in Acute Pancreatitis. *J Pediatr* 2015; **167**: 397-402.e1 [PMID: 26210842 DOI: 10.1016/j.jpeds.2015.05.030]

39 **Raizner A**, Phatak UP, Baker K, Patel MG, Husain SZ, Pashankar DS. Acute necrotizing pancreatitis in children. *J Pediatr* 2013; **162**: 788-792 [PMID: 23102790 DOI: 10.1016/j.jpeds.2012.09.037]

40 **Goh SK**, Chui CH, Jacobsen AS. Childhood acute pancreatitis in a children's hospital. *Singapore Med J* 2003; **44**: 453-456 [PMID: 14740774]

41 **Flores-Calderón J**, Exiga-Gonzaléz E, Morán-Villota S, Martín-Trejo J, Yamamoto-Nagano A. Acute pancreatitis in children with acute lymphoblastic leukemia treated with L-asparaginase. *J Pediatr Hematol Oncol* 2009; **31**: 790-793 [PMID: 19770681 DOI: 10.1097/MPH.0b013e3181b794e8]

42 **Wan X**, Wang W, Liu J, Tong T. Estimating the sample mean and standard deviation from the sample size, median, range and/or interquartile range. *BMC Med Res Methodol* 2014; **14**: 135 [PMID: 25524443 DOI: 10.1186/1471-2288-14-135]

43 **Morinville VD**, Barmada MM, Lowe ME. Increasing incidence of acute pancreatitis at an American pediatric tertiary care center: is greater awareness among physicians responsible? *Pancreas* 2010; **39**: 5-8 [PMID: 19752770 DOI: 10.1097/MPA.0b013e3181baac47]

44 **Lopez MJ**. The changing incidence of acute pancreatitis in children: a single-institution perspective. *J Pediatr* 2002; **140**:

622-624 [PMID: 12032533 DOI: 10.1067/mpd.2002.123880]
45 **Zsoldos F**, Párniczky A, Mosztbacher D, Tóth A, Lásztity N, Hegyi P. Pain in the Early Phase of Pediatric Pancreatitis (PINEAPPLE Trial): Pre-Study Protocol of a Multinational Prospective Clinical Trial. *Digestion* 2016; **93**: 121-126 [PMID: 26641250 DOI: 10.1159/000441352]
46 **Párniczky A**, Mosztbacher D, Zsoldos F, Tóth A, Lásztity N, Hegyi P. Analysis of Pediatric Pancreatitis (APPLE Trial): Pre-Study Protocol of a Multinational Prospective Clinical Trial. *Digestion* 2016; **93**: 105-110 [PMID: 26613586 DOI: 10.1159/000441353]

P- Reviewer: Cosen-Binker LI, Fujino Y, Luo HS, Peng SY, Sperti C
S- Editor: Gong ZM **L- Editor**: A **E- Editor**: Liu WX

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v23.i6.964

World J Gastroenterol 2017 February 14; 23(6): 964-975
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

Long-term culture-induced phenotypic difference and efficient cryopreservation of small intestinal organoids by treatment timing of Rho kinase inhibitor

Sung-Hoon Han, Sehwan Shim, Min-Jung Kim, Hye-Yun Shin, Won-Suk Jang, Sun-Joo Lee, Young-Woo Jin, Seung-Sook Lee, Seung Bum Lee, Sunhoo Park

Sung-Hoon Han, Sehwan Shim, Min-Jung Kim, Hye-Yun Shin, Won-Suk Jang, Sun-Joo Lee, Young-Woo Jin, Seung-Sook Lee, Seung Bum Lee, Sunhoo Park, Laboratory of Radiation Exposure and Therapeutics, National Radiation Emergency Medical Center, Korea Institute of Radiological and Medical Science, Seoul 01812, South Korea

Author contributions: Lee SB and Park S contributed equally to this work; Han SH performed the majority of experiments and analyzed the data; Shim S, Kim MJ, Shin HY and Jang WS participated in care/treatment of animals and performed the molecular biochemical investigations; Lee SJ, Jin YW and Lee SS designed and coordinated the research; Lee SB and Park S analyzed the data and wrote paper; all the authors contributed to this manuscript.

Supported by a grant of the Korea Institute of Radiological and Medical Sciences, funded by Ministry of Science, ICT and Future Planning, South Korea, No. 1711031810/50586-2016 and No. 1711031808/50581-2016.

Institutional review board statement: The study was revised and approved by the Korea Institute of Radiological and Medical Sciences Institutional Review Board.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Korea Institute of Radiological and Medical Sciences in Korea (IACUC protocol number: kirams2016-0043).

Conflict-of-interest statement: The authors declare that they have no potential conflicts of interest.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Correspondence to: Sunhoo Park, MD, PhD, Laboratory of Radiation Exposure and Therapeutics, National Radiation Emergency Medical Center, Korea Institute of Radiological and Medical Science, 75 Nowon-ro, Nowon-gu, Seoul 01812, South Korea. sunhoo@kirams.re.kr
Telephone: +82-2-9701274
Fax: +82-2-9702430

Received: September 5, 2016
Peer-review started: September 6, 2016
First decision: October 20, 2016
Revised: November 3, 2016
Accepted: November 23, 2016
Article in press: November 23, 2016
Published online: February 14, 2017

Abstract

AIM

To investigate a suitable long-term culture system and optimal cryopreservation of intestinal organoid to improve organoid-based therapy by acquiring large numbers of cells.

METHODS

Crypts were isolated from jejunum of C57BL/6 mouse. Two hundred crypts were cultured in organoid medium with either epidermal growth factor/Noggin/R-spondin1 (ENR) or ENR/CHIR99021/VPA (ENR-CV). For

subculture, organoids cultured on day 7 were passaged using enzyme-free cell dissociation buffer (STEMCELL Technologies). The passage was performed once per week until indicated passage. For cryopreservation, undissociated and dissociated organoids were resuspended in freezing medium with or without Rho kinase inhibitor subjected to different treatment times. The characteristics of intestinal organoids upon extended passage and freeze-thaw were analyzed using EdU staining, methyl thiazolyl tetrazolium assay, qPCR and time-lapse live cell imaging.

RESULTS

We established a three-dimensional culture system for murine small intestinal organoids using ENR and ENR-CV media. Both conditions yielded organoids with a crypt-villus architecture exhibiting $Lgr5^+$ cells and differentiated intestinal epithelial cells as shown by morphological and biochemical analysis. However, during extended passage (more than 3 mo), a comparative analysis revealed that continuous passaging under ENR-CV conditions, but not ENR conditions induced phenotypic changes as observed by morphological transition, reduced numbers of $Lgr5^+$ cells and inconsistent expression of markers for differentiated intestinal epithelial cell types. We also found that recovery of long-term cryopreserved organoids was significantly affected by the organoid state, *i.e.*, whether dissociation was applied, and the timing of treatment with the Rho-kinase inhibitor Y-27632. Furthermore, the retention of typical morphological characteristics of intestinal organoids such as the crypt-villus structure from freeze-thawed cells was observed by live cell imaging.

CONCLUSION

The maintenance of the characteristics of intestinal organoids upon extended passage is mediated by ENR condition, but not ENR-CV condition. Identified long-term cryopreservation may contribute to the establishment of standardized cryopreservation protocols for intestinal organoids for use in clinical applications.

Key words: Intestinal organoid; Rho kinase inhibitor; Three-dimensional culture; Cryopreservation; Long-term culture

Core tip: The phenotypes of intestinal organoids under epidermal growth factor/Noggin/R-spondin1 (ENR) medium were maintained over a long duration, whereas organoid under ENR/CHIR99021/VPA medium exhibited morphological change, reduced numbers of $Lgr5^+$ cells and inconsistent expression of markers for differentiated intestinal epithelial cell types upon extended passages. We also demonstrated an efficacious long-term cryopreservation method for intestinal organoids through optimization of the organoid state and timing of treatment with the Rho kinase inhibitor Y-27632. Thus, the suitable long-term culture system and optimal cryopreservation of small intestinal organoid may contribute to the establishment of standardized cryopreservation protocols for intestinal organoids and subsequent clinical applications of these cell sources.

Han SH, Shim S, Kim MJ, Shin HY, Jang WS, Lee SJ, Jin YW, Lee SS, Lee SB, Park S. Long-term culture-induced phenotypic difference and efficient cryopreservation of small intestinal organoids by treatment timing of Rho kinase inhibitor. *World J Gastroenterol* 2017; 23(6): 964-975 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i6/964.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i6.964

INTRODUCTION

The gastrointestinal (GI) tract is lined by a monolayer of epithelial cells that separates the intestinal lumen and underlying tissues. The epithelial cells of the small intestine are organized into villi and crypt structures. Intestinal stem cells (ISCs), which express leucine-rich repeat containing G protein-coupled receptor 5 (Lgr5), and their progenitors are located in crypts. ISCs generate daughter cells called transit-amplifying (TA) cells, which either return to stemness or differentiate into a secretory epithelial cells lineage, such as Paneth cells, goblet cells, enteroendocrine cells, or enterocytes[1,2]. The GI tract is highly vulnerable to the external environment, such as radiation. Exposure to high levels of ionizing radiation induces the clonogenic loss of crypt cells and villus depopulation and leads to malabsorption of nutrients and impaired physical barrier function. The resulting breach in the GI barrier, accompanied by immune suppression, results in a high risk of life-threatening infection[3-5]. Many studies have been focused on understanding the mechanisms of radiation-induced gastrointestinal syndrome (RIGS). However, *in vitro* analysis of RIGS has been hampered by the lack of a suitable culture system.

Long-term maintenance of crypts in traditional two-dimensional (2D) cultures of primary intestinal crypts is difficult due to the poor survival of crypts *in vitro*[6,7]. Based on three-dimensional (3D) culture systems, long-term cultures in which crypts are able to differentiate and recapitulate normal crypt-villus architecture have been established using crypts isolated from the mouse and human intestine using two different media[8,9]. Initial defined factors present in epidermal growth factor (EGF)/Noggin/R-spondin1 (ENR) medium, which are associated with growth requirements of intestinal epithelium, include EGF to enhance intestinal proliferation, bone morphogenic protein antagonists to induce expansion of crypt numbers, and Wnt agonists to increase crypt proliferation[8,10]. Additionally, small molecule screening showed

that ENR/CHIR99021/valproic acid (VPA) (ENR-CV) medium, which was associated with enrichment of intestinal stem cells ($Lgr5^+$) and growth of the intestinal epithelium, included a combination of ENR components and small molecules, such as CHIR99021 (a glycogen synthase kinase 3 inhibitor) and VPA [a histone deacetylase (HDAC) inhibitor][9]. Although both media can support the formation of organoid containing crypt-villus structures that recapitulate the native intestinal epithelium, there is little comparative study of the characteristics of the resulting cells, particularly after long-term continual passage.

In vitro expanded organoids have recently been applied to treat gastrointestinal diseases in preclinical models, supporting the establishment of potential organoid-based therapies for repairing damaged intestine[11,12]. Because clinical applications require large numbers of cells, it may be indispensable to *in vitro* expansion of organoids in long-term culture with retaining their initial characteristics. In addition, the cells should be capable of being preserved for prolonged periods, while maintaining cell functionality for off-the-shelf use. Cryopreservation may be an attractive technique for maintaining the functional properties and genetic characteristics of cells through long-term storage in order to facilitate the experimental and clinical applications of cell-based therapies[13-15]. However, although various methods have been developed for cryopreservation of different types of stem cells, such as mesenchymal, hematopoietic, and pluripotent stem cells[16-18], protocols for cryopreservation of intestinal organoids have not been described. Therefore, it is necessary to develop an efficient method for optimal cryopreservation of cultured organoids.

In the present study, we performed quantitative assessments to compare the characteristics (*e.g.*, cell morphological phenotype, proliferation, and composition of differentiated intestinal epithelial cell types) of small intestinal organoids subjected to long-term culture under two different media. We also sought to optimize the cryopreservation method by elucidation of the survival of cryopreserved small intestinal organoids through a combination of dissociation and treatment with a Rho kinase (ROCK) inhibitor during freezing. Our findings provided important insights into our understanding of 3D culture systems with similarities to the intestine and contribute to the establishment of standardized cryopreservation protocols for intestinal organoid for use in clinical applications.

MATERIALS AND METHODS

Isolation of small intestinal crypts from mice

All animal experiments were approved by the Animal Investigation Committee of the Korea Institute of Radiological and Medical Sciences in South Korea and were performed according to institutional guidelines and national animal protection laws. Isolation of small intestinal crypts from mice was conducted as described previously with some modifications[8]. Briefly, the jejunum (10 cm from the stomach) of C57BL/6 male mice (8-10 wk age, n = 4) was opened longitudinally, cut into 5-mm pieces, washed three times with cold phosphate-buffered saline (PBS), and incubated with 2 mmol/L ethylenediaminetetraacetic acid (EDTA) in PBS for 15 min at 37 ℃. After removal of EDTA solution, the supernatant containing villi was replaced with cold PBS. Crypts were isolated from the basal membrane by vigorous hand shaking for 1 min. This procedure was repeated until enriched crypts could be observed in the supernatant using microscopy. After collection of isolated crypts from tubes by centrifugation, the crypts were resuspended in 2% D-sorbitol (Sigma, St. Louis, MO, United States) in PBS, passed through a 70-μm cell strainer (BD Biosciences, Heidelberg, Germany), and centrifuged at 100 × g for 3 min at 4 ℃. The pellet was resuspended in 10 mL basic medium [advanced Dulbecco's modified Eagle's medium/F12, 2 mmol/L L-glutamine, 10 mmol/L HEPES, 100 mg/mL streptomycin, 100 U/mL penicillin, 1 mmol/L N-acetylcysteine, 1% B27, and N2 supplement], and crypt numbers were counted using microscopy.

3D culture of crypts and organoid passage

The isolated crypts were cultured in organoid medium with either ENR or ENR-CV, as previously reported[8,9]. Two hundred crypts in 50 μL matrigel (BD Biosciences) were seeded in each well of a pre-warmed 24-well flat-bottomed plate. Crypts were then incubated for 30 min at 37 ℃, and 500 μL of complete crypt culture medium was added. The ENR medium contained basic medium plus 50 ng/mL murine EGF (Invitrogen, Carlsbad, CA, United States), 100 ng/mL murine Noggin (Peprotech, Hamburg, Germany), and 500 ng/mL human R-spondin-1 (R&D Systems, Minneapolis, MN, United States), whereas the ENR-CV medium contained ENR medium plus 1 mmol/L valproic acid (Invitrogen) and 10 μmol/L CHIR99021 (Invitrogen). The crypts were cultured at 37 ℃ in an atmosphere containing 5% CO_2 for the indicated number of days. The medium was changed every 2-3 d. For subculture, the organoids cultured on day 7 were passaged using enzyme-free cell dissociation buffer (STEMCELL Technologies Inc., Vancouver, BC, Canada). Briefly, cultured organoids were washed with cold PBS, and 500 μL cell dissociation buffer was added to the wells and incubated for 5 min at 37 ℃. After washing with 0.1% BSA in PBS, dissociated organoids were passaged (a 1:5 ratio). Freshly prepared medium and Matrigel were then added for organoid culture. The passage of organoids cultured under ENR or ENR-CV medium was performed once per week until the indicated passage.

Cell proliferation and crypt viability

For analysis of cell proliferation in organoids by 5-ethynyl-2′-deoxyuridine (EdU) staining, the cultured organoids on the indicated day were incubated with fresh medium containing 10 μmol/L EdU (Molecular

Table 1 Primer sequences used in quantitative polymerase chain reaction analysis

Gene		Primer sequences (5′-3′)	Annealing temperatures (℃)
mLgr5	F	ACATTCCCAAGGGAGCGTTC	60
	R	ATGTGGTTGGCATCTAGGCG	
mLyz1	F	GCCAAGGTCTACAATCGTTGTGAGTTG	60
	R	CAGTCAGCCAGCTTGACACCACG	
mMuc2	F	ATGCCCACCTCCTCAAAGAC	60
	R	GTAGTTTCCGTTGGAACAGTGAA	
mChgA	F	CCCACTGCAGCATCCAGTT	60
	R	AGTCCGACTGACCATCATCTTTC	
mALP	F	AACTCACCTCATGGGCCTCTT	60
	R	GGGTTTCGGTTGGCATCATA	
mGAPDH	F	TCATCAACGGGAAGCCCATCAC	
	R	AGACTCCACGACATACTCAGCACCG	

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

Probes, Eugene, OR, United States) for 30 min and then fixed in 4% paraformaldehyde in PBS overnight at 4 ℃. The fixed organoids were permeabilized with 0.5% Triton X-100 for 1 h, and following steps were performed using a Click-iT EdU Imaging kit (Molecular Probes) according to the manufacturer's protocol. Hoechst (1:2000) was used for nuclear staining to facilitate cell counting. Images were acquired using an immunofluorescence microscope (Olympus, Shinjuku, Tokyo, Japan). For quantitative analysis of growing organoids, cultured crypts were examined at the indicated time point under bright-field of microscope. Organoids exhibiting at least two budding structures in each group were counted. Experiments were performed in triplicate. The data were expressed as the mean ± SD. For quantitative analysis of crypt viability after freezing and thawing, we performed methyl thiazolyl tetrazolium (MTT) assays as previously reported[19]. Briefly, on the indicated days, cultured organoids were incubated with 10% MTT (AMRESCO, Solon, OH, United States) for 2-3 h at 37 ℃. After cell lysis by treatment with 2% sodium dodecyl sulfate (SDS) and dimethyl sulfoxide (DMSO), the optical density (OD) value of the solution was measured at 562 nm using a Synergy HT (BioTek, Winooski, VT, United States). Experiments were performed in triplicate. The data were expressed as the mean ± SD.

Immunofluorescence staining

For immunofluorescence staining, cultured organoids were fixed in 4% paraformaldehyde in PBS overnight at 4 ℃. After washing with PBS, organoids were incubated with PBS containing 1% BSA and 0.5% Triton X-100 for 1 h at room temperature, followed by incubation with primary antibodies against lysozyme 1 (1:100; Abcam, Cambridge, MA, United States), mucin 2 (1:100; Dako, Carpenteria, CA, United States), or chromogranin A (1:200; Thermo Scientific). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG or anti-mouse IgG (1:100; Invitrogen) was used as a secondary antibody. Images were acquired using a Zeiss 710 confocal microscope (Carol Zeiss, Oberkochen, Germany) and analyzed by imaging software (Olympus America, Center Valley, PA, United States).

Quantitative real-time polymerase chain reaction

Total RNA was prepared from raw crypts (freshly isolated crypts from mice) and cultured crypts using an RNase mini kit (Qiagen, Valencia, CA, United States) according to the manufacturer's protocol. A total of 1 μg of RNA was reverse transcribed using an AccuPower RT PreMix kit (Bioneer, Seoul, South Korea). Real-time PCR was performed with FastStart Essential DNA Green Master Mix (Roche, Indianapolis, IN, United States). All reactions were performed in triplicate. mRNA expression was normalized to endogenous glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) expression and expressed relative to ENR-derived cells or raw crypts. The primers sequences are listed in Table 1.

Freezing-thawing of in vitro cultured organoids

For organoid cryopreservation, organoids cultured under ENR conditions were left intact or dissociated into single crypt-like colonies using enzyme-free cell dissociation buffer (STEMCELL Technologies Inc.). Undissociated and dissociated organoids were resuspended in freezing medium, *e.g.*, 10% DMSO and 10% fetal bovine serum or recovery cell culture freezing medium (RCCFM; Invitrogen). To determine the effects of Y-27632, a specific inhibitor of ROCK (STEMCELL Technologies Inc.) on the recovery of organoids, organoids were treated with the ROCK inhibitor for different times, including pretreatment for 30 min prior to freezing (before freezing), direct addition into freezing medium (during freezing), and post-thaw treatment for 3 d (after thawing). After storage in liquid nitrogen for 1-3 mo, vials were quickly thawed, and thawed organoids were then cultured for 7 d.

Time-lapse live cell imaging

Live cell imaging was performed on a JuLi stage system (NanoEnTek, Seoul, South Korea). A culture

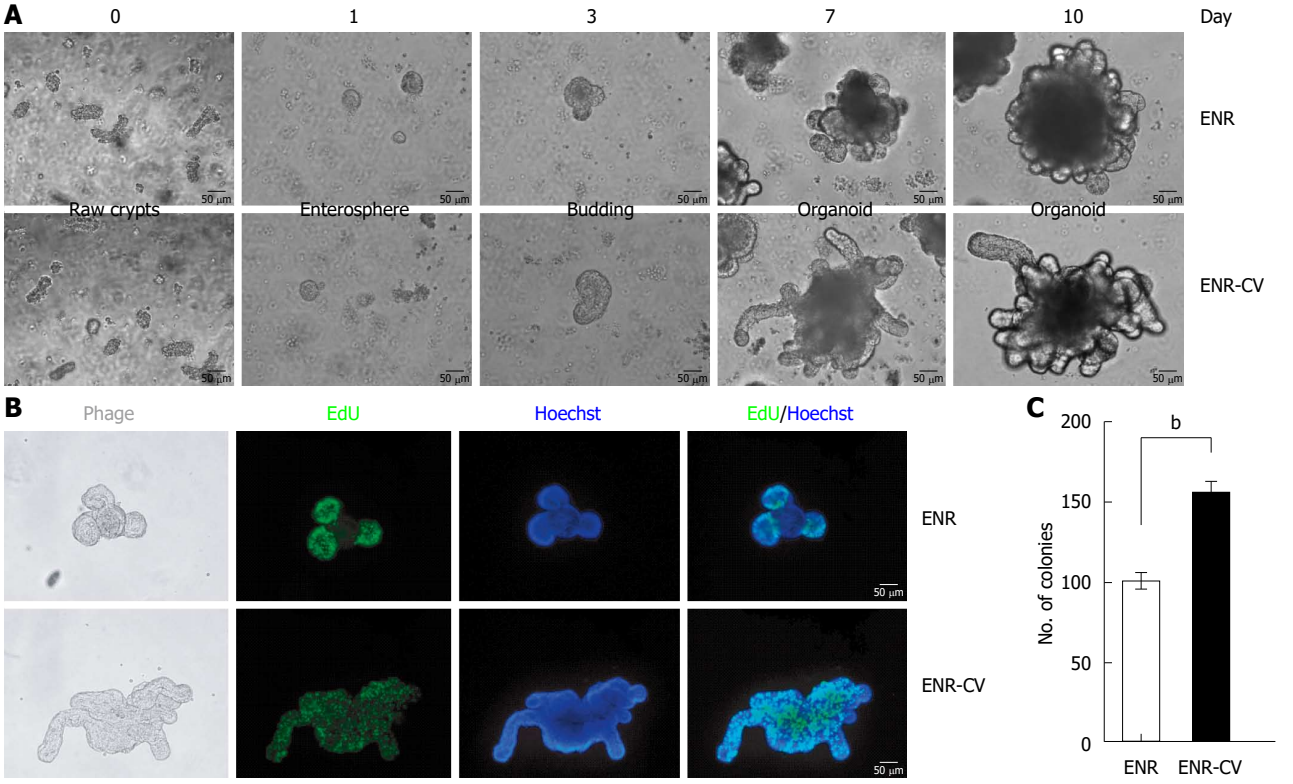

Figure 1 Establishment of small intestinal organoid culture under epidermal growth factor/noggin/r-spondin1 and epidermal growth factor/noggin/r-spondin1-/CHIR99021/VPA conditions. Crypts were isolated from the small intestines of C57/B6 mice at ages 9-12 wk and were resuspended in growth factor-reduced Matrigel. A: Time course of the growth of isolated crypts at passage 0 (P0) under two different culture media. Enterospheres formed on day 1, budding appeared on day 3, and robust budding was observed on days 5-10. Scale bars: 50 μm. B: Organoids were incubated with the thymidine analog EdU (green) for 1 h, and freshly isolated crypts were cultured for 6 d. Images were analyzed by fluorescence microscopy, and nuclei were double stained with Hoechst (blue). Scale bars: 50 μm. C: Numbers of organoids grown in two different media for 7 d. Organoids exhibiting at least two budding structures in each group were counted. The data are shown as means ± SDs of triplicate experiments ($^bP < 0.01$, Student's *t*-tests).

dish placed on the microscope stage was covered with a chamber in 5% CO_2 at 37 ℃. Images for the growth of crypts were an acquired at 60-min intervals. The data were processed using JuLi stage software v1.0 (NanoEnTek).

Animal care and use statement

All procedures involving were reviewed and approved by the Institutional Animal Care and Use Committee of the South Korea Institute of Radiological and Medical Sciences in Korea, and performed according to the Guidelines for Animal Experimentation of Korea Institute of Radiological and Medical Sciences. The animals were acclimatized to laboratory conditions (23 ℃ ± 1 ℃, 12 h/12 h light/dark, 50% ± 5% humidity and libitum access to food and water) for two, three or four weeks prior to experimentation. All appropriate protocols for study were taken to minimize pain and discomfort of animals.

Statistical analysis

Data are expressed as the mean ± SD or ± SEM of at least two independent samples. Statistical comparisons between groups were performed with two-tailed Student's *t*-tests or two-way analysis of variance (ANOVA) with Dunnett's T3 tests. Differences with *P* values of less than 0.05 were considered significant.

RESULTS

Establishment of a small intestinal organoid culture system using ENR and ENR-CV media

In an attempt to establish a conventional culture for intestinal organoids using two different conditions[8,9], freshly isolated crypts from the jejunum of C57/B6 mice were cultured in ENR or ENR-CV medium. Representative images of crypt growth into organoids are shown in Figure 1A. On day 1, crypts formed a round shape, called an enterosphere, and these structures became larger over time. Budding of enterospheres was observed beginning on day 3, and robust budding was observed on day 10, demonstrating a morphology typical of small intestinal organoids with a crypt-villus structure. We found that organoids cultured under ENR-CV conditions yielded increased budding length and size compared with those of organoids cultured under ENR conditions (Figure 1A). Consistent with the results of previous reports (Sato *et al*[8], 2009; Yin *et al*[9], 2014), ENR-based organoids exhibited proliferating cells within the crypt domains,

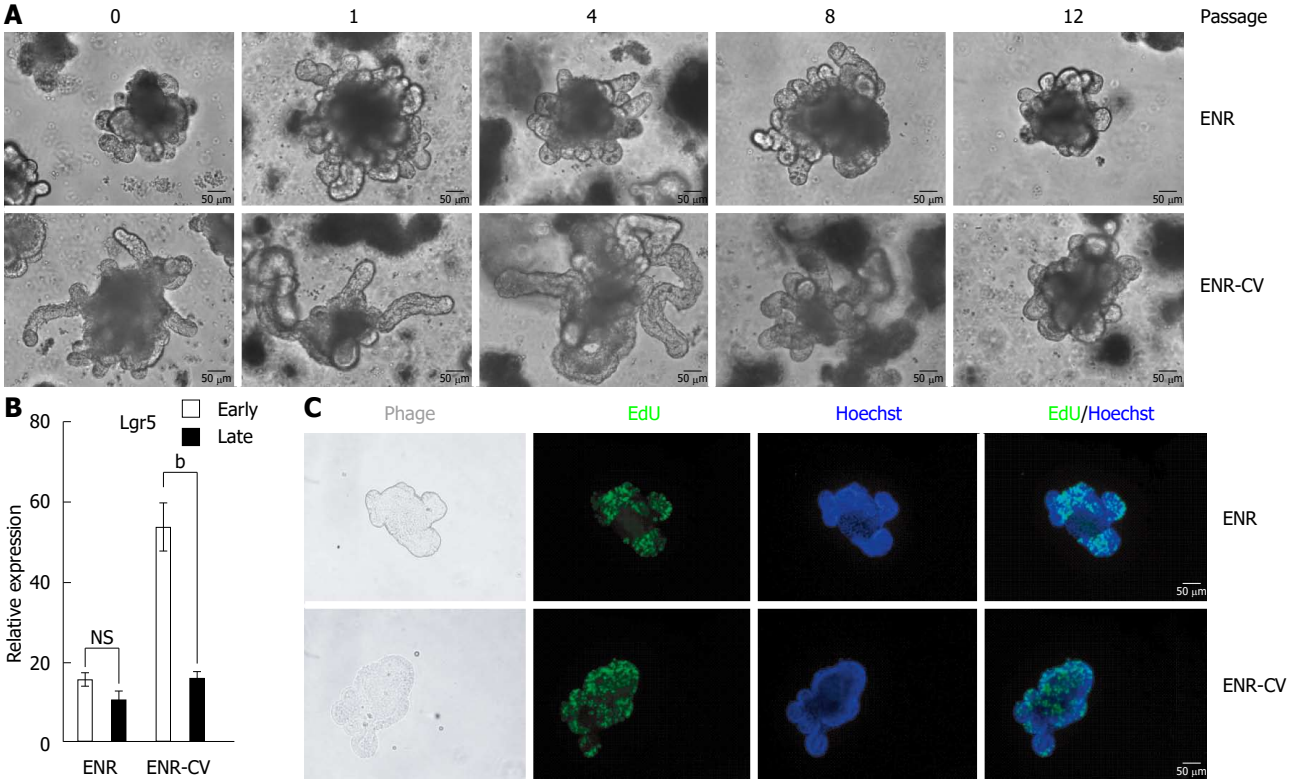

Figure 2 Phenotypic differences of intestinal organoids cultured under epidermal growth factor/noggin/r-spondin1-/CHIR99021/VPA or epidermal growth factor/noggin/r-spondin1 conditions upon continual passage. Organoids cultured for 7 d from freshly isolated crypt were split (1:4) and were cultured. Passage was performed once per week. A: Representative morphology of organoids cultured on day 7 under ENR or ENR-CV conditions upon continual passage. Scale bars; 50 μm. B: Quantitative real-time polymerase chain reaction analysis of relative mRNA expression levels of markers for intestinal stem cells (*Lgr5*) in organoids at early passage (P0-4) or late passage (P8-12) after culture for 6 d under ENR or ENR-CV conditions. *GAPDH* was used as an internal control. The data are shown as means ± SEMs of two independent experiments ($^bP < 0.01$, two-way analysis of variance with Dunnett's T3 tests) and normalized to the value for the ENR condition. Note that the mean of the sum from each passage with triplicate experiments in the indicated early and late passages was used. C: Organoids cultured on day 6 at late passage (P10) were incubated with the thymidine analog EdU (green) for 1 h. Images were analyzed by fluorescence microscopy. Nuclei were double stained with Hoechst (blue). Scale bars: 50 μm. *GAPDH:* Glyceraldehyde 3-phosphate dehydrogenase; ENR: Epidermal growth factor/Noggin/R-spondin1; ENR-CV: ENR/CHIR99021/VPA.

whereas proliferating cells were present throughout the organoids under ENR-CV conditions, as shown by EdU staining (Figure 1B). We further confirmed the effects of the ENR-CV medium on enhancement of cell proliferation within organoids by counting the numbers of organoids exhibiting at least two budding structures (Figure 1C).

Long-term culture induced phenotypic differences in organoids under ENR-CV culture conditions, but not ENR culture conditions

The two different types of medium used in this study have been shown to support long-term culture of intestinal organoids[8-10]. Thus, we aimed to confirm the long-term culture of organoids under our experimental conditions. As shown in Figure 2A, during continuous passage, the morphology of ENR-based organoids was constant, whereas the enhanced size and budding length of organoids in ENR-CV culture conditions were gradually diminished after passage 8 (P8). For a more extensive comparative analysis, we classified organoid into early phase (P0-4) and late phase (P8-12) based on morphological criteria, as shown in Figure 2A, and data are presented the mean of the sum of organoids from P0 to P4 or from P8 to P12 after performing two independent experiments with each passage.

To evaluate the characteristics of organoids at early and late passages, we analyzed the expression of Lgr5, known marker of ISCs[20], in organoids cultured in the two different media during continuous passage. At early passages, organoids cultured under ENR-CV conditions showed a dramatic increase in Lgr5 expression compared with that of organoids cultured under ENR conditions. These findings were consistent with a previous study showing that the expression level of Lgr5 was upregulated more than 3-fold in organoids cultured under ENR-CV conditions[9]. However, at later passages, Lgr5 expression under ENR-CV conditions was dramatically decreased to a level similar to that under ENR conditions. In contrast, Lgr5 expression levels in organoids cultured under ENR conditions were similar during both early and late passages (Figure 2B). Furthermore, reduced numbers of proliferating cells, which were generally positive for Lgr5, were observed in organoids cultured under ENR-CV conditions during the late phase, as observed by EdU staining (Figure

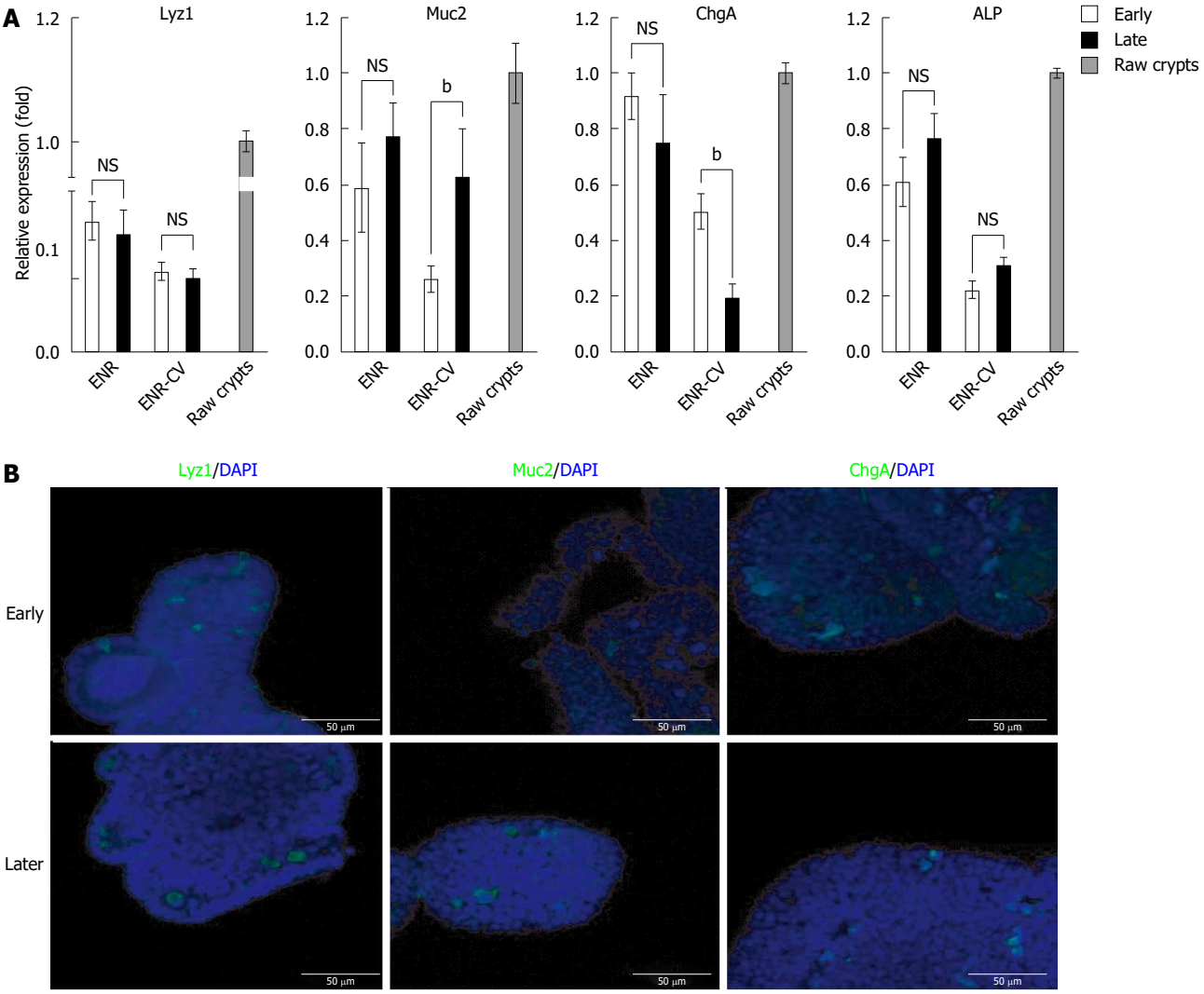

Figure 3 Comparison of the compositions of differentiated intestinal epithelial cells in long-term cultured intestinal organoids under two different media. A: Quantitative real-time polymerase chain reaction analysis of relative mRNA expression of markers for intestinal epithelial cells (*Muc2* for goblet cells, *ChgA* for enteroendocrine cells, *Alp* for enterocytes, and *Lyz1* for paneth cells) in organoids at early passage (P0-4) or late passage (P8-12) cultured for 6 d under ENR or ENR-CV conditions. *GAPDH* was used as an internal control. The data are shown as means ± SEMs of two independent experiments ($^{b}P < 0.01$, two-way analysis of variance with Dunnett's T3 test) and normalized to the ENR value. Note that the mean of the sum from each passage with triplicate experiments in the indicated early and late passages was used. Raw crypts: freshly isolated crypts from mice. B: Representative images show lysozyme (paneth cells), Muc2 (goblet cells), and ChgA (enteroendocrine cells) staining of organoids cultured for 6 d at passages 3 and 10 under ENR-CV conditions. Three-dimensional reconstructed confocal images are shown. Nuclei were double stained with DAPI (blue). Scale bars: 50 μm. *GAPDH:* Glyceraldehyde 3-phosphate dehydrogenase; ENR: Epidermal growth factor/Noggin/R-spondin1; ENR-CV: ENR/CHIR99021/VPA.

2C). Consistent with these results, we also detected decreased number of colonies in organoids cultured under ENR-CV conditions upon continual passage, but not in those cultured under ENR conditions, as shown by low-magnification observation of morphology and counting of organoid colonies (Supplementary Figure 1).

Next, we compared the compositions of intestinal epithelial cells in long-term cultured organoids under ENR and ENR-CV conditions. qPCR of intestinal epithelial marker expression showed that the expression levels of *Lyz* (a paneth cell marker), *Muc2* (a goblet cell marker), *ChgA* (an enteroendocrine cell marker), and *ALP* (an enterocyte marker) were low and unstable under ENR-CV conditions compared with that under ENR conditions, similar to the expression of epithelial markers in primary raw crypts (Figure 3A). Consistent with this, the result of immunostaining showed the reduced expression of some epithelial markers in organoids under ENR-CV conditions upon continual passage (Figure 3B). In contrast, no changes in these markers were observed in organoids cultured under ENR conditions (data not shown). Therefore, these findings suggested that ENR-CV culture conditions could be susceptible to phenotypic alterations in organoids upon extended passage and may be less relevant to the *in vivo* composition of intestine cell types.

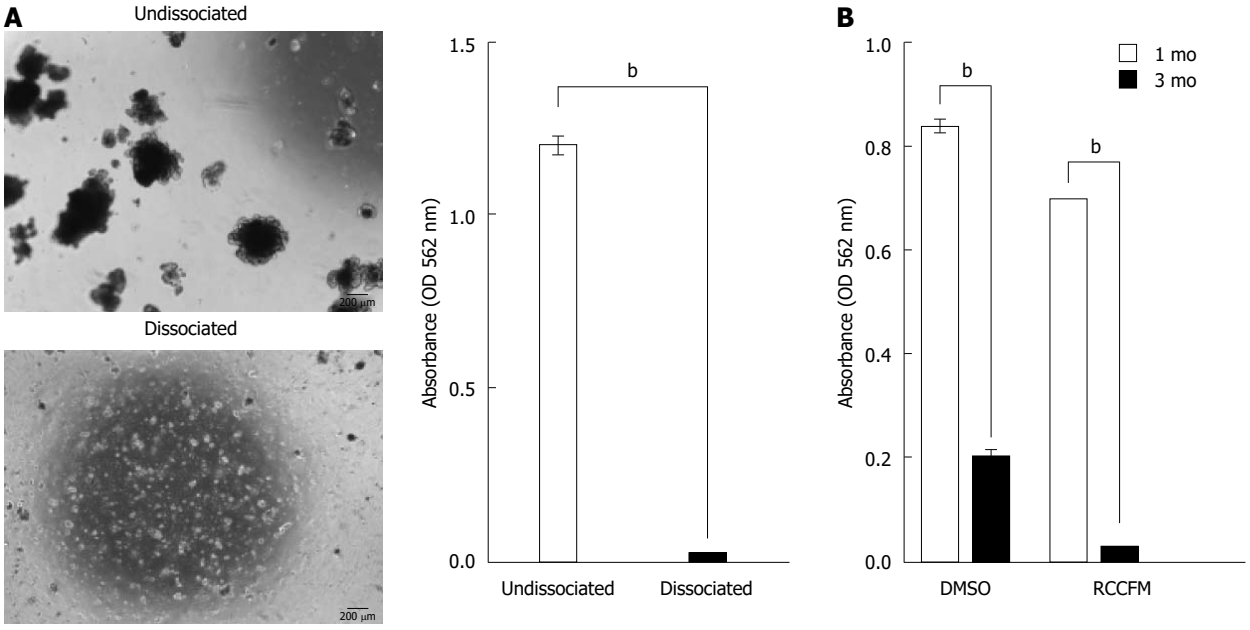

Figure 4 Low recovery of cryopreserved intestinal organoids upon long-term cryopreservation. Cultured organoids (P3) under ENR medium were subjected to dissociation or were left undissociated, followed by cryopreservation. A: Representative morphology (left panel) and quantification of recovery (right panel) for organoids on day 7 cultured under ENR conditions after thawing organoid cryopreserved for 1 mo in the presence of 10% DMSO as a cryoprotectant. Scale bars: 200 μm. The data are shown as the means ± SDs of triplicate MTT assays ($^bP < 0.01$, Student's *t*-tests). B: Quantification of recovery from cryopreserved organoid was performed using MTT assay. After 1 or 3 mo of cryopreservation with 10% DMSO or RCCFM (commercial freezing media), organoids were cultured for 7 d under ENR conditions. The data are shown as the mean ± SDs of triplicate experiments ($^bP < 0.01$, two-way analysis of variance with Dunnett's T3 tests). MTT: Methyl thiazolyl tetrazolium; ENR: Epidermal growth factor/Noggin/R-spondin1.

Direct addition of the ROCK inhibitor Y-27632 into freezing media was superior for the recovery of cryopreserved organoids without dissociation

Recent studies have reported the use of continuously cultured intestinal organoids to treat GI disease in mice[11,12], suggesting that organoid-based therapy may have applications in repairing damaged intestines. In order to improve therapeutic technologies, we have examined the optimal conditions for cryopreservation of organoids. To explore the cryopreservation of cultured intestinal organoids under ENR conditions, we first performed freezing-thawing of undissociated and dissociated organoids using 10% DMSO, a traditional cryopreservative[21]. After 1 mo, cryopreserved organoids were thawed in medium and incubated for 7 d. Organoids with a crypt-villus structure were visible from frozen stock only for undissociated organoids (Figure 4A), indicating that undissociated organoids showed better recovery from cryopreservation with 10% DMSO compared with that of dissociated organoids. Similar results were obtained from RCCFM (data not shown). We also extended the storage period of cryopreserved organoids up to 3 mo, which has been used for long-term cryopreservation in previous studies[21,22]. The viability of organoids was dramatically decreased, even in commercial freezing medium, in a time-dependent manner, as shown by MTT assays (Figure 4B).

The survival of various types of stem cells, including ISCs, is enhanced by ROCK inhibition during subculture[8,16]. In addition, ROCK activity and cytoskeletal phenotypes are almost completely inhibited by 10 μmol/L Y-27632[23]. Thus, in this study, we aimed to further optimize the cryopreservation of cultured organoids by examining the effects of Y-27632, a specific inhibitor of ROCK activity, on the recovery of organoids from cryopreserved stocks when added before freezing, during freezing, and after thawing of organoids. By evaluating the densities of grown organoids after freezing-thawing, we found that direct addition of Y-27632 into freezing medium during freezing resulted in superior recovery compared with that of untreated organoids, organoids pretreated with Y-27632, or organoids treated with Y-27632 after thawing (Figure 5A). Consistent with this, MTT analysis revealed that there was a higher rate of recovery from direct addition of Y-27632 during freezing (> 2.5 fold) upon cryopreservation, compare with that observed under other conditions (Figure 5B). We also observed similar effects of Y-27632 in commercial freezing medium when the drug was directly added during freezing (data not shown), and the typical organoid morphology with a crypt-villus structure was further confirmed by tracing the growth of organoids for 7 d after freezing-thawing, as shown by live-imaging analysis (Video data). In contrast to undissociated organoids, we did not observe improvements in dissociated organoids following treatment with Y-27632 (Supplementary Figure 2). Taken together, these results suggested that the recovery of cryopreserved intestinal organoids was significantly

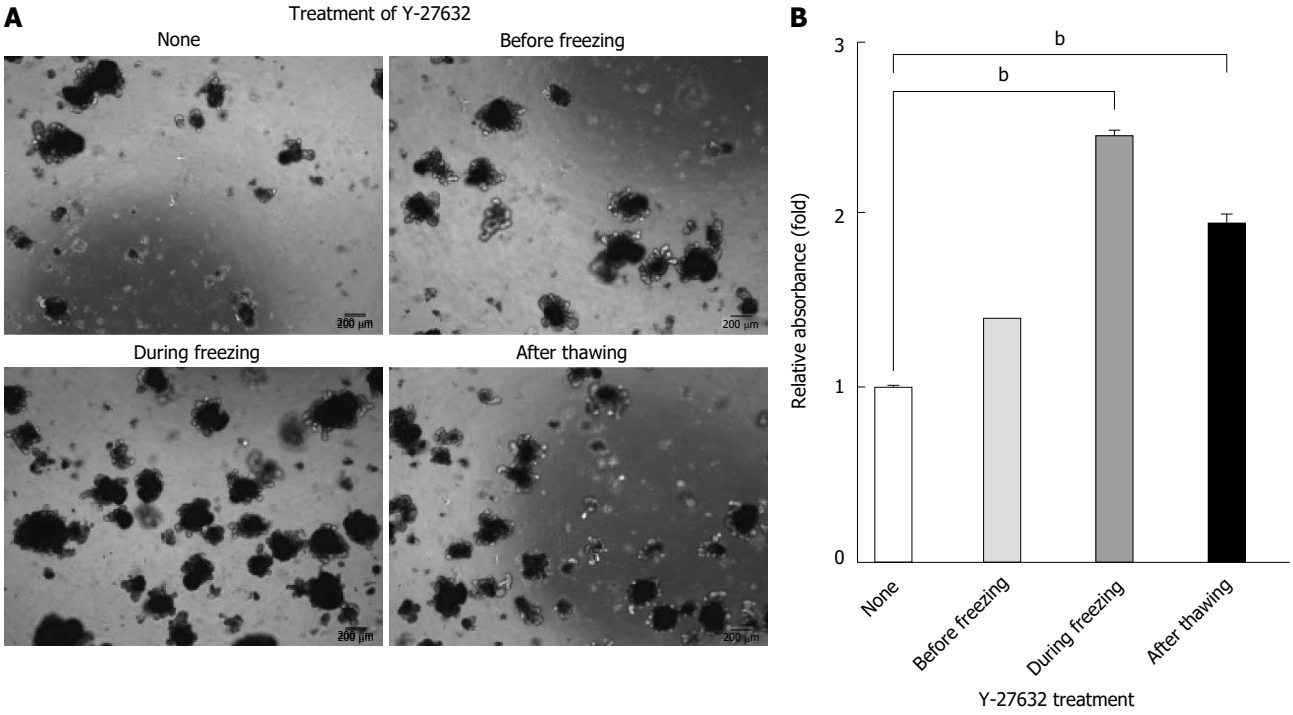

Figure 5 Enhanced recovery of long-term cryopreserved intestinal organoids was dependent on the timing of Y-27632 treatment. Undissociated organoids (P3) were treated with 10 μmol/L Y-27632 at the indicated time points. A: Representative morphology of organoids cultured on day 7 in the presence of ENR medium after thawing organoids cryopreserved for 3 mo in the presence of 10% DMSO. Scale bars, 200 μm. B: Quantification of recovery from cryopreserved organoids was performed using MTT assays. The data are shown as means ± SDs of triplicate experiments ($^bP < 0.01$, two-way analysis of variance with Dunnett's T3 tests) and normalized to untreated samples. MTT: Methyl thiazolyl tetrazolium; ENR: Epidermal growth factor/Noggin/R-spondin1.

improved when the ROCK inhibitor Y-27632 was used for treatment of undissociated organoids rather than dissociated organoids during freezing.

DISCUSSION

Previous studies reported that long-term culture of intestinal organoids could be supported through either ENR or ENR-CV medium in a 3D culture system with a Matrigel matrix[8,10,12] , and *in vitro* cultured intestinal organoids may have applications in organoid-based therapy as shown in studies investigating the repair of damaged intestines in mice[11,12]. Here, we have extended these studies to determine a suitable long-term culture system and optimal cryopreservation of small intestinal organoid. We found that the phenotypes of intestinal organoids under ENR media were maintained over a long duration, whereas organoid under ENR-CV media exhibited morphological alterations, reduced numbers of $Lgr5^+$ cells and inconsistent expression of markers for differentiated intestinal epithelial cell types upon extended passages. We also identified an efficacious cryopreservation method for expansion of undissociated intestinal organoids. For undissociated intestinal organoids, direct addition of the ROCK inhibitor Y-27632 during freezing permitted superior recovery of crypts after long-term cryopreservation.

Using established cultures of intestinal organoids under two different media, we confirmed that the characteristics of intestinal organoids under ENR-CV medium in the early passage were consistent with a previous report showing enrichment of $Lgr5^+$ expression, enhanced organoid size and budding length, and rapid proliferating cells[9]. However, upon extended passaging under ENR-CV conditions, but not ENR conditions, we observed phenotypic changes, such as reduced size and budding length of organoid, accompanied by reduced expression of Lgr5, an ISC marker, and upregulation of Muc2, a goblet cell marker (Figures 2 and 3). Although these findings are contradictory to the report by Yin *et al*[9] , who showed maintenance of $Lgr5^+$ stem cells during long-term passage, our findings were consistent with other reports demonstrating conversion of proliferating progenitors into secretory cells, along with loss of stem cells expressing Lgr5 in the context of inhibited Notch signaling[24]. The Notch signaling pathway contributes to enhancement of $Lgr5^+$ stem cell proliferation and suppresses the differentiation of these ISCs into secretory cells, such as goblet and enteroendocrine cells. In contrast, Wnt signaling is associated with the formation of paneth cells, which we found to be unaltered as shown by Figure 3A[25-27]. Thus, we analyzed the expression of Notch signaling-associated molecules, including Notch family members and Hes1, in ENR and ENR-CV cultured organoids upon extended passage. However, the gene expression patterns were similar for organoids cultured under both conditions (data not shown). This suggested that the Notch signaling pathway was not

involved in the observed changes under our culture conditions. Interestingly, although the expression of Lgr5 in intestinal organoids cultured in ENR-CV medium was reduced to a level similar to that of ENR-cultured intestinal organoids during late passages, indicating that these events may result from the reduced effects of small molecules, this relationship did not seem to be causal because the composition ratio of differentiated epithelial cells in long-term cultured intestinal organoids under ENR and ENR-CV conditions was not well correlated (Figure 3A). It is unclear why enhanced expression of Lgr5 was diminished upon continual passage in this study; however, a recent report demonstrated that loss of $Lgr5^+$ stem cells is often observed as an unexpected side effect in patients treated with HDAC inhibitors[28]. Therefore, it is likely that changes in the phenotype and composition ratio of functionally differentiated cells in intestinal organoids under ENR-CVin long-term culture may be attributed to prolonged treatment with valproic acid, a known HDAC inhibitor[29].

In order to determine the mechanisms underlying RIGS at the cellular level, in-depth characterization of intestinal epithelial cells within *in vitro* cultured intestinal organoids is necessary. A previous study compared the characteristics of these cells under two different media[9]. Moreover, our current findings further showed that both media could support the long-term culture of intestinal organoids, recapitulating the crypt-villus architecture *in vivo* with ISCs (Lgr5) and differentiated intestinal epithelial cells, consistent with previous reports[8,9]. Based on the comparative analysis in our study, including analysis of raw crypts, we found that the expression levels of most markers of differentiated intestinal epithelial cells in ENR-cultured organoid were higher than those in ENR-CV-cultured organoids, regardless of whether the organoids were cultured long term. Furthermore, upon continuous passaging, the expression levels of epithelial cell markers in intestinal organoids under ENR conditions were constant and similar to the expression levels of corresponding markers in primary raw crypts, suggesting that ENR conditions may be appropriate for long-term culture of intestinal organoids and that the characteristics of ENR culture were relevant to determining the *in vivo* composition of small intestine cell types. Given that the specialized cellular niche plays an important role in the maintenance of intestinal homeostasis by creating a unique environment *in vivo*[30], our data emphasized that the ENR-based intestinal organoid system may be useful for analysis of the mechanisms of radiation induced-intestinal cell death and that results obtained from the ENR-CV culture system, particularly for long-term culture, should be interpreted cautiously.

One of the most important findings in this study was that recovery of cryopreserved intestinal organoids was dependent on the timing of Y-27632 treatment and the absence of dissociation. We found that intact organoids, not dissociated organoids, were efficiently cryopreserved in the presence of 10% DMSO as standard components in slow-freezing protocols[15,21]. Among current cryopreservation methods, including slow or fast freezing (vitrification), conventional slow-freezing protocols are generally effective in presence of DMSO as a cryoprotectant, are less labor intensive, and allow for handling of bulk quantities of cells[15,31]. However, DMSO is known to be toxic to tissues and cells and is considered an appropriate cryoprotectant for short-term storage owing to its time-dependent toxicity[31]. Indeed, we observed that low survival rates after freeze-thaw of cryopreserved organoids following extended storage (Figure 4). Importantly, however, addition of Y-27632 at the time of freezing improved the recovery of freeze-thawed intestinal organoids. Although Y-27632 is known to be a potent inhibitor of apoptosis and to facilitate the survival of dissociated stem cells during subculture including ISCs[8,16,32], we did not observe efficient recovery of cryopreserved intestinal organoids when dissociated organoids were treated with ROCK inhibitor directly into the freezing medium (Supplementary Figure 2). These differences may be explained by the toxicity of DMSO, which varies from cell type to cell type during cryopreservation[31].

Our live-imaging data indicated the characteristics of long-term cryopreserved intestinal organoids by tracing the growth of organoids having a typical intestinal organoid phenotype with a crypt-villus structure. Further studies are required to determine whether subtle genetic alterations can be induced by cryopreservation with the ROCK inhibitor Y-27632. In the present study, undissociated intestinal organoids, but not dissociated organoids, were effectively cryopreserved and propagated after long-term cryopreservation by incorporating the ROCK inhibitor Y-27632 directly into the freezing medium.

In conclusion, using a comparative analysis of the characteristics of long-term cultured small intestinal organoids under two different culture conditions, we demonstrated that ENR-CV condition, but not ENR conditions, induced phenotypic transition in *in vitro* cultured small intestinal organoids upon extended passaging. We also identified an efficacious long-term cryopreservation method for intestinal organoids through optimization of the organoid state and timing of treatment with the ROCK inhibitor Y-27632. This method may contribute to the establishment of standardized cryopreservation protocols for intestinal organoids and subsequent clinical applications of these cell sources.

ACKNOWLEDGMENTS

The authors would like to thank Songwon Seo, Chief of the laboratory of Low Dose Risk Assessment, National Radiation Emergency Medical Center, Korea Institute of Radiological and Medical Science for his statistical support.

COMMENTS

Background

Recent studies have suggested that *in vitro* cultured intestinal organoids can be introduced to manage gastrointestinal diseases, supporting the development of promising organoid-based therapies for repair of damaged intestines. To improve organoid-based therapeutic technologies by acquiring large numbers of cells for clinical application, it is essential for long-term maintenance of characteristics and optimal cryopreservation method of intestinal organoid.

Research frontiers

Two different media [epidermal growth factor/Noggin/R-spondin1 (ENR) and ENR/CHIR99021/VPA (ENR-CV)] can support the formation of organoid containing crypt-villus structures that recapitulate the native intestinal epithelium. However, there is little comparative study of the characteristics of the resulting cells, particularly after long-term continual passage. In addition, it has not been well described for optimal cryopreservation methods for maintaining the functional properties of intestinal organoids in order to facilitate the experimental and clinical applications of organoid-based therapies.

Innovations and breakthroughs

This is the first study to report a continuous passages-induced phenotypic difference of intestinal organoid under ENR-CV condition, but not ENR condition which is suitable to long-term culture. The authors also demonstrate that efficient long-term cryopreservation of organoids is associated with a combination of organoid state and timing of treatment with the Rho kinase (ROCK) inhibitor.

Applications

This study provide important insights into our understanding of 3D culture systems for intestine-related organs and contribute to the establishment of standardized cryopreservation protocols for intestinal organoids on application of organoid-based therapy.

Peer-review

The manuscript by Han *et al* described that phenotypes of mouse intestinal organoids under ENR media were maintained over a long duration, and organoids under ENR-CV media exhibited morphological alterations. They also found that adding the ROCK inhibitor Y-27632 during freezing benefits recovery of undissociated intestinal organoids after long-term cryopreservation. The manuscript is succinct and the conclusions are well supported by the data.

REFERENCES

1 **Barker N**. Adult intestinal stem cells: critical drivers of epithelial homeostasis and regeneration. *Nat Rev Mol Cell Biol* 2014; **15**: 19-33 [PMID: 24326621 DOI: 10.1038/nrm3721]

2 **Clevers H**. The intestinal crypt, a prototype stem cell compartment. *Cell* 2013; **154**: 274-284 [PMID: 23870119 DOI: 10.1016/j.cell.2013.07.004]

3 **Baer AR**, Cheeseman CI, Thomson AB. The assessment of recovery of the intestine after acute radiation injury. *Radiat Res* 1987; **109**: 319-329 [PMID: 3027742 DOI: 10.2307/3576957]

4 **Shim S**, Jang WS, Lee SJ, Jin S, Kim J, Lee SS, Bang HY, Jeon BS, Park S. Development of a new minipig model to study radiation-induced gastrointestinal syndrome and its application in clinical research. *Radiat Res* 2014; **181**: 387-395 [PMID: 24786169 DOI: 10.1667/RR13207.1]

5 **Williams JP**, McBride WH. After the bomb drops: a new look at radiation-induced multiple organ dysfunction syndrome (MODS). *Int J Radiat Biol* 2011; **87**: 851-868 [PMID: 21417595 DOI: 10.3109/09553002.2011.560996]

6 **Grossmann J**, Maxson JM, Whitacre CM, Orosz DE, Berger NA, Fiocchi C, Levine AD. New isolation technique to study apoptosis in human intestinal epithelial cells. *Am J Pathol* 1998; **153**: 53-62 [PMID: 9665465 DOI: 10.1016/S0002-9440(10)65545-9]

7 **Kaeffer B**. Mammalian intestinal epithelial cells in primary culture: a mini-review. *In Vitro Cell Dev Biol Anim* 2002; **38**: 123-134 [PMID: 12026159 DOI: 10.1290/1071-2690(2002)038<0123:MIECIP>2.0.CO;2]

8 **Sato T**, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, van Es JH, Abo A, Kujala P, Peters PJ, Clevers H. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 2009; **459**: 262-265 [PMID: 19329995 DOI: 10.1038/nature07935]

9 **Yin X**, Farin HF, van Es JH, Clevers H, Langer R, Karp JM. Niche-independent high-purity cultures of Lgr5+ intestinal stem cells and their progeny. *Nat Methods* 2014; **11**: 106-112 [PMID: 24292484 DOI: 10.1038/nmeth.2737]

10 **Sato T**, Stange DE, Ferrante M, Vries RG, Van Es JH, Van den Brink S, Van Houdt WJ, Pronk A, Van Gorp J, Siersema PD, Clevers H. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* 2011; **141**: 1762-1772 [PMID: 21889923 DOI: 10.1053/j.gastro.2011.07.050]

11 **Shaker A**, Rubin DC. Stem cells: One step closer to gut repair. *Nature* 2012; **485**: 181-182 [PMID: 22575955 DOI: 10.1038/485181a]

12 **Yui S**, Nakamura T, Sato T, Nemoto Y, Mizutani T, Zheng X, Ichinose S, Nagaishi T, Okamoto R, Tsuchiya K, Clevers H, Watanabe M. Functional engraftment of colon epithelium expanded in vitro from a single adult Lgr5$^+$ stem cell. *Nat Med* 2012; **18**: 618-623 [PMID: 22406745 DOI: 10.1038/nm.2695]

13 **Davies OG**, Smith AJ, Cooper PR, Shelton RM, Scheven BA. The effects of cryopreservation on cells isolated from adipose, bone marrow and dental pulp tissues. *Cryobiology* 2014; **69**: 342-347 [PMID: 25127874 DOI: 10.1016/j.cryobiol.2014.08.003]

14 **Hubel A**. Parameters of cell freezing: implications for the cryopreservation of stem cells. *Transfus Med Rev* 1997; **11**: 224-233 [PMID: 9243775 DOI: 10.1053/tmrv.1997.0110224]

15 **Hunt CJ**. Cryopreservation of Human Stem Cells for Clinical Application: A Review. *Transfus Med Hemother* 2011; **38**: 107-123 [PMID: 21566712 DOI: 10.1159/000326623]

16 **Martin-Ibañez R**, Unger C, Strömberg A, Baker D, Canals JM, Hovatta O. Novel cryopreservation method for dissociated human embryonic stem cells in the presence of a ROCK inhibitor. *Hum Reprod* 2008; **23**: 2744-2754 [PMID: 18716037 DOI: 10.1093/humrep/den316]

17 **Son JH**, Heo YJ, Park MY, Kim HH, Lee KS. Optimization of cryopreservation condition for hematopoietic stem cells from umbilical cord blood. *Cryobiology* 2010; **60**: 287-292 [PMID: 20138169 DOI: 10.1016/j.cryobiol.2010.01.007]

18 **Yong KW**, Pingguan-Murphy B, Xu F, Abas WA, Choi JR, Omar SZ, Azmi MA, Chua KH, Wan Safwani WK. Phenotypic and functional characterization of long-term cryopreserved human adipose-derived stem cells. *Sci Rep* 2015; **5**: 9596 [PMID: 25872464 DOI: 10.1038/srep09596]

19 **Grabinger T**, Luks L, Kostadinova F, Zimberlin C, Medema JP, Leist M, Brunner T. Ex vivo culture of intestinal crypt organoids as a model system for assessing cell death induction in intestinal epithelial cells and enteropathy. *Cell Death Dis* 2014; **5**: e1228 [PMID: 24832600 DOI: 10.1038/cddis.2014.183]

20 **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 [PMID: 17934449 DOI: 10.1038/nature06196]

21 **Katkov II**, Kim MS, Bajpai R, Altman YS, Mercola M, Loring JF, Terskikh AV, Snyder EY, Levine F. Cryopreservation by slow cooling with DMSO diminished production of Oct-4 pluripotency marker in human embryonic stem cells. *Cryobiology* 2006; **53**: 194-205 [PMID: 16839540 DOI: 10.1016/j.cryobiol.2006.05.005]

22 **Lee YA**, Kim YH, Kim BJ, Kim BG, Kim KJ, Auh JH, Schmidt JA, Ryu BY. Cryopreservation in trehalose preserves functional capacity of murine spermatogonial stem cells. *PLoS One* 2013; **8**: e54889 [PMID: 23349986 DOI: 10.1371/journal.pone.0054889]

23 **Olson MF**. Applications for ROCK kinase inhibition. *Curr Opin*

Cell Biol 2008; **20**: 242-248 [PMID: 18282695 DOI: 10.1016/j.ceb.2008.01.002]

24 **Pellegrinet L**, Rodilla V, Liu Z, Chen S, Koch U, Espinosa L, Kaestner KH, Kopan R, Lewis J, Radtke F. Dll1- and dll4-mediated notch signaling are required for homeostasis of intestinal stem cells. *Gastroenterology* 2011; **140**: 1230-1240.e1-7 [PMID: 21238454 DOI: 10.1053/j.gastro.2011.01.005]

25 **Andreu P**, Peignon G, Slomianny C, Taketo MM, Colnot S, Robine S, Lamarque D, Laurent-Puig P, Perret C, Romagnolo B. A genetic study of the role of the Wnt/beta-catenin signalling in Paneth cell differentiation. *Dev Biol* 2008; **324**: 288-296 [PMID: 18948094 DOI: 10.1016/j.ydbio.2008.09.027]

26 **Farin HF**, Van Es JH, Clevers H. Redundant sources of Wnt regulate intestinal stem cells and promote formation of Paneth cells. *Gastroenterology* 2012; **143**: 1518-1529.e7 [PMID: 22922422 DOI: 10.1053/j.gastro.2012.08.031]

27 **VanDussen KL**, Carulli AJ, Keeley TM, Patel SR, Puthoff BJ, Magness ST, Tran IT, Maillard I, Siebel C, Kolterud Å, Grosse AS, Gumucio DL, Ernst SA, Tsai YH, Dempsey PJ, Samuelson LC. Notch signaling modulates proliferation and differentiation of intestinal crypt base columnar stem cells. *Development* 2012; **139**: 488-497 [PMID: 22190634 DOI: 10.1242/dev.070763]

28 **Zimberlin CD**, Lancini C, Sno R, Rosekrans SL, McLean CM, Vlaming H, van den Brink GR, Bots M, Medema JP, Dannenberg JH. HDAC1 and HDAC2 collectively regulate intestinal stem cell homeostasis. *FASEB J* 2015; **29**: 2070-2080 [PMID: 25648995 DOI: 10.1096/fj.14-257931]

29 **Dokmanovic M**, Clarke C, Marks PA. Histone deacetylase inhibitors: overview and perspectives. *Mol Cancer Res* 2007; **5**: 981-989 [PMID: 17951399 DOI: 10.1158/1541-7786.MCR-07-0324]

30 **Yen TH**, Wright NA. The gastrointestinal tract stem cell niche. *Stem Cell Rev* 2006; **2**: 203-212 [PMID: 17625256 DOI: 10.1007/s12015-006-0048-1]

31 **Thirumala S**, Goebel WS, Woods EJ. Clinical grade adult stem cell banking. *Organogenesis* 2009; **5**: 143-154 [PMID: 20046678 DOI: 10.4161/org.5.3.9811]

32 **Claassen DA**, Desler MM, Rizzino A. ROCK inhibition enhances the recovery and growth of cryopreserved human embryonic stem cells and human induced pluripotent stem cells. *Mol Reprod Dev* 2009; **76**: 722-732 [PMID: 19235204 DOI: 10.1002/mrd.21021]

P- Reviewer: Pan QW **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Liu WX

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v23.i6.976

World J Gastroenterol 2017 February 14; 23(6): 976-985
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

MicroRNA-155 promotes the pathogenesis of experimental colitis by repressing SHIP-1 expression

Zhan-Jun Lu, Jian-Jiong Wu, Wei-Liang Jiang, Jun-Hua Xiao, Kai-Zhong Tao, Lei Ma, Ping Zheng, Rong Wan, Xing-Peng Wang

Zhan-Jun Lu, Jian-Jiong Wu, Wei-Liang Jiang, Kai-Zhong Tao, Lei Ma, Rong Wan, Xing-Peng Wang, Department of Gastroenterology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200080, China

Jun-Hua Xiao, Ping Zheng, Department of Gastroenterology, Shanghai East Hospital, School of Medicine, Tong Ji University, Shanghai 200092, China

Author contributions: Lu ZJ and Wu JJ contributed equally to this study; Lu ZJ and Wu JJ performed the majority of experiments; Wang XP designed and supervised this research; Jiang WL and Xiao JH were responsible for part of molecular studies *in vitro*; while Tao KZ and Ma L conducted the establishment of animal models and treatment; Zheng P and Wan R did the data process and analyzation; Lu ZJ and Wu JJ wrote this paper.

Institutional review board statement: This study (2015KY154) was approved by the Institutional Review Board of Shanghai First People's Hospital Affiliated to Nanjing Medical University.

Institutional animal care and use committee statement: All protocols concerning laboratory animal usage in this study (2015KY154) were validated by the Animal Care Ethics Committee of Shanghai First People's Hospital.

Conflict-of-interest statement: The authors declare no conflict of interest.

Data sharing statement: Technical appendix, statistical code, and dataset are available from the corresponding author at richardwxp@163.com.

Manuscript source: Invited manuscript

Correspondence to: Xing-Peng Wang, MD, PhD, Professor, Department of Gastroenterology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, No 100, Haining Road, Shanghai 200080, China. richardwxp@163.com
Telephone: +86-21-63240090
Fax: +86-21-63241377

Received: June 29, 2016
Peer-review started: July 1, 2016
First decision: August 19, 2016
Revised: November 27, 2016
Accepted: December 16, 2016
Article in press: December 19, 2016
Published online: February 14, 2017

Abstract

AIM

To explore the mechanism by which microRNA-155 (miR-155) regulates the pathogenesis of experimental colitis.

METHODS

A luciferase assay was performed to confirm the binding of miR-155 to the SHIP-1 3′-UTR. MiR-155 mimics, negative controls and SHIP-1 expression/knockdown vectors were established and then utilized in gain- and loss-of-function studies performed in raw264.7 cells and primary bone marrow-derived macrophages (BMDMs). Thereafter, dextran sulfate sodium (DSS)-induced colitis mouse model with or without antagomiR-155 treatment was established, and the levels of miR-155 and SHIP-1, as well as the pro-inflammatory capabilities, were measured by western blot, quantitative polymerase chain reaction, and immunohistochemistry.

RESULTS

MiR-155 directly bound to the 3′-UTR of *SHIP-1* mRNA and induced a significant decrease in SHIP-1 expression in both raw264.7 cells and primary BMDMs. MiR-155 markedly promoted cell proliferation and pro-inflammatory secretions including IL-6, TNF-α, IL-1β, and IFN-γ, whereas these effects could be reversed by the restoration of SHIP-1 expression. *In vivo* studies showed that antagomiR-155 administration could alleviate DSS-induced intestinal inflammation in Balb/c mice. Moreover, significantly increased SHIP-1 expression, as well as decreased Akt activation and inflammatory response, were observed in the antagomiR-155-treated mice.

CONCLUSION

MiR-155 promotes experimental colitis by repressing SHIP-1 expression. Thus, the inhibition of miR-155 might be a promising strategy for therapy.

Key words: Experimental colitis; Inflammatory bowel disease; MicroRNA-155; SHIP-1

Core tip: Our present study identifies SHIP-1 as the functional target of microRNA-155 (miR-155) in macrophages. The up-regulation of miR-155 during colitis led to a significant decrease in SHIP-1 expression as well as a marked enhancement in cell proliferation and pro-inflammatory secretions, whereas the restoration of SHIP-1 expression partly reversed these changes. We further confirmed that antagomiR-155 treatment effectively alleviates dextran sulfate sodium-induced intestinal inflammation in mice, correlated with a significant elevation in SHIP-1 expression levels. Our findings indicate a novel mechanism by which miR-155 influences colitis progression.

Lu ZJ, Wu JJ, Jiang WL, Xiao JH, Tao KZ, Ma L, Zheng P, Wan R, Wang XP. MicroRNA-155 promotes the pathogenesis of experimental colitis by repressing SHIP-1 expression. *World J Gastroenterol* 2017; 23(6): 976-985 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i6/976.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i6.976

INTRODUCTION

Inflammatory bowel disease (IBD) is characterized by idiopathic, chronic, recurrent, inflammatory conditions of the human bowel triggered by multi-factorial causes that are not completely understood. IBD predominantly includes ulcerative colitis (UC) and Crohn's disease (CD)[1,2]. Although its etiology remains unclear, studies have indicated that the combination of the dysfunction of the intestinal mucosal immune system, the imbalanced constitution of the gut flora, genetic susceptibility and environmental factors all contribute to the pathogenesis of IBD[3,4]. Thus far, certain molecular changes in gene and protein expression patterns have been identified during the chronic inflammation process of IBD, and unraveling the molecular events involved in these intracellular signaling transduction pathways may be helpful for IBD diagnosis and treatment.

MicroRNAs are endogenous small non-coding RNAs that regulate gene expression by binding to the 3′-UTR of target messenger RNAs, either targeting the transcripts for degradation or blocking their translation[5,6]. MicroRNA-155 (miR-155), whose expression is induced by inflammatory cytokines and toll-like receptor ligands, has been reported to be involved in tissue development, immune responses, hematopoiesis, and a number of other important physiological functions[7-9]. Because the dysregulation of these same physiological functions is frequently observed in various inflammation or inflammation-induced human diseases[10-12], miR-155 has received a great deal of interest. Recent studies have demonstrated that miR-155 is up-regulated in both UC and CD patients[13,14]; conversely, its deficiency protects mice from experimental colitis[15], although the underlying mechanism stills needs to be elucidated.

Previous studies have proven that the 145-kDa protein Src homology 2 domain-containing inositol 5′-phosphatase-1 (SHIP-1) is a primary target of miR-155[16,17]. The direct repression of SHIP-1 by miR-155 has been demonstrated in many mammalian cell types[18,19]. In fact, the phenotype observed in mice over-expressing miR-155 is closely related to that of SHIP-1 knockout mice[16]. Ubiquitously expressed in hematopoietic cells, SHIP-1 is at the nexus of intracellular signaling pathways in immune cells that mediate the immune response, production of inflammatory and immunosuppressive cytokines, immunoregulatory cell formation, autoimmune diseases, and immune cancers[20-22]. For example, the PI3K-Akt pathway is a crucial intracellular signaling pathway that mediates many biological processes, and SHIP-1 negatively regulates the PI3K-Akt cascade through the dephosphorylation of PIP3[23]. Recent evidence has shown that SHIP-1 is significantly decreased in leukemias and lymphomas[24,25], as well as in some chronic inflammatory diseases such as clinical and experimental arthritis[26]. However, there are few reports on SHIP-1 and IBD, and the currently available studies give inconsistent or even opposing results. In this study, we sought to determine the detailed relationship between miR-155, SHIP-1, and the pathogenesis of IBD by *in vitro* studies using raw264.7 cells and primary bone marrow-derived macrophages (BMDMs) and by *in vivo* studies using an experimental colitis mouse model induced by dextran sulfate sodium (DSS).

MATERIALS AND METHODS

Cell culture, isolation, and lipopolysaccharide challenge

The raw264.7 cell line was obtained from the American Type Culture Collection and was maintained in low-glucose Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, United States) supplemented with 10% fetal bovine serum and 1% Pen/Strep. Cells were incubated at 37 ℃ and in 5% CO_2/95% air. BMDMs were isolated by flushing the femurs and tibias of Balb/c mice (female, 6-8 wk, Laboratory Animal Center, Chinese Academy of Sciences, Shanghai, China). Detailed procedures were performed as described previously[27]. BMDM phenotype and purity was determined by FACS analysis for macrophage specific antigen F4/80 (Abcam, United Kingdom). Before function studies, cells were exposed to *E. coli* lipopolysaccharide (LPS; 1 μg/mL; Sigma, St. Louis, MO, United States) for 24 h.

Vectors and cell transfection

MiR-155 mimics (UUAAUGCUAAUUGUGAUAGGGGU) and negative controls (CCUACGCCACCAAUUUCGU) were provided by GenePharma (Shanghai, China). To express the murine *SHIP-1* gene (*Inpp5d*), the coding sequence of *Inpp5d* was amplified from cDNA and was then subcloned into a pcDNA3.1 plasmid (Thermo Fisher Scientific, Waltham, MA), while silencing of SHIP-1 expression was achieved by designing a small-hairpin RNA targeting its coding sequence (shSHIP-1) and inserting this sequence into the vector. Transfection was performed in 6-well plates (5×10^6 cells/well), and the cells were mixed with Lipofectamine2000 reagent (Invitrogen, Carlsbad, CA). Cells were harvested 48 h after transfection for further analyses.

Induction of colitis and treatment

Forty pathogen-free female Balb/c mice were randomly separated into four groups (group 1, group 2, group 3, and group 4). Five mice per cage were maintained in an individual ventilated cage. All protocols concerning laboratory animal usage were submitted and validated by the Animal Care Ethics Committee of Shanghai First People's Hospital and Nanjing Medical University. Groups 2, 3, and 4 were treated by oral administration of 4.0% (w/v) DSS (MP Biomedicals, Aurora, OH) dissolved in drinking water for 7 d, while group 1 was used as the control group and given normal drinking water. On day 2 and day 5, mice in group 4 were treated with antagomiR-155 (GenePharma) by tail vein injection at doses of 45 mg/kg in 100 μL volumes. Meanwhile, group 3 was treated with a negative control (GenePharma) and group 2 was untreated. The sequences of antagimiR-155 and the negative control were as follows: antagomiR-155: 5'-AsCsCCCUAUCACAAUUAGCAUsUsAsAs-Cholesterol-3'; negative control: 5'-UsUsUGUACUACACAAAAGUAsCsUsGs-Cholesterol-3'.

During the induction phase, weight loss, stool character and bleeding were recorded daily to monitor the disease activity, and the disease activity index (DAI) was determined as previously described[28]. Mice were sacrificed under deep anesthesia at the end of day 7. The colon tissues were stored in 10% buffered formalin or at -80 ℃ in liquid nitrogen after the colon length was measured and photographed.

Histological evaluation and immunohistochemistry

Histological examination of the distal colon was performed on paraffin-embedded sections by hematoxylin-eosin (HE) staining. The inflammatory damage score was determined as previously described[29] and was the sum of inflammation infiltrations, depth of lesions, destruction of crypt, and width of lesions. Immunohistochemistry for SHIP-1 was performed using the peroxidase-conjugated avidin-biotin method. Deparaffinized and rehydrated sections were incubated with rabbit polyclonal anti-SHIP-1 (1:300, Santa Cruz, CA, United States) followed by biotinylated secondary antibody (Mai Bio, Shanghai, China). Positive staining was indicated by gray and brown particles. Ten visual fields (× 400 magnification) were chosen randomly in each section for evaluation of stained cells. The final score was the product of the number of stained cells and staining intensities. Detailed counting methods are listed in Supplementary Table 1.

Quantitative real-time RT-PCR

Total RNA was extracted from cells or tissues by the TriPure Reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. Reverse transcription was performed using the Transcriptor First Strand cDNA Synthesis kit (Roche). The single-stranded cDNA served as the template for SYBR real-time polymerase chain reaction (PCR) using SYBR-Green PCR Master Mix (Takara Bio, Kyoto, Japan). All reactions were run in triplicate on the MasterCycler Real-Time PCR Detection System (Eppendorf, Hamburg, Germany). Supplementary Table 2 lists all primer sequences used in the study. The fold change of gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. The expression level of miR-155 was normalized to U6 snRNA, and the expression levels of other genes were normalized to *GAPDH*.

Western blot assay

Cells or colon tissues (stored at -80 ℃) were harvested and extracted using the lysis buffer, and an equal amount of protein was separated on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. Separated protein bands were transferred into PVDF membranes and then blocked in 5% skim milk powder. The primary antibody against SHIP-1 (Santa Cruz) was diluted according to the manufacturer's instructions and incubated with the membrane overnight at 4 ℃, followed by incubation with secondary antibodies (1:1000 dilution; Mai Bio) at room temperature for 2 h. The immunoreactive bands were visualized using

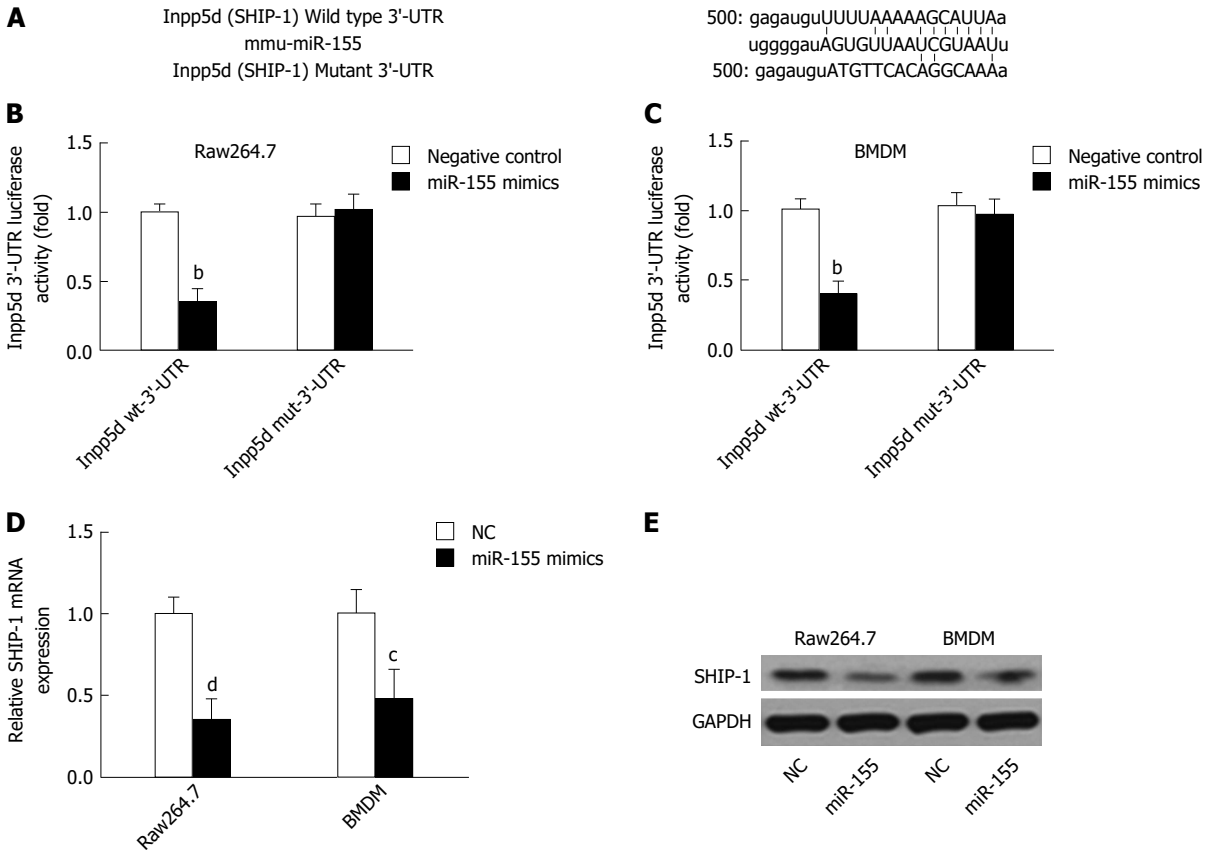

Figure 1 SHIP-1 is targeted by microRNA-155 in mouse macrophages. A: A mouse Inpp5d 3'-UTR fragment containing the wild-type or mutant miR-155-binding sequence was cloned downstream to the luciferase reporter gene; B and C: The luciferase activity of Inpp5d 3'-UTR in raw264.7 cells (B) or primary mouse bone marrow-derived macrophages (BMDMs) (C) after transfection with miR-155 mimics or negative controls. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the wild-type Inpp5d 3'-UTR luciferase activity in cells transfected with NCs; D: The mRNA level of SHIP-1 was significantly down-regulated in raw 254.7 cells and BMDMs after cells were transfected with miR-155 mimics. $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* controls. B: The protein level of SHIP-1 also reduced after miR-155 mimics transfection.

an ECL-PLUS kit (Piscataway, NJ). The relative protein expression levels were normalized to GAPDH.

Luciferase reporter assay

The murine *Inpp5d* target site and its mutant version were amplified by primers. The target site was predicted by three databases (miRBase, PicTar and miRanda). The PCR products were cloned downstream of the luciferase gene in psiCHECK-2 luciferase vector (Promega, WI, United States). The constructs were transfected together with miR-155 mimics or the negative controls. Luciferase activity was measured using the Dual-Luciferase Reporter Assay (Promega) 24 h after transfection. Each treatment was performed in triplicate.

Cell proliferation assay

Cell proliferation was analyzed using an MTT assay. Briefly, 1×10^3 cells per well were seeded into a 96-well plate and incubated for three days. At the indicated time point, 20 μL of MTT (5 mg/mL) (Sigma-Aldrich) was added into each well and incubated for 4 h. Then, the supernatants were removed and 150 μL of DMSO (Sigma-Aldrich) was added to terminate the reaction. The absorbance value (OD) was measured at 570 nm.

Enzyme-linked immunosorbent assay

The levels of TNF-α, IL-6, IL-1β and IFN-γ in cell lysate supernatants were measured using corresponding enzyme-linked immunosorbent assay (ELISA) kits (Mai Bio) according to the manufacturer's instructions.

Statistical analysis

SPSS 21.0 and GraphPad Prism 5 were used for statistical analyses and the rendering of figures. One-way analysis of variance (ANOVA) was used to analyze the differences between groups. The Student-Newman-Keuls method of multiple comparisons was used when the ANOVA analysis resulted in statistical significance. Data are expressed as the means ± SD. Statistical significance was set at $P < 0.05$.

RESULTS

MiR-155 directly targets the 3'-UTR of SHIP-1 and inhibits its expression in murine macrophages

Because SHIP-1 is a well-established target of miR-155, we first performed a dual-luciferase reporter assay by constructing luciferase reporter constructs containing the wild-type or mutant SHIP-1 3'-UTR and co-transfecting them with miR-155 mimics or negative controls into cells (Figure 1A). We found that miR-155

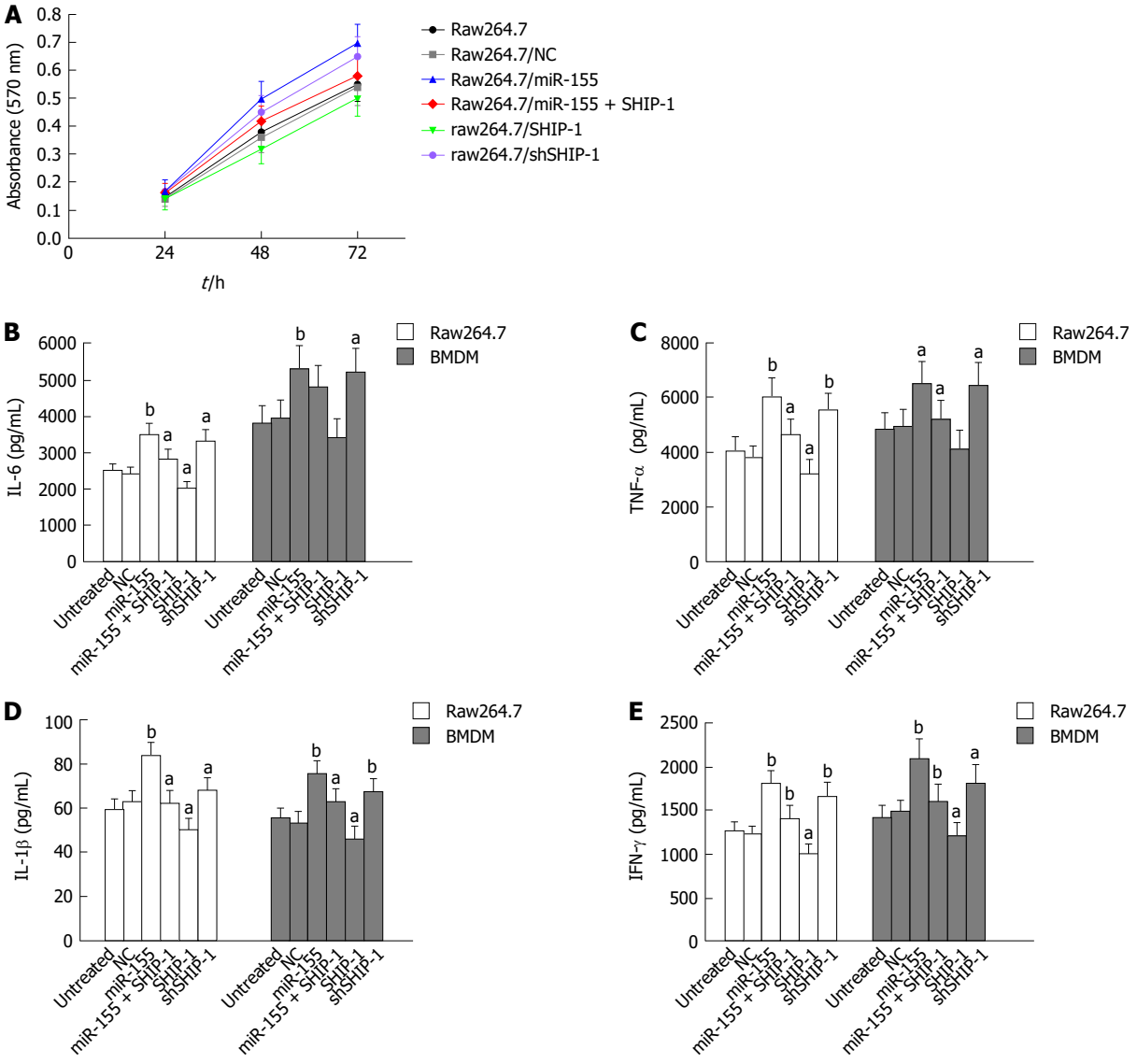

Figure 2 Effects of microRNA-155 and its target SHIP-1 on cell proliferation and pro-inflammatory capabilities. A: The proliferation rate of raw264.7 cells transfected with miR-155 mimics, the negative controls, miR-155 mimics + SHIP-1 vectors, SHIP-1 expression vectors, or SHIP-1 knockdown vectors was detected by MTT assay. $^{a}P < 0.05$ indicated there was significant difference between groups. B-E: ELISA analyses of the secretion of IL-6 (B), TNF-α (C), IL-1β (D), and IFN-γ (E) in both the raw264.7 cells and mouse bone marrow-derived macrophages (BMDMs) and the respective cells transfected with miR-155 mimics, the negative controls, miR-155 mimics + SHIP-1 vectors, SHIP-1 expression vectors, or SHIP-1 knockdown vectors. Comparison was conducted between groups: Raw264.7/miR-155, raw264.7/SHIP-1, raw264.7/shSHIP-1 *vs* Raw264.7, Raw264.7/NC; Raw264.7/miR-155+SHIP-1 *vs* Raw264.7/miR-155. $^{a}P < 0.05$, $^{b}P < 0.01$.

directly bound to the wild-type but not the mutant 3′-UTR of *SHIP-1 mRNA* and caused the significant reduction of luciferase activities in both the murine macrophage cell line raw264.7 and the primarily isolated BMDM cells (Figure 1B and C). Then, we focused on the expression of SHIP-1 in miR-155 over-expressing raw264.7 cells and BMDMs. As shown in Figure 1D and E, both the mRNA and the protein levels of SHIP-1 were significantly decreased after cells were transfected with 60 nmol/L miR-155 mimics, which confirmed that SHIP-1 is the direct target of miR-155 in murine macrophages.

Effects of miR-155 and SHIP-1 on the cell proliferation and pro-inflammatory secretion of murine macrophages

An MTT assay was performed to test the effects of miR-155 and its target SHIP-1 on the proliferation of raw264.7 cells. Cell proliferation was significantly elevated following the over-expression of miR-155 and was decreased after the up-regulation of SHIP-1 (Figure 2A). ELISA analysis was then conducted to determine whether miR-155 and SHIP-1 affected the pro-inflammatory secretions of LPS-stimulated raw264.7 cells and primary BMDMs. After exposure to LPS (1 μg/mL) for 24 h, both the raw264.7 cells and BMDMs showed remarkable secretion levels of IL-6, TNF-α, IL-1β, and IFN-γ, which represent the most important pro-inflammatory cytokines in IBD. The cells that over-expressed miR-155 exhibited the highest levels of secretion of these factors, while SHIP-1 restoration could inhibit the over-production of IL-6, TNF-α, IL-1β, and IFN-γ in these two cell types (Figure

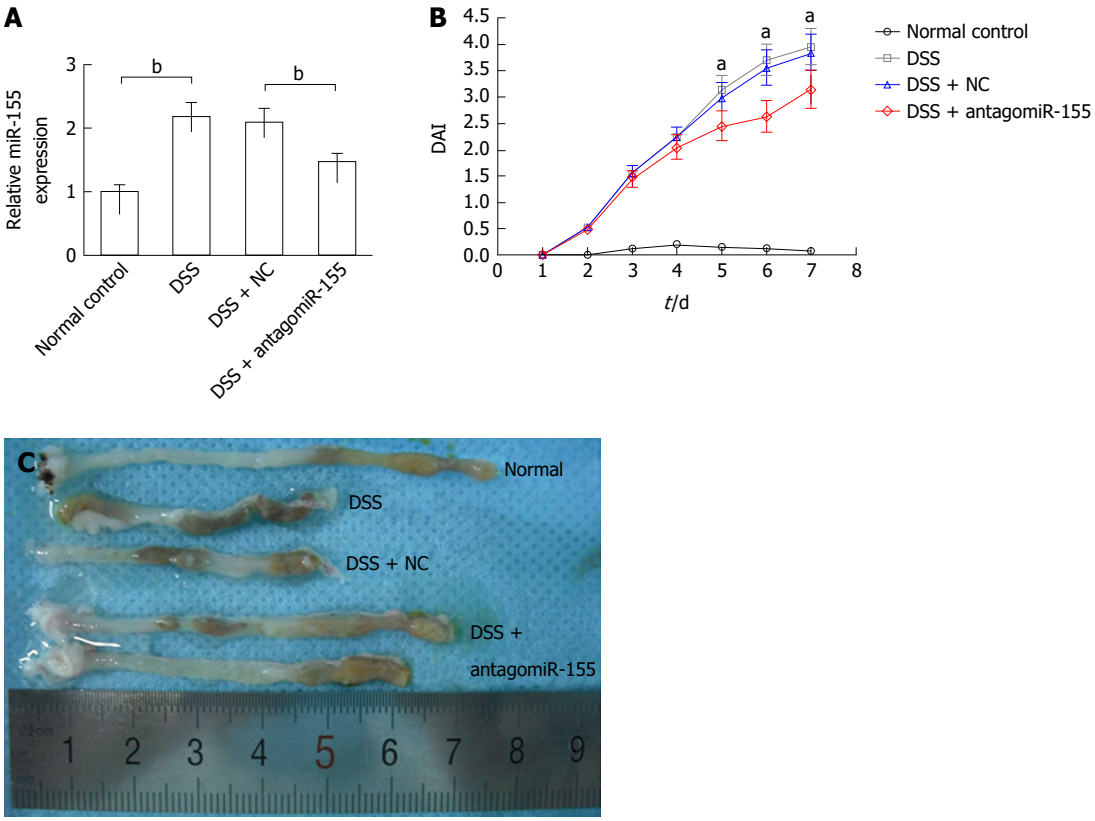

Figure 3 Inhibition of microRNA-155 alleviated mouse experimental colitis. A: AntagomiR-155 treatment significantly reduced the expression level of miR-155 in colon tissues. $^{b}P < 0.01$, dextran sulfate sodium (DSS) *vs* control, DSS + antagomiR-155 *vs* DSS + NC; B: Inhibition of miR-155 markedly decreased the disease activity index of experimental colitis. $^{a}P < 0.05$, DSS + antagomiR-155 *vs* DSS + NC, DSS; C: AntagomiR-155 treatment alleviated the shortening of colon induced by DSS.

2B-E). These results indicate that miR-155 serves its pro-inflammatory function by repressing SHIP-1 expression in macrophages.

Inhibition of miR-155 significantly alleviates murine intestinal inflammation induced by DSS

DSS-induced experimental colitis was established in Balb/c mice to determine the role of miR-155 and SHIP-1 *in vivo*. On day 2 and day 5, one group of mice was injected with 45 mg/kg antagomiR-155 through the tail vein, while another group was treated with the same dose of negative controls. Our PCR analysis confirmed that the level of miR-155 in murine colons was significantly reduced by antagomiR-155 treatment (Figure 3A). During the 7-d DSS induction, changes in body weight, occult blood, and gross bleeding were assessed and scored for the determination of DAI scores. As shown in Figure 3B, the DSS-treated groups exhibited higher DAI scores compared to the normal distilled water-treated group. In the three DSS-treated groups, it was observed that the mice that received antagomiR-155 injection exhibited significantly lower DAI compared with the mice that received random antagomiR treatment and the untreated mice. Additionally, the colon length of DSS-treated mice was markedly shortened compared to controls (8.23 ± 1.35 cm *vs* 5.45 ± 1.29 cm, $P < 0.05$), and the inhibition of miR-155 could partly abate such shortening (6.82 ± 1.41 cm *vs* 8.23 ± 1.35 cm, $P > 0.05$) (Figure 3C).

Thereafter, we evaluated the histological changes in colon sections by HE staining and found that the DSS-treated mice exhibited the typical characteristics of intestinal inflammation compared with the normal control mice (Figure 4A). The mice co-treated with antagomiR-155 displayed remarkably reduced levels of colon inflammation, including neutrophil infiltration, epithelial damage, depletion of goblet cells, and distortion of crypt architectures, compared to the mice treated with only DSS or the mice treated with DSS and random antagomiRs (Figure 4B and C).

Inhibition of miR-155 leads to increased SHIP-1 expression and decreased inflammatory responses in experimental colitis

We performed an expression analysis of SHIP-1 in distal colon tissues and found that both the RNA and protein levels of SHIP-1 were significantly increased with antagomiR-155 administration (Figure 5A and B), whereas activity of its major functional target, the Akt signaling pathway, was decreased due to the enhancement of the negative regulation of SHIP-1 upon p-Akt activation (Figure 5B). IHC analysis showed that SHIP-1 was mainly expressed in lymphocytes, neutrophils, and other hematopoietic cells in the inflamed mucosa (Figure 5C). Similarly, the mice co-treated with antagomiR-155 exhibited

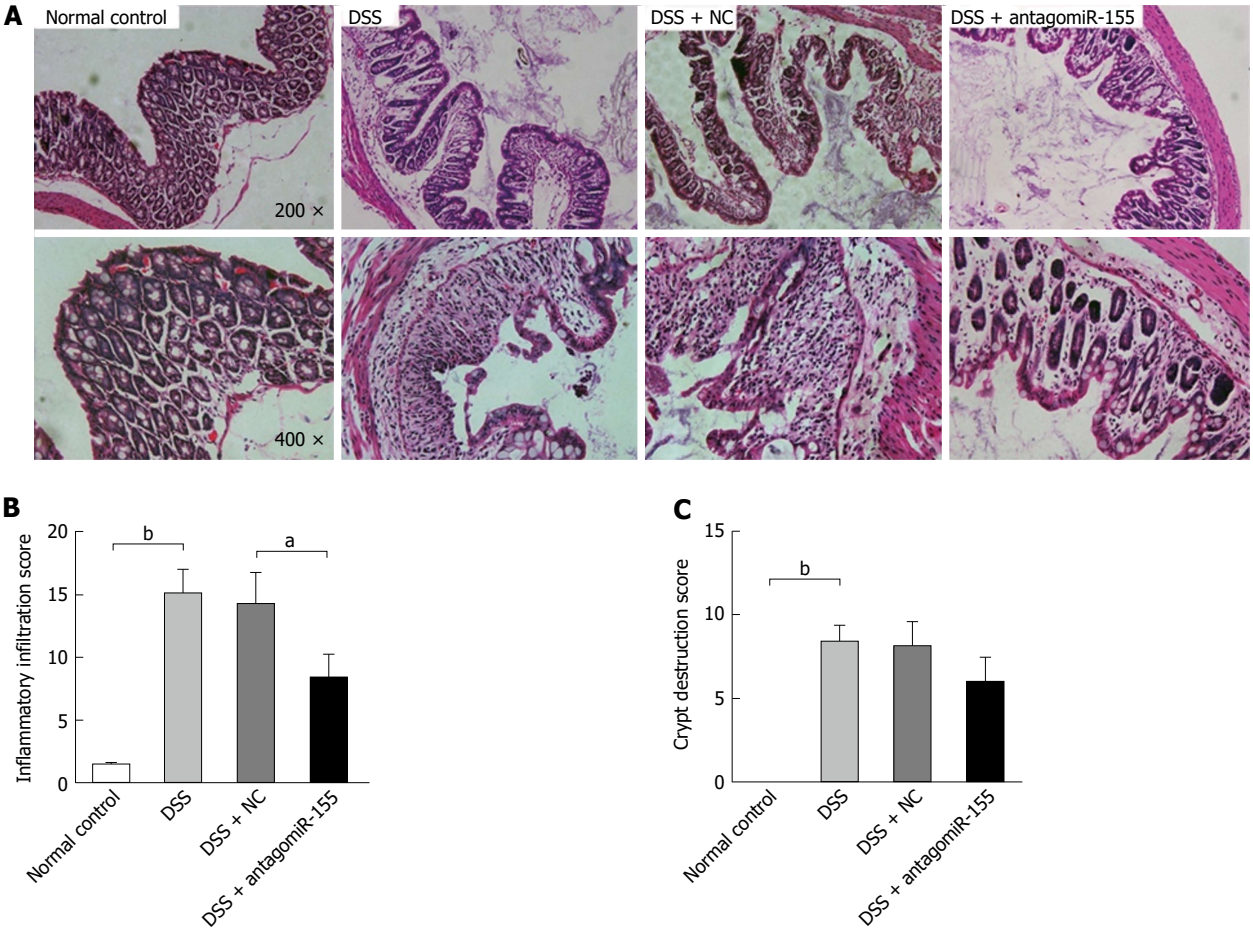

Figure 4 Effects of antagomiR-155 on dextran sulfate sodium-induced histological changes in intestinal mucosa. A: Representative histological images of colon tissues from mice in differently treated groups (original magnification, upper panel, × 200; lower panel, × 400); B: Microscopic inflammatory infiltration score. $^{b}P < 0.01$, DSS *vs* control; $^{a}P < 0.05$, DSS + antagomiR-155 *vs* DSS + NC; C: Microscopic crypt destruction score. $^{b}P < 0.01$, DSS *vs* control. DSS: Dextran sulfate sodium.

higher positive staining of SHIP-1 than the mice in other groups. Since the activation of Akt is implicated in cell proliferation, survival, and pro-inflammatory release[30], we then investigated whether the change in SHIP-1 expression and downstream Akt signaling was associated with the inflammatory response in murine colon mucosa. PCR analysis revealed that the main pro-inflammatory mediators in colitis, including IL-6, TNF-α, IL-1β, IFN-γ, and IL-17, were all suppressed to a large degree after antagomiR-155 treatment; however, the paramount anti-inflammatory factor IL-10 demonstrated an opposite trend in expression (Figure 5D), suggesting that the inhibition of miR-155 and the over-expression of SHIP-1 could be an effective strategy to alleviate or suppress the inflammatory cascade in colitis.

DISCUSSION

Understanding the underlying mechanisms that regulate gene expression and the complex interplay of pathogenic factors is essential to develop novel therapeutics in IBD. Thus far, the ability of microRNAs to target functional genes and intracellular biological signaling pathways has drawn great attention from bench to bedside[31,32]. Over the past few decades, the identification of microRNAs in IBD has made great progress as an initial step in this regard. As a multi-functional microRNA, miR-155 plays an important role in the etiology of autoimmune diseases, and its ectopic up-regulation has been reported in both UC and CD. However, the detailed mechanism by which miR-155 influences the pathogenesis of colitis remains to be elucidated. Since SHIP-1, an important cytoplasmic phosphatase that regulates the number and function of immune cells, has been demonstrated as the direct target of miR-155, we therefore investigated the possible role of miR-155 and SHIP-1 in colitis in the present study.

We first determined that SHIP-1 was directly regulated by miR-155 in murine macrophages including raw264.7 cells and primary BMDMs. As it is well known that macrophages serve as the core regulator of innate immune response during gut inflammation or infection, here we proved that SHIP-1 might play a role in a miR-155-triggered inflammatory cascade during colitis. Singh *et al*[15] reported that miR-155 deficiency protects mice from experimental colitis by reducing T cell responses, and Min *et al*[33] found that miR-155 contributes to cytokine secretion in colitis

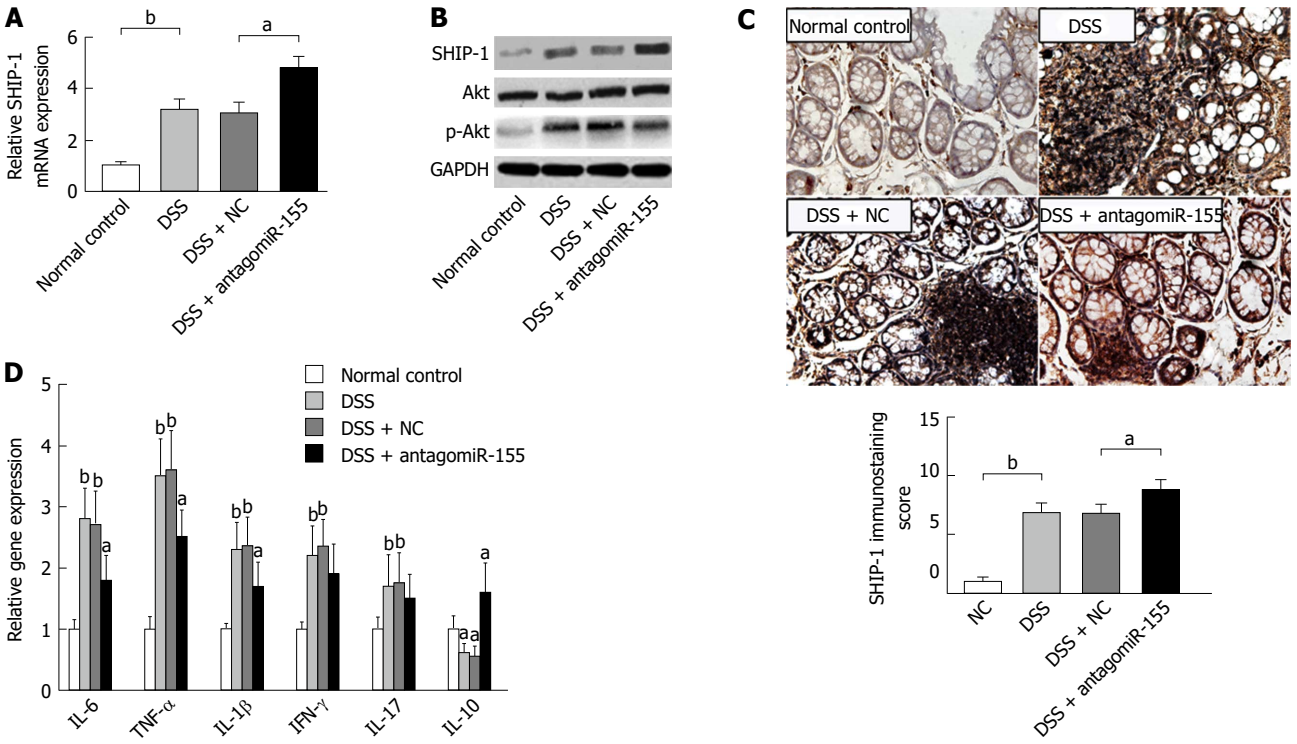

Figure 5 Inhibition of microRNA-155 alleviates colitis by regulating the SHIP-1/Akt signaling pathway. A: AntagomiR-155 treatment elevated the mRNA expression of SHIP-1. $^{b}P < 0.01$, DSS *vs* control; $^{a}P < 0.05$, DSS + antagomiR-155 *vs* DSS + NC; B: Western blot analysis of the protein levels of SHIP-1, Akt, and p-Akt after antagomiR-155 treatment, with normalized to GAPDH; C: Immunohistochemistry staining and semi-quantification for SHIP-1 in mice colon tissues. $^{b}P < 0.01$, DSS *vs* control; $^{a}P < 0.05$, DSS + antagomiR-155 *vs* DSS + NC. D: The mRNA expression levels of key factors involved in colitis-related inflammatory response. Comparison was conducted between groups: DSS, DSS + NC *vs* control; and DSS + antagomiR-155 *vs* DSS, DSS + NC. $^{a}P < 0.05$, $^{b}P < 0.01$. DSS: Dextran sulfate sodium.

by targeting FOXO3a. In this study, we confirmed the pro-proliferation and pro-inflammation capabilities of miR-155 in murine macrophages. Furthermore, we found that these effects were accompanied by a marked decrease in SHIP-1 expression and that the restoration of SHIP-1 could effectively inhibit or reverse these effects. Since it was first cloned and characterized in 1996, the role of SHIP-1 in immunity and other physiological or pathological processes has gradually emerged from numerous studies[20,21]. Thus far, the dysregulation of SHIP-1 has been described in several chronic inflammation and autoimmune disorders. There have been reports concerning SHIP-1 silencing in immune cells or knockout in animal models leading to the increased release of inflammatory cytokines[34]. Kerr *et al*[35] in 2011 reported that *Ship-1*$^{-/-}$ mice develop spontaneous CD-like ileitis, which could be corrected by adoptive transfer of bone marrow from wildtype mice. They further proposed that this type of colitis probably resulted from the imbalance of intestinal immune cells caused by SHIP-1 deprivation. Most recently, Jin *et al*[36] identified that the miR-155-mediated down-regulation of SHIP-1 promotes gouty arthritis. All of these findings point towards a pivotal role of SHIP-1 in regulation of immune response in the body. Our analysis demonstrated that the anti-inflammation effect of SHIP-1 is possibly *via* the inhibition of the Akt signaling pathway, both *in vitro* and *in vivo*. The pro-inflammatory secretion of cytokines by macrophages was significantly suppressed upon the up-regulation of SHIP-1 expression, indicating a potential for its clinical utility in the future. Although there was a report documenting that the level of SHIP-1 is increased in the intestinal mucosa samples of IBD patients[37], we speculate that this finding was due to the presence of more lymphocytes, monocytes, and neutrophils infiltrating into the colorectal mucosa during colitis.

Previous studies have identified a number of microRNAs as diagnostic biomarkers or potential targets for IBD treatment, such as miR-21 and miR-31[32]. However, to date, no therapeutic manipulation of microRNAs in IBD has been reported in cell lines or animal models. In regards to miR-155, although its aberrant expression in colitis is well established, the prospect of a miR-155-targeted strategy has not been fully investigated. In the present study, we established a DSS-induced colitis model and treated it with antagomiR-155. As expected, the inhibition of miR-155 significantly alleviated the disease activity, the degree of intestinal inflammation, and the release of pro-inflammatory cytokines. We also demonstrated that these curative effects are closely associated with an increase in SHIP-1 expression. These data provide a strong proof-of-concept for miR-155- and SHIP-1-based therapeutic approaches that could modulate

inflammation in IBD. Nevertheless, experimental data in a chronic colitis animal model should be provided for further validation.

In conclusion, our current study demonstrated that miR-155 contributes to the pathogenesis of colitis by targeting SHIP-1 expression. Therefore, the inhibition of miR-155 and the restoration of SHIP-1 could effectively alleviate intestinal inflammation and cytokine secretion. Although some other effects of this miR-155 targeting strategy still need to be considered and studied, we cannot help speculating that this promising therapeutic concept may emerge in the near future.

COMMENTS

Background

Inflammatory bowel disease (IBD) is one of the major threats to human digestive health and causes a significant increase in the incidence of colorectal cancer. However, thus far, the pathogenesis of IBD remains unclear, highlighting the need for a thorough understanding of its underlying mechanism.

Research frontiers

MicroRNAs play important roles in IBD pathogenesis. microRNA-155 (miR-155) has been reported to be upregulated in human IBD samples and animal colitis models, and emerging lines of evidence are unraveling its functional targets, including SHIP-1.

Innovations and breakthroughs

The authors focus on the molecular mechanisms of miR-155 in the immunopathogenesis of IBD using a mouse model of dextran sulfate sodium-induced colitis. This work adds evidence to clarify that the reduction in SHIP-1 levels resulting from increased miR-155 expression is the reason why IBD patients have high levels of miR-155.

Applications

This study on the potential role and particularly the mechanisms of miR-155 in IBD is important for the clinical management of the disease and the development of novel therapeutic modalities.

Peer-review

This work offers new insight into the understanding of the inflammatory mechanisms in IBD.

REFERENCES

1 **Ordás I**, Eckmann L, Talamini M, Baumgart DC, Sandborn WJ. Ulcerative colitis. *Lancet* 2012; **380**: 1606-1619 [PMID: 22914296 DOI: 10.1016/s0140-6736(12)60150-0]

2 **Baumgart DC**, Sandborn WJ. Crohn's disease. *Lancet* 2012; **380**: 1590-1605 [PMID: 22914295 DOI: 10.1016/s0140-6736(12)60026-9]

3 **Marchesi JR**, Adams DH, Fava F, Hermes GD, Hirschfield GM, Hold G, Quraishi MN, Kinross J, Smidt H, Tuohy KM, Thomas LV, Zoetendal EG, Hart A. The gut microbiota and host health: a new clinical frontier. *Gut* 2016; **65**: 330-339 [PMID: 26338727 DOI: 10.1136/gutjnl-2015-309990]

4 **de Souza HS**, Fiocchi C. Immunopathogenesis of IBD: current state of the art. *Nat Rev Gastroenterol Hepatol* 2016; **13**: 13-27 [PMID: 26627550 DOI: 10.1038/nrgastro.2015.186]

5 **Mo YY**. MicroRNA regulatory networks and human disease. *Cell Mol Life Sci* 2012; **69**: 3529-3531 [PMID: 22926413 DOI: 10.1007/s00018-012-1123-1]

6 **Xiao C**, Rajewsky K. MicroRNA control in the immune system: basic principles. *Cell* 2009; **136**: 26-36 [PMID: 19135886 DOI: 10.1016/j.cell.2008.12.027]

7 **Mashima R**. Physiological roles of miR-155. *Immunology* 2015; **145**: 323-333 [PMID: 25829072 DOI: 10.1111/imm.12468]

8 **Vigorito E**, Kohlhaas S, Lu D, Leyland R. miR-155: an ancient regulator of the immune system. *Immunol Rev* 2013; **253**: 146-157 [PMID: 23550644 DOI: 10.1111/imr.12057]

9 **Faraoni I**, Antonetti FR, Cardone J, Bonmassar E. miR-155 gene: a typical multifunctional microRNA. *Biochim Biophys Acta* 2009; **1792**: 497-505 [PMID: 19268705 DOI: 10.1016/j.bbadis.2009.02.013]

10 **Marques-Rocha JL**, Samblas M, Milagro FI, Bressan J, Martínez JA, Marti A. Noncoding RNAs, cytokines, and inflammation-related diseases. *FASEB J* 2015; **29**: 3595-3611 [PMID: 26065857 DOI: 10.1096/fj.14-260323]

11 **Tili E**, Michaille JJ, Croce CM. MicroRNAs play a central role in molecular dysfunctions linking inflammation with cancer. *Immunol Rev* 2013; **253**: 167-184 [PMID: 23550646 DOI: 10.1111/imr.12050]

12 **Tili E**, Croce CM, Michaille JJ. miR-155: on the crosstalk between inflammation and cancer. *Int Rev Immunol* 2009; **28**: 264-284 [PMID: 19811312 DOI: 10.1080/08830180903093796]

13 **Béres NJ**, Szabó D, Kocsis D, Szűcs D, Kiss Z, Müller KE, Lendvai G, Kiss A, Arató A, Sziksz E, Vannay Á, Szabó AJ, Veres G. Role of Altered Expression of miR-146a, miR-155, and miR-122 in Pediatric Patients with Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2016; **22**: 327-335 [PMID: 26752469 DOI: 10.1097/mib.0000000000000687]

14 **Svrcek M**, El-Murr N, Wanherdrick K, Dumont S, Beaugerie L, Cosnes J, Colombel JF, Tiret E, Fléjou JF, Lesuffleur T, Duval A. Overexpression of microRNAs-155 and 21 targeting mismatch repair proteins in inflammatory bowel diseases. *Carcinogenesis* 2013; **34**: 828-834 [PMID: 23288924 DOI: 10.1093/carcin/bgs408]

15 **Singh UP**, Murphy AE, Enos RT, Shamran HA, Singh NP, Guan H, Hegde VL, Fan D, Price RL, Taub DD, Mishra MK, Nagarkatti M, Nagarkatti PS. miR-155 deficiency protects mice from experimental colitis by reducing T helper type 1/type 17 responses. *Immunology* 2014; **143**: 478-489 [PMID: 24891206 DOI: 10.1111/imm.12328]

16 **O'Connell RM**, Chaudhuri AA, Rao DS, Baltimore D. Inositol phosphatase SHIP1 is a primary target of miR-155. *Proc Natl Acad Sci USA* 2009; **106**: 7113-7118 [PMID: 19359473 DOI: 10.1073/pnas.0902636106]

17 **Pedersen IM**, Otero D, Kao E, Miletic AV, Hother C, Ralfkiaer E, Rickert RC, Gronbaek K, David M. Onco-miR-155 targets SHIP1 to promote TNFalpha-dependent growth of B cell lymphomas. *EMBO Mol Med* 2009; **1**: 288-295 [PMID: 19890474 DOI: 10.1002/emmm.200900028]

18 **Wu R**, Li Y, Guo Z, Gong J, Zhu W, Li N, Li J. Triptolide ameliorates ileocolonic anastomosis inflammation in IL-10 deficient mice by mechanism involving suppression of miR-155/SHIP-1 signaling pathway. *Mol Immunol* 2013; **56**: 340-346 [PMID: 23911388 DOI: 10.1016/j.molimm.2013.05.006]

19 **Costinean S**, Sandhu SK, Pedersen IM, Tili E, Trotta R, Perrotti D, Ciarlariello D, Neviani P, Harb J, Kauffman LR, Shidham A, Croce CM. Src homology 2 domain-containing inositol-5-phosphatase and CCAAT enhancer-binding protein beta are targeted by miR-155 in B cells of Emicro-MiR-155 transgenic mice. *Blood* 2009; **114**: 1374-1382 [PMID: 19520806 DOI: 10.1182/blood-2009- 05-220814]

20 **Kerr WG**. Inhibitor and activator: dual functions for SHIP in immunity and cancer. *Ann N Y Acad Sci* 2011; **1217**: 1-17 [PMID: 21155837 DOI: 10.1111/j.1749-6632.2010.05869.x]

21 **Condé C**, Gloire G, Piette J. Enzymatic and non-enzymatic activities of SHIP-1 in signal transduction and cancer. *Biochem Pharmacol* 2011; **82**: 1320-1334 [PMID: 21672530 DOI: 10.1016/j.bcp.2011.05.031]

22 **Kalesnikoff J**, Sly LM, Hughes MR, Büchse T, Rauh MJ, Cao LP, Lam V, Mui A, Huber M, Krystal G. The role of SHIP in cytokine-induced signaling. *Rev Physiol Biochem Pharmacol* 2003; **149**: 87-103 [PMID: 12692707 DOI: 10.1007/s10254-003-0016-y]

23 **Viernes DR**, Choi LB, Kerr WG, Chisholm JD. Discovery and development of small molecule SHIP phosphatase modulators.

Med Res Rev 2014; **34**: 795-824 [PMID: 24302498 DOI: 10.1002/med.21305]
24 **Hamilton MJ**, Ho VW, Kuroda E, Ruschmann J, Antignano F, Lam V, Krystal G. Role of SHIP in cancer. *Exp Hematol* 2011; **39**: 2-13 [PMID: 21056081 DOI: 10.1016/j.exphem.2010.11.002]
25 **Fukuda R**, Hayashi A, Utsunomiya A, Nukada Y, Fukui R, Itoh K, Tezuka K, Ohashi K, Mizuno K, Sakamoto M, Hamanoue M, Tsuji T. Alteration of phosphatidylinositol 3-kinase cascade in the multilobulated nuclear formation of adult T cell leukemia/lymphoma (ATLL). *Proc Natl Acad Sci USA* 2005; **102**: 15213-15218 [PMID: 16217039 DOI: 10.1073/pnas.0507184102]
26 **Kurowska-Stolarska M**, Alivernini S, Ballantine LE, Asquith DL, Millar NL, Gilchrist DS, Reilly J, Ierna M, Fraser AR, Stolarski B, McSharry C, Hueber AJ, Baxter D, Hunter J, Gay S, Liew FY, McInnes IB. MicroRNA-155 as a proinflammatory regulator in clinical and experimental arthritis. *Proc Natl Acad Sci USA* 2011; **108**: 11193-11198 [PMID: 21690378 DOI: 10.1073/pnas.1019536108]
27 **Lee CM**, Hu J. Cell density during differentiation can alter the phenotype of bone marrow-derived macrophages. *Cell Biosci* 2013; **3**: 30 [PMID: 23895502 DOI: 10.1186/2045-3701-3-30]
28 **Li X**, Xu Y, Zhang C, Deng L, Chang M, Yu Z, Liu D. Protective Effect of Calculus Bovis Sativus on Dextran Sulphate Sodium-Induced Ulcerative Colitis in Mice. *Evid Based Complement Alternat Med* 2015; **2015**: 469506 [PMID: 26579201 DOI: 10.1155/2015/469506]
29 **Cui Y**, Wei H, Lu F, Liu X, Liu D, Gu L, Ouyang C. Different Effects of Three Selected Lactobacillus Strains in Dextran Sulfate Sodium-Induced Colitis in BALB/c Mice. *PLoS One* 2016; **11**: e0148241 [PMID: 26840426 DOI: 10.1371/journal.pone.0148241]
30 **Patel RK**, Mohan C. PI3K/AKT signaling and systemic autoimmunity. *Immunol Res* 2005; **31**: 47-55 [PMID: 15591622]
31 **Kalla R**, Ventham NT, Kennedy NA, Quintana JF, Nimmo ER, Buck AH, Satsangi J. MicroRNAs: new players in IBD. *Gut* 2015; **64**: 504-517 [PMID: 25475103 DOI: 10.1136/gutjnl-2014-307891]
32 **Fisher K**, Lin J. MicroRNA in inflammatory bowel disease: Translational research and clinical implication. *World J Gastroenterol* 2015; **21**: 12274-12282 [PMID: 26604636 DOI: 10.3748/wjg.v21.i43.12274]
33 **Min M**, Peng L, Yang Y, Guo M, Wang W, Sun G. MicroRNA-155 is involved in the pathogenesis of ulcerative colitis by targeting FOXO3a. *Inflamm Bowel Dis* 2014; **20**: 652-659 [PMID: 24583476 DOI: 10.1097/mib.0000000000000009]
34 **Kalesnikoff J**, Baur N, Leitges M, Hughes MR, Damen JE, Huber M, Krystal G. SHIP negatively regulates IgE + antigen-induced IL-6 production in mast cells by inhibiting NF-kappa B activity. *J Immunol* 2002; **168**: 4737-4746 [PMID: 11971024 DOI: 10.4049/jimmunol.168.9.4737]
35 **Kerr WG**, Park MY, Maubert M, Engelman RW. SHIP deficiency causes Crohn's disease-like ileitis. *Gut* 2011; **60**: 177-188 [PMID: 20940287 DOI: 10.1136/gut.2009.202283]
36 **Jin HM**, Kim TJ, Choi JH, Kim MJ, Cho YN, Nam KI, Kee SJ, Moon JB, Choi SY, Park DJ, Lee SS, Park YW. MicroRNA-155 as a proinflammatory regulator via SHIP-1 down-regulation in acute gouty arthritis. *Arthritis Res Ther* 2014; **16**: R88 [PMID: 24708712 DOI: 10.1186/ar4531]
37 **Arijs I**, De Hertogh G, Lemmens B, Van der Goten J, Vermeire S, Schuit F, Rutgeerts P. Intestinal expression of SHIP in inflammatory bowel diseases. *Gut* 2012; **61**: 956-957 [PMID: 22052065 DOI: 10.1136/gutjnl-2011-301256]

P- Reviewer: Bian ZX, Chen WX, Zhu YL **S- Editor**: Qi Y
L- Editor: A **E- Editor**: Zhang FF

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v23.i6.986

World J Gastroenterol 2017 February 14; 23(6):986-998
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

Melatonin, a novel selective ATF-6 inhibitor, induces human hepatoma cell apoptosis through COX-2 downregulation

Li-Jia Bu, Han-Qing Yu, Lu-Lu Fan, Xiao-Qiu Li, Fang Wang, Jia-Tao Liu, Fei Zhong, Cong-Jun Zhang, Wei Wei, Hua Wang, Guo-Ping Sun

Li-Jia Bu, Han-Qing Yu, Lu-Lu Fan, Xiao-Qiu Li, Fang Wang, Jia-Tao Liu, Fei Zhong, Cong-Jun Zhang, Hua Wang, Guo-Ping Sun, Department of Oncology, the First Affiliated Hospital of Anhui Medical University, Hefei 230022, Anhui Province, China

Jia-Tao Liu, Department of Pharmacy, the First Affiliated Hospital of Anhui Medical University, Hefei 230022, Anhui Province, China

Wei Wei, Institute of Clinical Pharmacology, Anhui Medical University, Hefei 230032, Anhui Province, China

Hua Wang, Institute for Liver Diseases of Anhui Medical University, Hefei 230032, Anhui Province, China

Author contributions: Bu LJ, Yu HQ and Fan LL contributed equally to this work; Bu LJ, Yu HQ and Fan LL performed select experiments and analyzed data; Li XQ and Wang F performed select experiments; Liu JT and Zhong F performed select histopathological experiments; Zhang CJ and Wei W read the manuscript and gave important intellectual suggestions; Wang H and Sun GP prepared the manuscript, and designed and supervised the project.

Supported by grants from the National Natural Science Foundation of China, No. 81572430 and No. 81272739.

Institutional review board statement: The study was carried out in accordance with a protocol approved by the Ethics Committee of Anhui Medical University (Anhui, China).

Conflict-of-interest statement: No conflicts of interest exist for any of the authors.

Manuscript source: Unsolicited manuscript

Correspondence to: Dr. Guo-Ping Sun, Department of Oncology, the First Affiliated Hospital of Anhui Medical University, No. 218 Jixi Road, Hefei 230022, Anhui Province, China. sunguoping@ahmu.edu.cn
Telephone: +86-551-62923509
Fax: +86-551-62923509

Received: August 31, 2016
Peer-review started: August 31, 2016
First decision: September 28, 2016
Revised: October 11, 2016
Accepted: October 27, 2016
Article in press: October 27, 2016
Published online: February 14, 2017

Abstract

AIM

To clarify the mechanisms involved in the critical endoplasmic reticulum (ER) stress initiating unfolded protein response pathway modified by melatonin.

METHODS

Hepatoma cells, HepG2, were cultured *in vitro*. Flow cytometry and TUNEL assay were used to measure HepG2 cell apoptosis. Western blotting and quantitative reverse transcription-polymerase chain reaction methods were used to determine the protein and messenger RNA levels of ER stress and apoptosis related genes' expression, respectively. Tissue microarray construction from patients was verified by immunohistochemical analysis.

RESULTS

In the present study, we first identified that melatonin

selectively blocked activating transcription factor 6 (ATF-6) and then inhibited cyclooxygenase-2 (COX-2) expression, leading to enhanced liver cancer cell apoptosis under ER stress condition. Dramatically increased CCAAT-enhancer-binding protein homologous protein level, suppressed COX-2 and decreased Bcl-2/Bax ratio by melatonin or ATF-6 siRNA contributed the enhanced HepG2 cell apoptosis under tunicamycin (an ER stress inducer) stimulation. In clinical hepatocellular carcinoma patients, the close relationship between ATF-6 and COX-2 was further confirmed.

CONCLUSION

These findings indicate that melatonin as a novel selective ATF-6 inhibitor can sensitize human hepatoma cells to ER stress inducing apoptosis.

Key words: Melatonin; Endoplasmic reticulum stress; Activating transcription factor 6; Cyclooxygenase-2; Hepatocellular carcinoma

Core tip: Endoplasmic reticulum (ER) stress plays an important role in tumor growth and resistance to treatment. Our previous studies have already shown that melatonin sensitizes the human hepatocellular carcinoma cell to ER stress-induced apoptosis and attenuates ER stress-induced doxorubicin resistance. In this study, we first identified that melatonin selectively blocked ER stress downstream activating transcription factor 6 (ATF-6) and then inhibited cyclooxygenase-2 expression, leading to enhanced liver cancer cell apoptosis under ER stress condition. Our findings indicate that melatonin as a novel selective ATF-6 inhibitor can sensitize human hepatoma cells to ER stress inducing apoptosis.

Bu LJ, Yu HQ, Fan LL, Li XQ, Wang F, Liu JT, Zhong F, Zhang CJ, Wei W, Wang H, Sun GP. Melatonin, a novel selective ATF-6 inhibitor, induces human hepatoma cell apoptosis through COX-2 downregulation. *World J Gastroenterol* 2017; 23(6): 986-998 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i6/986.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i6.986

INTRODUCTION

Hepatocellular carcinoma (HCC) was the fifth most frequently diagnosed cancer worldwide and the third cause of cancer-related death, responsible for approximately 700000 human deaths annually worldwide[1,2]. China accounts for the majority of total HCC incidence in the world[1]. Most patients with HCC are diagnosed at a late stage, when there is no chance of surgical therapy. Unfortunately, there are only a few effective chemotherapy agents for this type of malignant tumor. One of the major reasons for untreatable HCC is that the liver cancer cell has much greater tolerance towards a number of cellular stress conditions, such as endoplasmic reticulum (ER) stress, hypoxia, nutrient deprivation and so on[3,4]. Thus, approaches to overcoming this superior tolerance have been emerging as potential drug targets for the treatment of HCC[5].

All types of stress, including oncogenetic stress that interferes with ER function, cause accumulation of unfolded proteins in the ER lumen, referred to as ER stress, and activate a homeostatic signaling network known as the unfolded protein response (UPR)[6,7]. The three main ER transmembrane sensors that elicit the UPR are protein kinase RNA-like ER kinase (PERK), inositol-requiring enzyme-1 (IRE-1), and activating transcription factor 6 (ATF-6). In general, ER stress initiated by IRE-1a, PERK, and ATF-6 and activated in solid tumors is crucial for tumor growth and aggressiveness, as well as for microenvironment remodeling or drug resistance[3,4].

Melatonin (N-acetyl-5-methoxytryptamine), which is produced in the human pineal gland during the night phase of the light-dark cycle, plays important roles in physiological and pharmacological functions, such as circadian rhythms and antioxidants. More interestingly to us, however, is the fact that melatonin exerts anticancer effects through interplay with ER stress[8-10]. Our previous study demonstrated that melatonin could sensitize the human HCC cell line HepG2 to ER stress-induced apoptosis *via* the inhibition of cyclooxygenase-2 (COX-2)[11]. Furthermore, we found that melatonin attenuates ER stress-induced resistance to doxorubicin through reversing tunicamycin-induced ER stress[12]. However, the mechanisms involved in the critical UPR pathway modified by melatonin must still be clarified.

In the present study, we first identified that melatonin can selectively block ATF-6 and then inhibit COX-2 expression, leading to enhanced liver cancer cell apoptosis. In clinical HCC patients, the close relationship between ATF-6 and COX-2 was further confirmed. Our study explored the more detailed mechanisms of melatonin enhancing ER stress-induced apoptosis in human hepatoma cells *via* inhibition of COX-2 by selectively targeting ATF-6.

MATERIALS AND METHODS

Reagents

Melatonin (M5250) and tunicamycin (T7765) were obtained from Sigma Chemical (St. Louis, MO, United States). DMEM was purchased from Gibco-BRL Life Technologies (Grand Island, NY, United States). Anti-COX-2 (ab179800), anti-Bax (ab32503) and anti-CCAAT/enhancer-binding protein homologous protein (CHOP) (ab11419) were obtained from Abcam (Cambridge, MA, United States). Anti-ATF-6 (BS6476), anti-PERK (BS2156) and anti-Bcl-2 (BS3711) were obtained from Bioworld Technology Inc. (St Louis

Park, MN, United States). The terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) system was purchased from Roche (Indianapolis, IN, United States). The annexin V-FITC kit was obtained from Shanghai Bestbio (Shanghai, China). The reverse transcription kit (A3500) and TRIzol (15596-026) were obtained from Promega Inc. (Madison, WI, United States). The SYBR Green qPCR kit (11744-100) was obtained from Invitrogen Life Technologies (Grand Island, NY, United States).

HCC specimens

Tissue samples were obtained from 100 patients with HCC. These 100 patients underwent surgery at the First Affiliated Hospital of Anhui Medical University between 2001 and 2007. Patients with HCC who had accepted chemotherapy or radiation therapy before surgery were excluded from the study. The pathohistological diagnosis of the specimens was consistent with HCC in accordance with the World Health Organization Guidelines. A total of 85 HCC patients were male, and 15 HCC patients were female. The median age of the HCC patient population was 50.7 years, ranging from 18 to 84 years. These 100 HCC patients were staged according to UICC as follows: 3 HCC patients were Stage Ⅰ (3%), 73 HCC patients were Stage Ⅱ (73%), 9 HCC patients were Stage Ⅲ (9%), and 15 patients were Stage Ⅳ (15%). Tumors were pathologically graded according to WHO guidelines: 4 HCC patients were well-differentiated, 91 HCC patients were moderately differentiated, and 5 HCC patients were poorly differentiated. All the clinical specimens were collected from patients after obtaining written informed consent. The study was carried out in accordance with a protocol approved by the Ethics Committee of Anhui Medical University (Anhui, China).

Tissue microarray construction

Formalin-fixed paraffin-embedded specimens were obtained from the archives of the Department of Pathology at the First Affiliated Hospital of Anhui Medical University. Hematoxylin and eosin-stained tissue sections were reviewed for identification of the target area for tissue microarray construction. Three to five representative 1-mm cores were obtained from each sample and inserted in a grid pattern into a new recipient paraffin block using a manual tissue arrayer (Hengtai Instruments, Liaoning, China).

Immunohistochemical analysis

Tissue microarray sections were deparaffinized, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min and heated in 0.01 mol/L sodium citrate buffer (pH 6.0) for 10 min for antigen retrieval. Subsequently, the sections were incubated with a primary antibody in a moist chamber for 1 h at ambient temperature. Then, the sections were washed in phosphate buffered saline (PBS) (pH 7.2), incubated with a biotinylated secondary antibody and then incubated with peroxidase-conjugated streptavidin. To observe positive binding of the antigen, the sections were incubated with diaminobenzidine solution and then counterstained with hematoxylin. Next, the sections were viewed under a microscope and scored on the basis of staining intensity and the percentage of stained cells relative to the background: > 10% of tumor cells stained was considered positive staining.

Cell culture

The human hepatoma cell line HepG2 was obtained from the Shanghai Cell Bank (Chinese Academy of Sciences, Shanghai, China). The cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell culture was carried out at 37 ℃ in a humidified 5% CO_2 atmosphere.

Flow cytometry

After treatment, cells were detached from the 6-well plates by using 0.2% trypsin, harvested, washed twice with PBS, and centrifuged twice at 300 g for 5 min at 4 ℃. Then, the supernatant was discarded, and the pellet was resuspended in 400 μL Annexin V binding buffer at 20 ℃ for at least 12 h. Cells were subsequently treated in PBS with RNase A for 30 min at room temperature and stained with propidium iodide (PI). Flow cytometric analysis was performed using an EPICS XL-MCL model counter (Beckman Coulter, Fullerton, CA, United States). A total of 1×10^6 cells/mL were analyzed for each sample, and the experiment was repeated at least three times.

TUNEL assay

Cells were cultured on coverslips in 6-well plates overnight. After treatment with various concentrations of the indicated compounds for each time period, the coverslips were washed twice with cold PBS and fixed in a 4% paraformaldehyde solution for 1 h at room temperature. Apoptotic cells were detected by the TUNEL assay (TUNEL System Kit; Roche, Basel, Switzerland), which was performed according to the manufacturer's instructions. The TUNEL assay results were quantitatively analyzed through the biological image analysis system from the Nikon ECLIPSE 80i biology microscope, Nikon Digital Camera DXM 1200F, ACT-1 version 2.63 software (Tokyo, Japan).

Western blotting

After drug treatment for the indicated time periods and concentrations, cells were lysed in RIPA lysis buffer [50 mmol/L Tris-HCl, (pH 7.4), 150 mmol/L NaCl, 10 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L ethylene diamine tetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate (SDS), 1%

Table 1 Expression of cyclooxygenase-2 was more likely to be associated with the expression of activating transcription factor 6 than with inositol-requiring enzyme-1 and protein kinase RNA-like endoplasmic reticulum kinase in hepatocellular carcinoma patients

COX-2	AFP-6				IRE-1				PERK			
	-	+	*r*	*P* value	-	+	*r*	*P* value	-	+	*r*	*P* value
Negative	16	39	0.198	0.011	30	25	0.135	0.086	29	26	0.017	0.134
Positive	14	95			44	65			44	65		

ATF-6: Activating transcription factor 6; COX-2: Cyclooxygenase-2; IRE: Inositol-requiring enzyme; PERK: Protein kinase RNA-like endoplasmic reticulum kinase.

Triton X-100 and 1% sodium deoxycholate] for 20-30 min on ice. Protein concentrations were determined by the Lowry protein assay. Lysates were incubated with 2 × Laemmli sample buffer (Bio-Rad, Hercules, CA, United States) and heated for 10 min at 95 ℃. The proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, United States) and incubated with blocking buffer [Tris-buffered saline/Tween 20/5% nonfat dry milk] overnight at 4 ℃. Immunoblots were incubated with the indicated primary antibody followed by the appropriate horseradish peroxidase-conjugated secondary antibody and visualized with enhanced chemiluminescence (Pierce, Rockford, IL, United States) using hydrogen peroxide and luminol as substrate with Kodak X-AR film. Autoradiographs were scanned using a GS-700 Imaging Densitometer (Bio-Rad).

Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from HepG2 cells using the Trizol reagent, and 1 mg RNA was reverse transcribed to cDNA using the Reverse Transcription System A3500 (Fermentas, Burlington, Canada). To determine the quantity of mRNA, the cDNA was amplified by real-time PCR with a SYBR Green PCR master mix kit (Invitrogen), and the housekeeping gene GAPDH was used as the internal control. The SYBR Green assays were performed in triplicate on a 7500 real-time instrument (Applied Biosystems Inc, Foster City, CA, United States). The primers to detect mRNA were 5′-CTGTATCCCGCCCTGCTGGTG-3′ and 5′-ACTTGCGTTG ATGGTGGCTGTCTT-3′ for COX-2 and 5′-AGAAGGCTGG GGCTCATTTG-3′ and 5′-AGGGGCCATCCACAGTCTTC-3′ for GADPH. All samples were normalized to internal controls, and fold-changes were calculated by relative quantification. The conditions for quantitative reverse transcription polymerase chain reaction (qRT-PCR) were as follows: 5 min at 94 ℃, and then 50 cycles of 94 ℃ for 30 s, 59 ℃ for 30 s, and 72 ℃ for 1 min.

Statistical analysis

Three or more separate experiments were performed for each experiment. Statistical analysis was performed by Student's *t*-test or ANOVA. Data are presented as the mean ± SD. Significance was noted at $P < 0.05$.

RESULTS

COX-2 is the inducible form of cyclooxygenase and is frequently highly expressed in tumor tissues, including liver cancer, playing an important role in tumor development. To determine which UPR pathway was associated with COX-2 expression under ER stress in HCC patients, we analyzed the paraffin-embedded, formalin-fixed HCC specimens by immunohistochemical staining. For all 100 specimens, COX-2, ATF-6, IRE-1 and PERK were stained in the nuclei and/or cytoplasm of tumor cells. We observed that the expression of COX-2 was more likely to be associated with the expression of ATF-6 than with IRE-1 and PERK, which reached statistical significance ($P = 0.011$) (Table 1). These data suggest the existence of a close relationship between ATF-6 and COX-2 (Figure 1).

There is limited information about the effects of melatonin under ER stress conditions and the impact of melatonin on these three UPR pathways. To address this, we next evaluated the effects of melatonin on the UPR pathways *in vitro*. To mimic the ER stress condition, HepG2 cells were first pretreated with tunicamycin for 8 h; then, the cells were treated with melatonin at four different concentrations (10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} mmol/L) for another 24 h. The expression of all three UPR pathways was evaluated by western blotting method. As illustrated in Figure 2A, and consistent with previous reports, the downstream signaling molecules ATF-6, IRE-1 and PERK were detected after ER stress activator tunicamycin treatment. Melatonin at concentrations between 10^{-7} to 10^{-3} mmol/L markedly inhibited ATF-6 expression. However, only high-concentration melatonin (10^{-3} mmol/L) slightly decreased the expression of IRE-1. Meanwhile, melatonin had no effect on the expression of PERK. These results indicate melatonin prominently affects the ATF-6 pathway under ER stress condition.

To investigate the underlying mechanisms of which pathway is associated with the expression of COX-2 under the condition of ER stress, we used RNA interference to knockdown the mRNA in all three UPR pathways and then observed the changes in COX-2 levels by western blotting. First, each UPR siRNA has

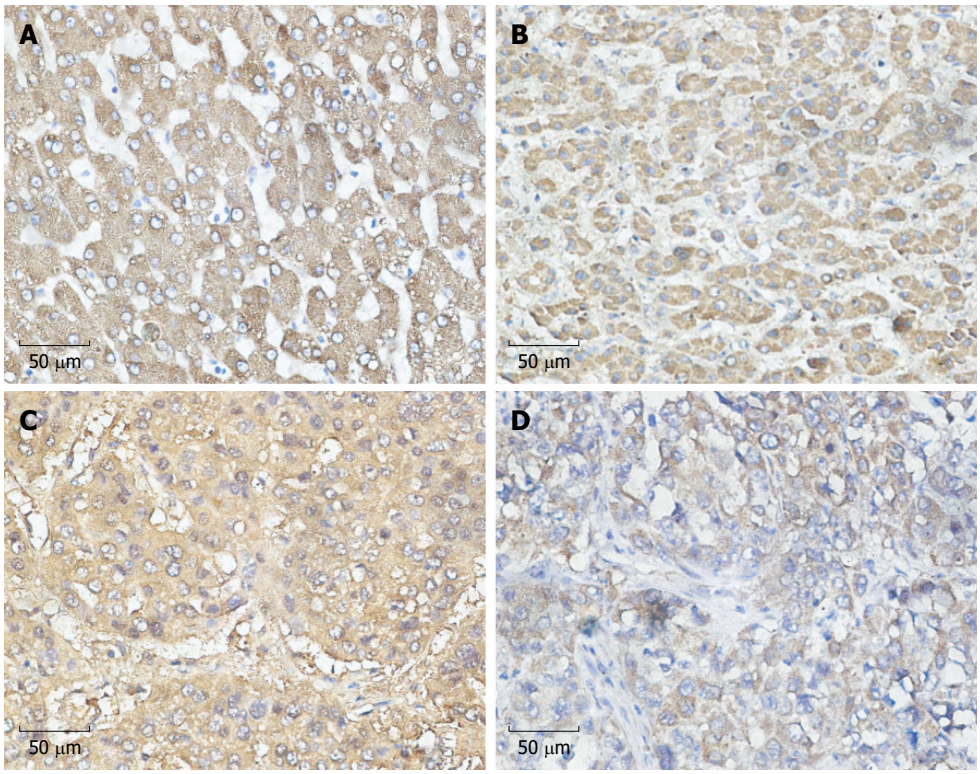

Figure 1 Relationship between cyclooxygenase-2 and unfolded protein response pathways. Expression of unfolded protein response (UPR) pathways and COX-2 in hepatocellular carcinoma (HCC) (streptavidin-peroxidase × 400). A: Expression of COX-2 in HCC; B: Expression of ATF-6 in HCC; C: Expression of IRE-1 in HCC; D: Expression of PERK in HCC. COX-2: Cyclooxygenase-2.

three candidate sequences, and we chose the most effective ones by western blot staining (Figure 3A-C). As shown in Figure 4A and B, the COX-2 protein expression is downregulated after blocking the ATF-6 mRNA under ER stress condition. In addition, the mRNA level of COX-2 was also decreased by si-ATF-6 after ER stress activator tunicamycin pre-treatment. Thus, we concluded that COX-2 can be regulated by the ATF-6 pathway under ER stress.

To further determine whether blocking the ATF-6 pathway influences ER stress-induced apoptosis, FACS analysis and TUNEL staining were performed using HepG2 cells. As shown in Figure 5, tunicamycin slightly but significantly induced tumor cell apoptosis to around 10%-20%. Interestingly, the percentage of apoptotic cells markedly increased to around 30% when combined with si-ATF-6. This result suggests that blocking ATF-6 pathway under ER stress can further aggravate liver tumor cell apoptosis.

As has already been found, melatonin likely acts as an ER stress inhibitor by selectively blocking the ATF-6 pathway; thus, we next asked whether melatonin also can aggravate liver tumor cell apoptosis in a manner similar to si-ATF-6 after ER stress activation. To further determine whether melatonin influences ER stress-induced apoptosis, FACS analysis and TUNEL staining were performed in HepG2 cells *in vitro*. Similar to the results from si-ATF-6, and as shown in Figure 6, treatment with melatonin for 24 h after pretreatment with tunicamycin led to an obvious increase in apoptotic tumor cells. The morphological changes indicative of apoptosis were also assessed by TUNEL staining, as shown in Figures 5 and 6. Treatment with ATF-6-siRNA and melatonin resulted in a dramatic increase in the number of apoptotic HepG2 cells. This also suggested that melatonin can effectively downregulate ATF-6 and lead to an increased number of HepG2 cells, which supports the results of FACS.

Further mechanism study, shown in Figure 7, showed that melatonin or si-ATF-6 inhibits COX-2 expression while increasing CHOP and the Bax/Bcl-2 ratio to induce cancer cell apoptosis. These data suggest that COX-2 expression may be directly involved in the adaptation of human hepatoma cells to ER stress-induced apoptosis. Based on the data of the relationship between COX-2 and UPR pathways, we concluded that melatonin can obviously knockdown ATF-6 and reduce apoptosis under ER stress by downregulating COX-2.

CHOP, also called GADD153, is one of the primary effectors of ER stress-mediated cell apoptosis. As shown in Figure 7A, the expression of CHOP was markedly increased in the presence of melatonin and ATF-6 siRNA. Similarly, the levels of the anti-apoptosis factor, Bcl-2, were decreased, and the levels of pro-apoptosis factor, Bax, were increased when cells were exposed to melatonin or ATF-6 siRNA. The Bcl-2/Bax ratio also decreased (Figure 7B). These data indicate

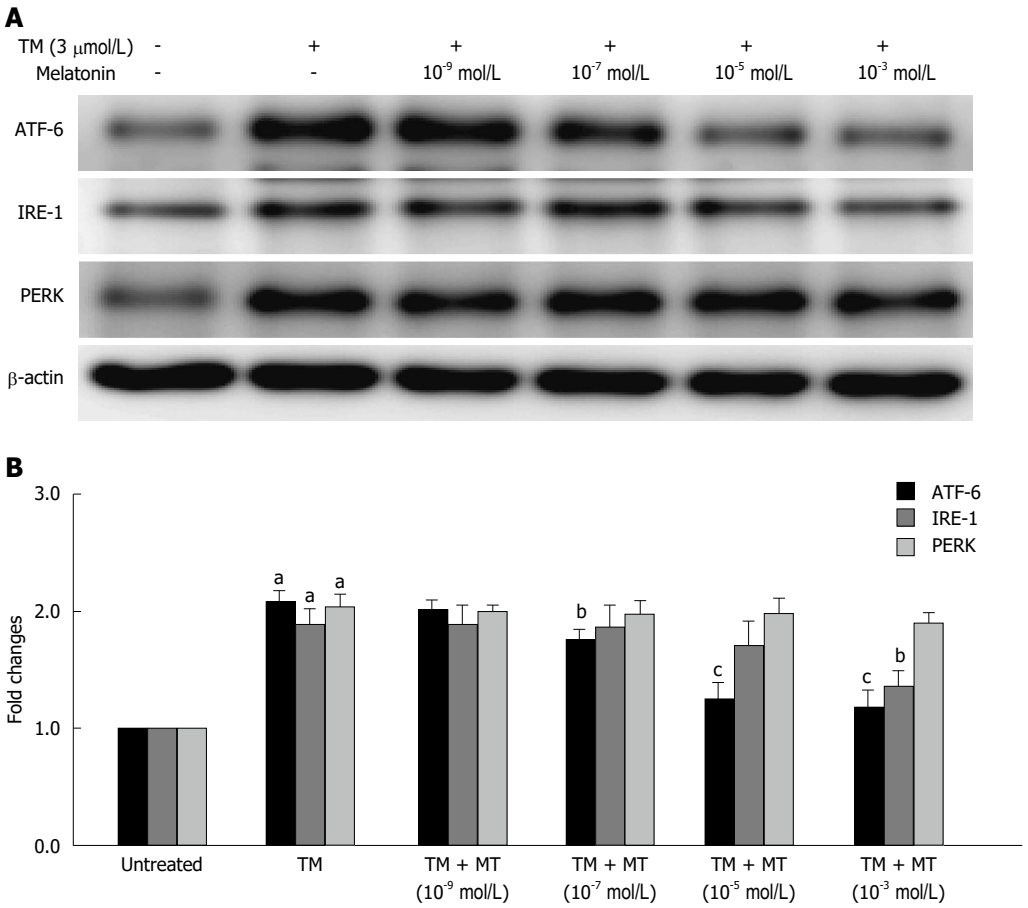

Figure 2 Selection of the proper melatonin concentration. The effects of melatonin (MT) on the three unfolded protein response (UPR) pathways of HepG2 cells induced by tunicamycin (TM). HepG2 cells were exposed to different concentrations of melatonin (10^{-9}, 10^{-7}, 10^{-5} and 10^{-3} mol/L) for 24 h. A: Equal protein amounts of cell lysates were subjected to western blot assay using anti-ATF-6, anti-IRE-1 and anti-PERK. β-actin in the same HepG2 cells extract was used as an internal reference; B: Optical density reading values of specific proteins are represented as fold differences relative to the loading control protein, β-actin. $^{a}P < 0.01$ *vs* negative control (NC); $^{b}P < 0.05$ *vs* positive control (TM); $^{c}P < 0.01$ *vs* positive control (TM). ATF-6: Activating transcription factor 6; IRE: Inositol-requiring enzyme; PERK: Protein kinase RNA-like endoplasmic reticulum kinase.

that inhibition of COX-2 with melatonin by knocking down ATF-6 mRNA increases the number of apoptotic cells by upregulating the expression of CHOP.

In conclusion, the results of this study indicate that under tunicamycin-induced ER stress, melatonin can inhibit the expression COX-2 by downregulating one of the UPR pathways, ATF-6, which increases the apoptosis of HepG2 cells *via* CHOP and Bcl-2/Bax pathway.

DISCUSSION

ER stress produced by tumor cells exposed to intrinsic and external factors often causes tumor growth and resistance to treatment[4,5]. Targeting ER stress signaling in cancer is a potential therapeutic method. Our previous studies and other lab's data show that melatonin exerts its anticancer actions through modulation of the ER stress response[8,9,11,12]. However, there is limited information focused on the effect of melatonin under ER stress conditions and the impact of melatonin on the UPR pathways. In the present study, we obtained evidence suggesting that melatonin can selectively target ATF-6 signal, an important pathway of UPR response initiated by ER stress. We found that melatonin induced hepatoma cell apoptosis through inhibiting the ATF-6 pathway. Our studies demonstrated that downregulation of ATF-6 contributes to the increased susceptibility of liver cancer cells to melatonin treatment under ER stress condition.

HCC is one of the most common hepatobiliary malignant tumors, causing increased cancer mortalities worldwide. As the fifth most common cancer in the world, HCC is always associated with poor prognosis; the 5-year survival rate is less than 17%[2]. Hepatic resection and liver transplantation offers treatment to only 20% because most patients are diagnosed at a late stage[13]. To eliminate the early stages of HCC, local ablation, surgical resection, or liver transplantation was applied to the clinical treatment of HCC. Patients who suffer late stage HCC always present with distant metastasis and liver dysfunctions, and the tumor size no longer allows surgical management. Thus, there are few effective treatments for HCC patients to date.

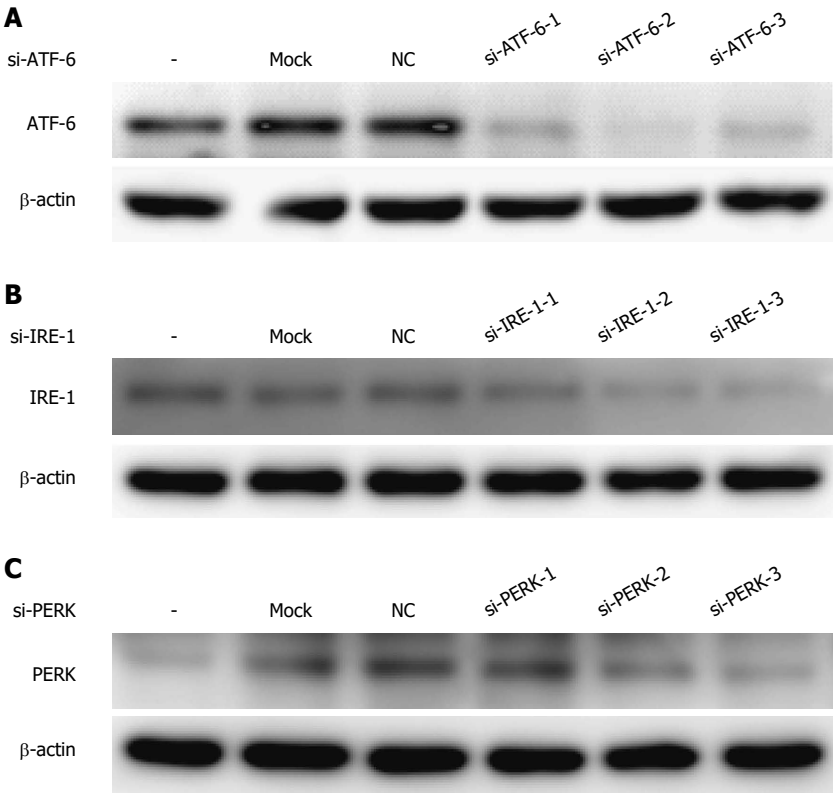

Figure 3 Selection of the most effective sequences of each unfolded protein response pathways siRNA (A-C). All of the candidate sequences of the three unfolded protein response (UPR) pathways were examined by RNA interference, and the expression of UPR proteins was evaluated by western blotting to select the most effective one to interfere with the UPR pathway. ATF-6: Activating transcription factor 6; NC: Negative control; IRE: Inositol-requiring enzyme; PERK: Protein kinase RNA-like endoplasmic reticulum kinases.

A major obstacle that we have yet to overcome is chemotherapy resistance and we still need to clarify the underlying mechanisms. Among the complex mechanisms involved in HCC development and progress, ER stress induction associated with COX-2 is emerging as a very important contributor. COX-2 is a well-known inducible form of cyclooxygenase considered as a good drug target, which is frequently elevated in variety kinds of cancer tissues including HCC[14,15].

There is increasing evidence that shows COX-2 induction as closely associated with ER stress[16,17]. One research group observed that COX-2 and the eIF2α-ATF-4 pathway of ER stress were induced by heavy metal cadmium in both kidney tissues and cultured cells[16]. This finding indicates the ER stress eIF2α-ATF-4 pathway mediates COX-2 overexpression. In the present study, we found that the expression of COX-2 was more likely to be associated with the expression of ATF-6 but not IRE1 and PERK. An *in vitro* cell experiment in which the ATF-6 pathway was blocked using si-ATF-6 showed significant repression of COX-2 expression. These data suggest the existence of a close relationship between ATF-6 and COX-2. Thus, it is rational to search for specific inhibitors of ATF-6 as potential candidates for use as new therapeutic agents for HCC.

As HCC is resistant to systemic chemotherapy, identification of a new intervention or targeted therapy is urgently needed for patients[13]. One of the mechanisms by which liver cancer cells gain resistance to chemotherapy is ER stress[3,4,6]. The ER stress response is a process that can be activated by a number of cellular stress conditions, such as hypoxia, nutrient deprivation, alterations in glycosylation status, and disturbances of calcium flux[3,4,6]. Those conditions always cause imbalances in intracellular homeostasis.

ER stress plays an important role in post-translational modifications[3,4,6]. The ER responds to stress conditions by activating a range of stress response signaling pathways, which is referred to as the UPR. The UPR is fundamentally a cytoprotective response, but excessive or prolonged activation of the UPR can result in apoptosis. In the present study, single tunicamycin-induced ER stress only slightly induced HepG2 cell apoptosis, whereas combination with si-ATF-6 strongly increased the percentage of HepG2 cells undergoing apoptosis. This finding suggests that targeting the ATF-6 pathway in HepG2 cells enhances sensitivity to the apoptosis inducer.

Melatonin is mainly secreted by the human pineal gland, and it has been detected in many other tissues or as being secreted by other organs. Melatonin is a highly lipophilic molecule that can easily cross cell membranes to reach subcellular compartments, including mitochondria, where it exists in high concentrations[18]. Melatonin is able to prevent oxidative

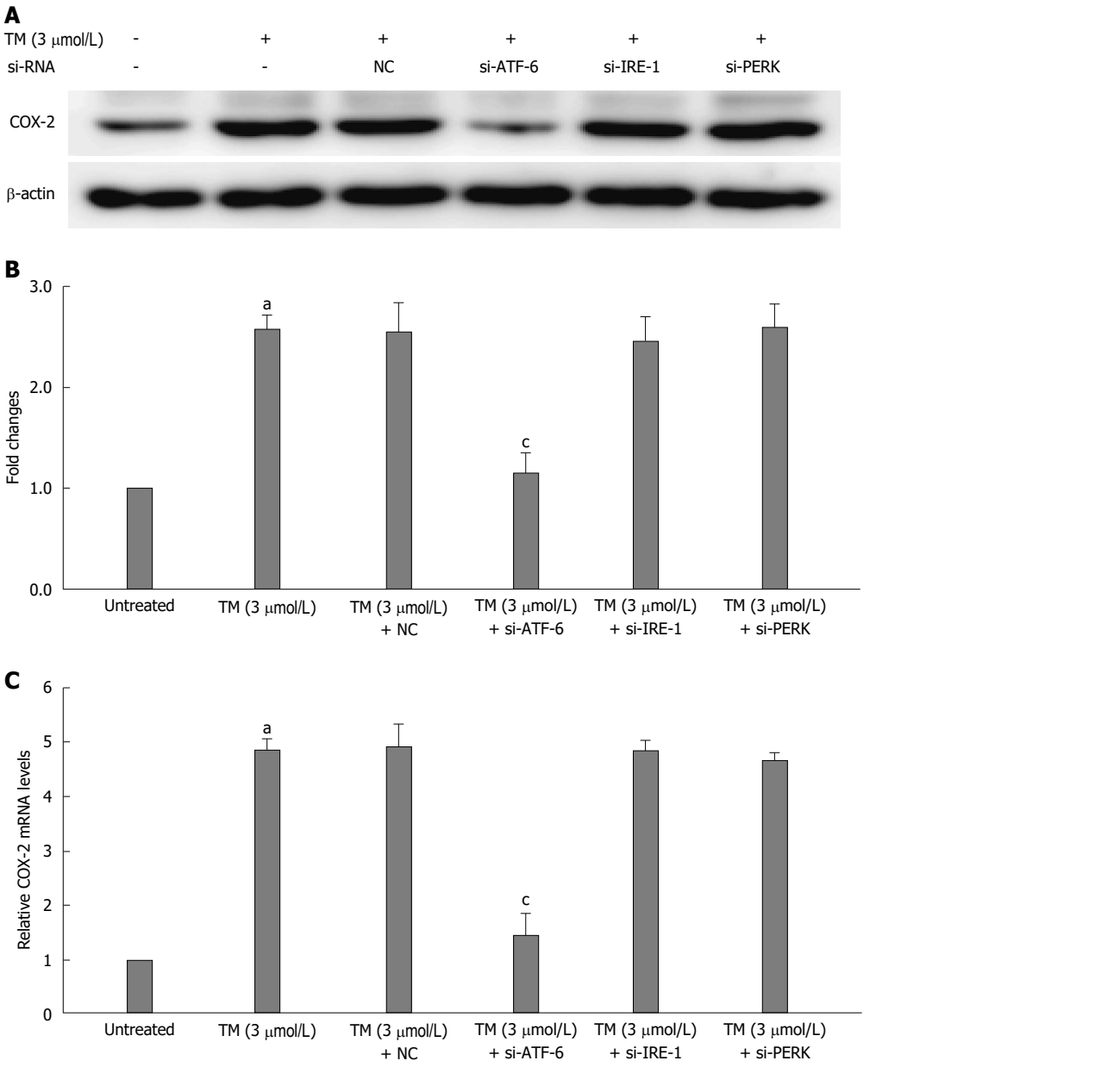

Figure 4 Relationship between activating transcription factor 6 and cyclooxygenase-2 under endoplasmic reticulum stress. The mRNA of the unfolded protein response (UPR) pathways was interfered by ATF-6, IRE-1 and PERK siRNA after pretreatment by tunicamycin (TM) for 8 h. A: Equal protein amounts of cell lysates were subjected to western blot assay using anti-COX-2. β-actin in the same HepG2 cell extract was used as an internal reference. B: Optical density reading values of specific proteins are represented as fold-differences relative to the loading control protein, β-actin. $^{a}P < 0.01$ *vs* the negative control. RNA was harvested and gene expression examined by qRT-PCR in the same condition above. C: The qRT-PCR fold-changes were normalized using the expression of housekeeping gene (GAPDH) and *vs* those obtained from untreated HepG2 cells. $^{a}P < 0.01$ *vs* the negative control; $^{c}P < 0.01$ *vs* the untreated HepG2 cells. ATF-6: Activating transcription factor 6; NC: Negative control; COX-2: Cyclooxygenase-2; IRE: Inositol-requiring enzyme; PERK: Protein kinase RNA-like endoplasmic reticulum kinase.

stress through both its free radical scavenging effect and by directly increasing antioxidant activity[19-25], and different studies have demonstrated its protective role against oxidative damage induced by drugs, toxins, and different diseases[26].

In addition, melatonin also acts upon complex functions through specific nuclear and plasma membrane receptors[27,28]. Melatonin MT1 and MT2 receptors are G protein coupled receptors expressed in various parts of the central nervous system and in peripheral organs, which mediate intracellular effects depending on the changes in intracellular cyclic nucleotides (cAMP, cGMP) and calcium levels, activation of certain protein kinase C subtypes, intracellular localization of steroid hormone receptors and regulation of G protein signaling proteins. Alterations in melatonin receptor expression and the following abnormal signaling pathway, as well as changes in endogenous melatonin production, contribute to the pathophysiology of various diseases, including sleep disorders, depression and Alzheimer's disease[27,28].

Interestingly, experimental and clinical studies

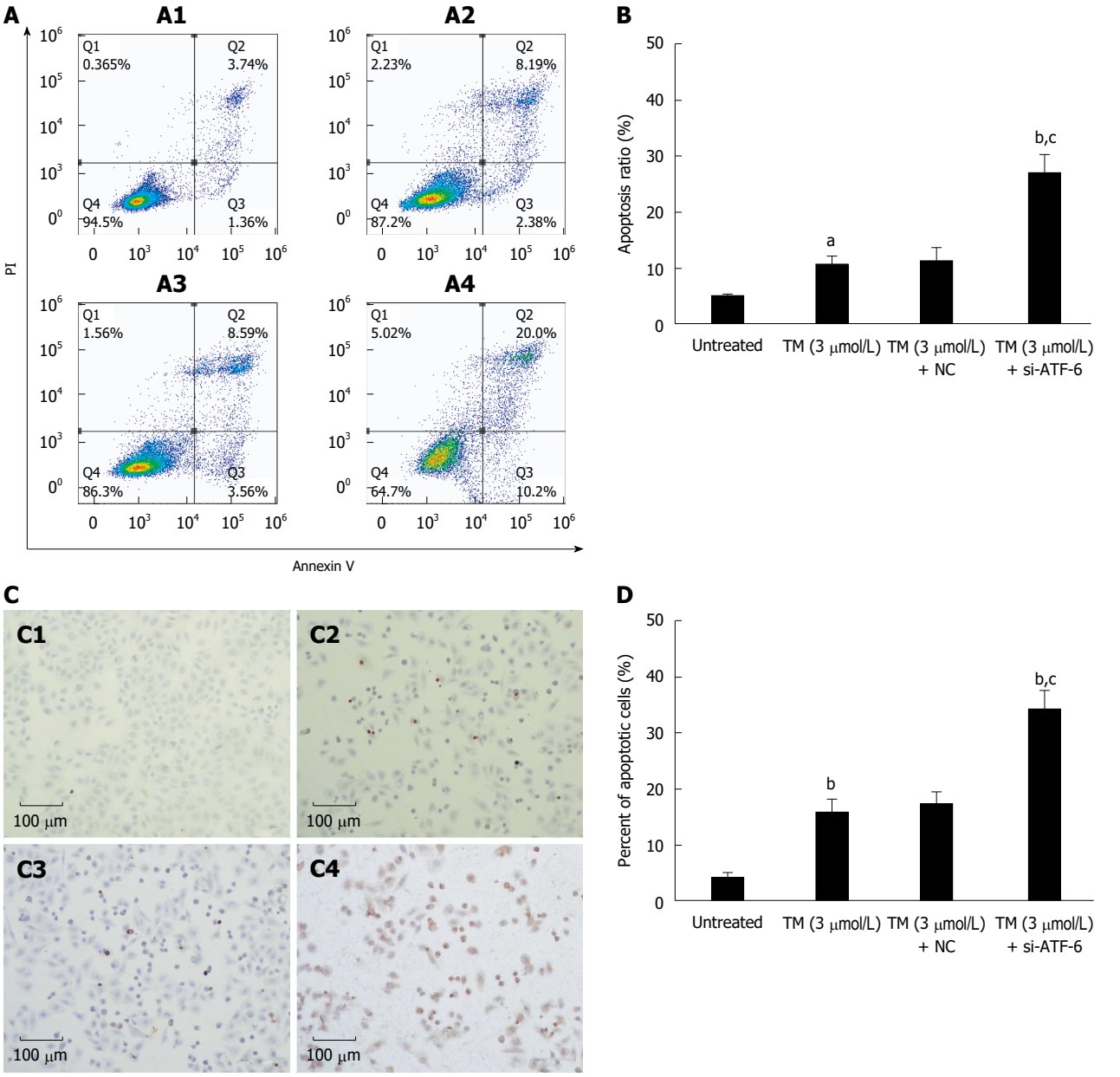

Figure 5 Effects of activating transcription factor 6 siRNA on cell apoptosis in HepG2 cells under endoplasmic reticulum stress. A: HepG2 cells were transfected with ATF-6 siRNA for 24 h after pretreatment by tunicamycin (TM) for 8 h. Apoptotic cells were determined by FACS, and the data are expressed as the mean ± SD. A1, Untreated HepG2 cells; A2, HepG2 cells treated by TM only; A3, HepG2 cells treated by combination of TM and ATF-6 siRNA negative control; A4, HepG2 cells treated by ATF-6 siRNA and melatonin; B: Data are presented as the mean ± SD for the independent experiments ($^aP < 0.05$ *vs* untreated HepG2 cells; $^bP < 0.01$ *vs* untreated HepG2 cells; $^cP < 0.01$ *vs* HepG2 cells treated with TM and ATF-6 siRNA negative control); C: Cell morphology and percentage of apoptotic cells was examined by TUNEL staining. C1, Untreated HepG2 cells; C2, HepG2 cells treated by TM only; C3, HepG2 cells treated by combination of TM and ATF-6 siRNA negative control; C4, HepG2 cells treated by ATF-6 siRNA and melatonin; D: Data are presented as the mean ± SD for the independent experiments ($^bP < 0.01$ *vs* untreated HepG2 cells; $^cP < 0.01$ *vs* HepG2 cells treated with TM and ATF-6 siRNA negative control). ATF-6: Activating transcription factor 6; NC: Negative control.

recommend an increase in the awareness of melatonin as a therapeutic agent in cancers including gastrointestinal tract cancer[10,29,30]. Our previous studies and other labs' findings suggest that melatonin exerts its anticancer action through suppressing COX-2 and attenuating ER stress-induced drug resistance[11,12,31,32]. A recent study also showed that melatonin inhibits the expression of proangiogenic proteins HIF-1α and VEGF in conditions of normoxia and hypoxia using the HepG2 cell line[33].

ER stress induced by hepatitis B virus X (HBx) protein enhances COX-2 expression *via* activating transcription factor 4 (ATF-4). Further experiment showed that ATF-4 binding to the COX-2 promoter plays a critical role in HBx-mediated COX-2 induction[34]. In addition, melatonin enhances antitumor function through upregulation of the pro-apoptotic protein BimBim expression and downregulation of COX-2 expression in tunicamycin-treated breast carcinoma MDA-MB-231 cells[35].

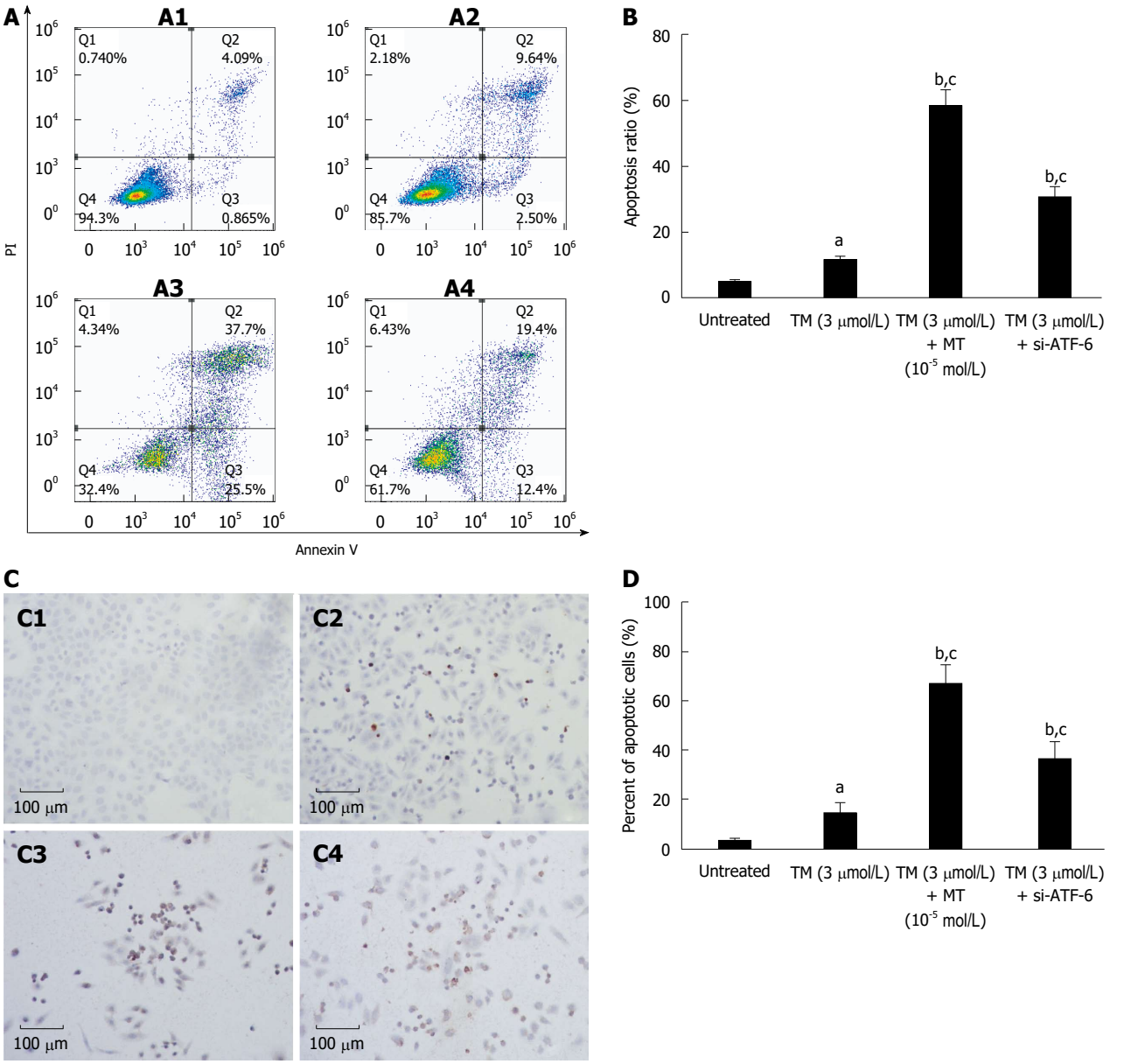

Figure 6 Comparison between melatonin and activating transcription factor 6 siRNA transfection on cell apoptosis in HepG2 cells under endoplasmic reticulum stress. A: HepG2 cells were exposed to melatonin (10^{-5} mmol/L) for 24 h after pretreatment by tunicamycin (TM) for 8 h or were transfected with ATF-6 siRNA for 6 h after pretreatment by tunicamycin TM for 8 h. Apoptotic cells were determined by FACS, and the data are expressed as the mean ± SD. A1, Untreated HepG2 cells; A2, HepG2 cells treated by TM only; A3, HepG2 cells treated by combination of TM and melatonin (10^{-5} mmol/L); A4, HepG2 cells treated by ATF-6 siRNA and melatonin; B: Data are presented as the mean ± SD for the independent experiments ($^{a}P < 0.05$ *vs* untreated HepG2 cells; $^{b}P < 0.01$ *vs* untreated HepG2 cells; $^{c}P < 0.01$ HepG2 cells treated by TM and melatonin *vs* HepG2 cells treated with TM and ATF-6 siRNA negative control); C: Cell morphology and percentage of apoptotic cells were examined by TUNEL staining. C1, Untreated HepG2 cells; C2, HepG2 cells treated by TM only; C3, HepG2 cells treated by combination of TM and melatonin (10^{-5} mmol/L); C4, HepG2 cells treated by ATF-6 siRNA and melatonin; D: Data are presented as the mean ± SD for the independent experiments ($^{a}P < 0.05$ *vs* untreated HepG2 cells; $^{b}P < 0.01$ *vs* untreated HepG2 cells; $^{c}P < 0.01$ HepG2 cells treated by TM and melatonin *vs* HepG2 cells treated with TM and ATF-6 siRNA negative control). ATF-6: Activating transcription factor 6; MT: Melatonin.

The present study further explored the new mechanism of specific effect of melatonin on ATF-6, one of the UPR responses. We found that melatonin selectively inhibited the ATF-6 expression (Figure 1). As it is well-known that melatonin has an inhibitory effect on COX-2 activity[36], it is easy to speculate and understand that si-ATF-6 has a similar COX-2 suppression as melatonin (Figure 4). Furthermore, we confirmed that downregulation of ATF-6 by melatonin contributes to the increased susceptibility of liver cancer cells to melatonin treatment under ER stress condition (Figure 6). Dramatically increased CHOP level led to suppressed COX-2 and decreased Bcl-2/Bax ratio by melatonin, and ATF-6 siRNA contributed to the enhanced HepG2 cell apoptosis under ER stress stimulation (Figure 7). These findings indicate that melatonin, as a selective ATF-6 inhibitor, can sensitize human hepatoma cells to ER stress-induced apoptosis.

In summary, our study provides the new mechanism by which melatonin downregulates COX-2

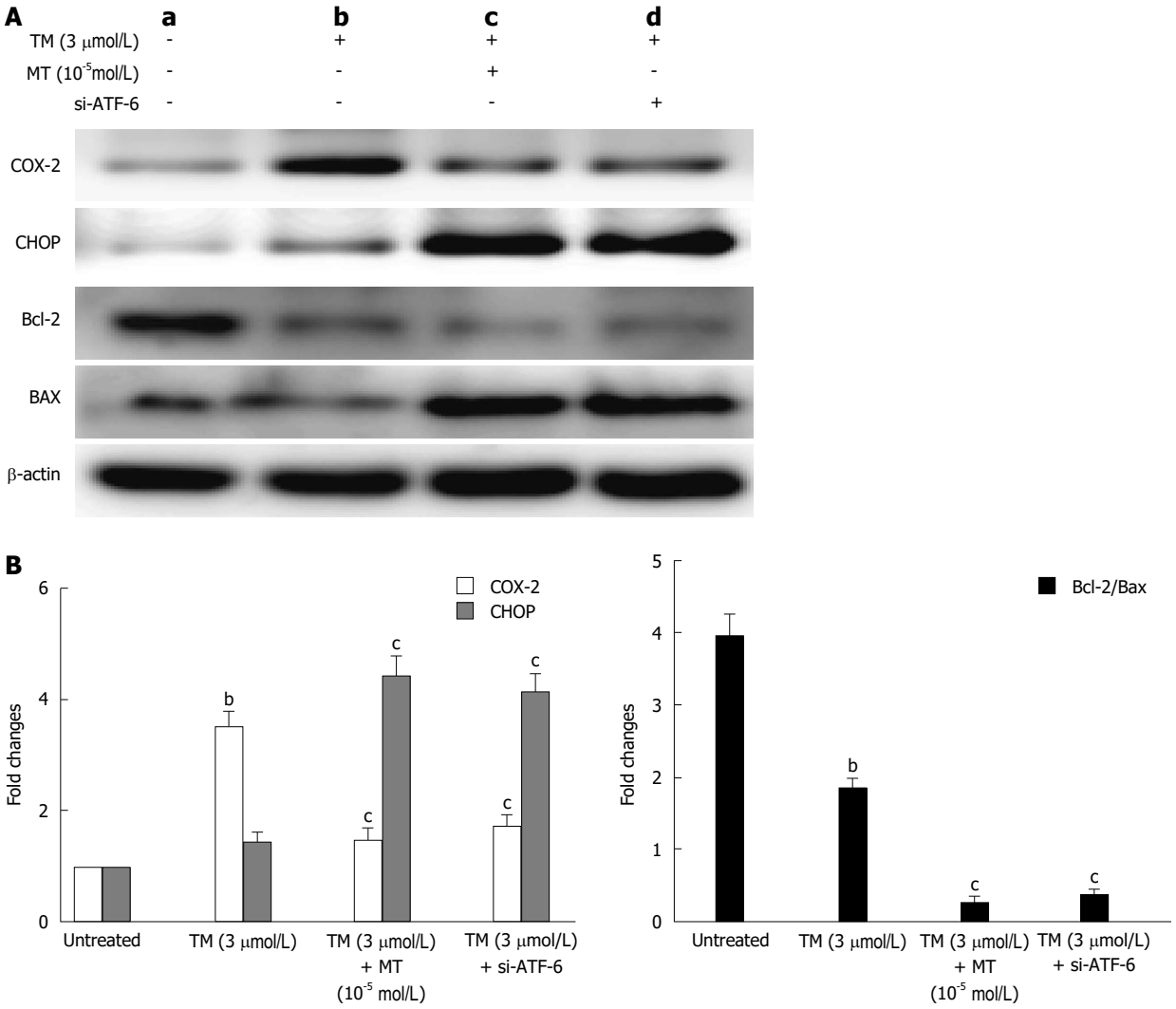

Figure 7 Pathway by which melatonin sensitized HepG2 to endoplasmic reticulum stress-induced apoptosis. A: a, Untreated HepG2 cells; b, HepG2 cells treated by tunicamycin (TM) only; c, HepG2 cells treated by TM, melatonin (10^{-5} mmol/L); d: HepG2 cells treated by TM and ATF-6 siRNA. Equal protein amounts of cell lysates were subjected to western blot assay using anti-COX-2, anti-CHOP, anti-Bcl-2, and anti-Bax. β-actin in the same HepG2 cell extract was used as an internal reference; B: Optical density reading values of specific proteins are represented as fold-differences relative to the loading control protein, β-actin. $^{c}P < 0.01$, CHOP *vs* the Bcl-2 and Bax expression, $^{b}P < 0.01$, *vs* the untreated HepG2 cells. ATF-6: Activating transcription factor 6; COX-2: Cyclooxygenase-2; CHOP: CCAAT-enhancer-binding protein homologous protein; MT: Melatonin.

expression and sensitizes apoptosis by selectively targeting ATF-6 in human HCC cells under ER stress. Our results raise the possibility that melatonin may be a promising approach in targeting ER stress-induced apoptosis as a therapeutic strategy for the treatment of HCC and other cancers. We also identified that of the three UPR pathways, ATF-6 was positively associated with COX-2 in HCC patient samples. Therefore, if there are any agents that can knockdown ATF-6, like melatonin, they can be used to treat HCC. However, we still need to investigate whether this effect of melatonin in ER stress-induced tumor apoptosis *in vitro* will also work well *in vivo*. Because of the low toxicity and well-documented oncostatic effects of melatonin, we believe melatonin has a promising future in the treatment of HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is a frequently diagnosed cancer in China and has a high lethality rate. Yet, there are few effective chemotherapy agents for this malignant tumor. One of the major reasons of untreatable HCC is that the liver cancer cell has greater tolerance towards a number of cellular stress conditions, especially endoplasmic reticulum (ER) stress. The published works by other authors have shown that melatonin can sensitize the human HCC cell to ER stress-induced apoptosis and that melatonin attenuates ER stress-induced resistance to doxorubicin. However, the precise mechanisms involved in the critical unfolded protein response (UPR) pathway modified by melatonin still need to be investigated.

Research frontiers

To the best knowledge of the authors, this is the first study to identify melatonin as a selective ATF-6 blocker, thereby inhibiting COX-2 and leading to enhanced cell apoptosis in liver cancer.

Innovations and breakthroughs

This is the first study investigating the precise mechanisms of the critical UPR pathways modified by melatonin.

Applications

The promising findings presented in the current report suggest that melatonin, as a novel selective ATF-6 inhibitor, can sensitize human hepatoma cells to ER stress inducing apoptosis, which may be considered as a therapeutic strategy for the treatment of HCC and other cancers.

Terminology

Melatonin plays important roles in human physiological and pharmacological functions, such as circadian rhythms and antioxidants. Interestingly, melatonin exerts anticancer effects through interplay with ER stress. The published studies show that melatonin sensitizes the human HCC cell to ER stress-induced apoptosis. Furthermore, melatonin also attenuates ER stress-induced resistance to doxorubicin through reversing tunicamycin-induced ER stress. In the current study, the authors first demonstrated that melatonin selectively blocks ATF-6 and then inhibits COX-2 expression, leading to enhanced liver cancer cell apoptosis. In clinical HCC patients, the close relationship between ATF-6 and COX-2 was further confirmed. The results presented raise the possibility that melatonin may be used in the treatment of HCC and other cancers.

Peer-review

In general, the enclosed set of data are very interesting. The article will be interesting for readers of this journal.

REFERENCES

1 **Chen W**, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J. Cancer statistics in China, 2015. *CA Cancer J Clin* 2016; **66**: 115-132 [PMID: 26808342 DOI: 10.3322/caac.21338]

2 **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin* 2016; **66**: 7-30 [PMID: 26742998 DOI: 10.3322/caac.21332]

3 **Binet F**, Sapieha P. ER Stress and Angiogenesis. *Cell Metab* 2015; **22**: 560-575 [PMID: 26278049 DOI: 10.1016/j.cmet.2015.07.010]

4 **Yadav RK**, Chae SW, Kim HR, Chae HJ. Endoplasmic reticulum stress and cancer. *J Cancer Prev* 2014; **19**: 75-88 [PMID: 25337575 DOI: 10.15430/JCP.2014.19.2.75]

5 **Chevet E**, Hetz C, Samali A. Endoplasmic reticulum stress-activated cell reprogramming in oncogenesis. *Cancer Discov* 2015; **5**: 586-597 [PMID: 25977222 DOI: 10.1158/2159-8290.CD-14-1490]

6 **Hiramatsu N**, Chiang WC, Kurt TD, Sigurdson CJ, Lin JH. Multiple Mechanisms of Unfolded Protein Response-Induced Cell Death. *Am J Pathol* 2015; **185**: 1800-1808 [PMID: 25956028 DOI: 10.1016/j.ajpath.2015.03.009]

7 **Kato H**, Nishitoh H. Stress responses from the endoplasmic reticulum in cancer. *Front Oncol* 2015; **5**: 93 [PMID: 25941664 DOI: 10.3389/fonc.2015.00093]

8 **Fernández A**, Ordóñez R, Reiter RJ, González-Gallego J, Mauriz JL. Melatonin and endoplasmic reticulum stress: relation to autophagy and apoptosis. *J Pineal Res* 2015; **59**: 292-307 [PMID: 26201382 DOI: 10.1111/jpi.12264]

9 **Wu SM**, Lin WY, Shen CC, Pan HC, Keh-Bin W, Chen YC, Jan YJ, Lai DW, Tang SC, Tien HR, Chiu CS, Tsai TC, Lai YL, Sheu ML. Melatonin set out to ER stress signaling thwarts epithelial mesenchymal transition and peritoneal dissemination via calpain-mediated C/EBPβ and NFκB cleavage. *J Pineal Res* 2016; **60**: 142-154 [PMID: 26514342 DOI: 10.1111/jpi.12295]

10 **Xin Z**, Jiang S, Jiang P, Yan X, Fan C, Di S, Wu G, Yang Y, Reiter RJ, Ji G. Melatonin as a treatment for gastrointestinal cancer: a review. *J Pineal Res* 2015; **58**: 375-387 [PMID: 25752643 DOI: 10.1111/jpi.12227]

11 **Zha L**, Fan L, Sun G, Wang H, Ma T, Zhong F, Wei W. Melatonin sensitizes human hepatoma cells to endoplasmic reticulum stress-induced apoptosis. *J Pineal Res* 2012; **52**: 322-331 [PMID: 22225575 DOI: 10.1111/j.1600-079X.2011.00946.x]

12 **Fan L**, Sun G, Ma T, Zhong F, Lei Y, Li X, Wei W. Melatonin reverses tunicamycin-induced endoplasmic reticulum stress in human hepatocellular carcinoma cells and improves cytotoxic response to doxorubicin by increasing CHOP and decreasing survivin. *J Pineal Res* 2013; **55**: 184-194 [PMID: 23711089 DOI: 10.1111/jpi.12061]

13 **Bruix J**, Reig M, Sherman M. Evidence-Based Diagnosis, Staging, and Treatment of Patients With Hepatocellular Carcinoma. *Gastroenterology* 2016; **150**: 835-853 [PMID: 26795574 DOI: 10.1053/j.gastro.2015.12.041]

14 **Breinig M**, Schirmacher P, Kern MA. Cyclooxygenase-2 (COX-2)--a therapeutic target in liver cancer? *Curr Pharm Des* 2007; **13**: 3305-3315 [PMID: 18045183]

15 **Misra S**, Sharma K. COX-2 signaling and cancer: new players in old arena. *Curr Drug Targets* 2014; **15**: 347-359 [PMID: 24467618]

16 **Luo B**, Lin Y, Jiang S, Huang L, Yao H, Zhuang Q, Zhao R, Liu H, He C, Lin Z. Endoplasmic reticulum stress eIF2α-ATF4 pathway-mediated cyclooxygenase-2 induction regulates cadmium-induced autophagy in kidney. *Cell Death Dis* 2016; **7**: e2251 [PMID: 27253415 DOI: 10.1038/cddis.2016.78]

17 **Hung JH**, Su IJ, Lei HY, Wang HC, Lin WC, Chang WT, Huang W, Chang WC, Chang YS, Chen CC, Lai MD. Endoplasmic reticulum stress stimulates the expression of cyclooxygenase-2 through activation of NF-kappaB and pp38 mitogen-activated protein kinase. *J Biol Chem* 2004; **279**: 46384-46392 [PMID: 15319438 DOI: 10.1074/jbc.M403568200]

18 **Paradies G**, Petrosillo G, Paradies V, Reiter RJ, Ruggiero FM. Melatonin, cardiolipin and mitochondrial bioenergetics in health and disease. *J Pineal Res* 2010; **48**: 297-310 [PMID: 20433638 DOI: 10.1111/j.1600-079X.2010.00759.x]

19 **Galano A**, Tan DX, Reiter RJ. Melatonin as a natural ally against oxidative stress: a physicochemical examination. *J Pineal Res* 2011; **51**: 1-16 [PMID: 21752095 DOI: 10.1111/j.1600-079X.2011.00916.x]

20 **Tomás-Zapico C**, Coto-Montes A.A proposed mechanism to explain the stimulatory effect of melatonin on antioxidative enzymes. *J Pineal Res* 2005; **39**: 99-104 [PMID: 16098085 DOI: 10.1111/j.1600-079X.2005.00248.x]

21 **Kleszczyński K**, Zillikens D, Fischer TW. Melatonin enhances mitochondrial ATP synthesis, reduces reactive oxygen species formation, and mediates translocation of the nuclear erythroid 2-related factor 2 resulting in activation of phase-2 antioxidant enzymes (γ-GCS, HO-1, NQO1) in ultraviolet radiation-treated normal human epidermal keratinocytes (NHEK). *J Pineal Res* 2016; **61**: 187-197 [PMID: 27117941 DOI: 10.1111/jpi.12338]

22 **Kleszczyński K**, Tukaj S, Kruse N, Zillikens D, Fischer TW. Melatonin prevents ultraviolet radiation-induced alterations in plasma membrane potential and intracellular pH in human keratinocytes. *J Pineal Res* 2013; **54**: 89-99 [PMID: 22856627 DOI: 10.1111/j.1600-079X.2012.01028.x]

23 **Fischer TW**, Kleszczyński K, Hardkop LH, Kruse N, Zillikens D. Melatonin enhances antioxidative enzyme gene expression (CAT, GPx, SOD), prevents their UVR-induced depletion, and protects against the formation of DNA damage (8-hydroxy-2'-deoxyguanosine) in ex vivo human skin. *J Pineal Res* 2013; **54**: 303-312 [PMID: 23110400 DOI: 10.1111/jpi.12018]

24 **Fischer TW**, Scholz G, Knöll B, Hipler UC, Elsner P. Melatonin suppresses reactive oxygen species induced by UV irradiation in leukocytes. *J Pineal Res* 2004; **37**: 107-112 [PMID: 15298669 DOI: 10.1111/j.1600-079X.2004.00142.x]

25 **Slominski A**, Pisarchik A, Zbytek B, Tobin DJ, Kauser S, Wortsman J. Functional activity of serotoninergic and melatoninergic systems expressed in the skin. *J Cell Physiol* 2003; **196**: 144-153 [PMID: 12767050 DOI: 10.1002/jcp.10287]

26 **García JJ**, López-Pingarrón L, Almeida-Souza P, Tres A, Escudero P, García-Gil FA, Tan DX, Reiter RJ, Ramírez JM, Bernal-Pérez M. Protective effects of melatonin in reducing oxidative stress and in preserving the fluidity of biological membranes: a review. *J Pineal Res* 2014; **56**: 225-237 [PMID: 24571249 DOI: 10.1111/jpi.12128]

27 **Pandi-Perumal SR**, Trakht I, Srinivasan V, Spence DW, Maestroni GJ, Zisapel N, Cardinali DP. Physiological effects of melatonin:

role of melatonin receptors and signal transduction pathways. *Prog Neurobiol* 2008; **85**: 335-353 [PMID: 18571301 DOI: 10.1016/j.pneurobio.2008.04.001]

28 **Emet M**, Ozcan H, Ozel L, Yayla M, Halici Z, Hacimuftuoglu A. A Review of Melatonin, Its Receptors and Drugs. *Eurasian J Med* 2016; **48**: 135-141 [PMID: 27551178 DOI: 10.5152/eurasianjmed.2015.0267]

29 **Mills E**, Wu P, Seely D, Guyatt G. Melatonin in the treatment of cancer: a systematic review of randomized controlled trials and meta-analysis. *J Pineal Res* 2005; **39**: 360-366 [PMID: 16207291 DOI: 10.1111/j.1600-079X.2005.00258.x]

30 **Reiter RJ**, Rosales-Corral SA, Manchester LC, Liu X, Tan DX. Melatonin in the biliary tract and liver: health implications. *Curr Pharm Des* 2014; **20**: 4788-4801 [PMID: 24251672]

31 **Carbajo-Pescador S**, Steinmetz C, Kashyap A, Lorenz S, Mauriz JL, Heise M, Galle PR, González-Gallego J, Strand S. Melatonin induces transcriptional regulation of Bim by FoxO3a in HepG2 cells. *Br J Cancer* 2013; **108**: 442-449 [PMID: 23257900 DOI: 10.1038/bjc.2012.563]

32 **Carbajo-Pescador S**, García-Palomo A, Martín-Renedo J, Piva M, González-Gallego J, Mauriz JL. Melatonin modulation of intracellular signaling pathways in hepatocarcinoma HepG2 cell line: role of the MT1 receptor. *J Pineal Res* 2011; **51**: 463-471 [PMID: 21718361 DOI: 10.1111/j.1600-079X.2011.00910.x]

33 **Colombo J**, Maciel JM, Ferreira LC, DA Silva RF, Zuccari DA. Effects of melatonin on HIF-1α and VEGF expression and on the invasive properties of hepatocarcinoma cells. *Oncol Lett* 2016; **12**: 231-237 [PMID: 27347130 DOI: 10.3892/ol.2016.4605]

34 **Cho HK**, Cheong KJ, Kim HY, Cheong J. Endoplasmic reticulum stress induced by hepatitis B virus X protein enhances cyclo-oxygenase 2 expression via activating transcription factor 4. *Biochem J* 2011; **435**: 431-439 [PMID: 21244365 DOI: 10.1042/BJ20102071]

35 **Woo SM**, Min KJ, Kwon TK. Melatonin-mediated Bim up-regulation and cyclooxygenase-2 (COX-2) down-regulation enhances tunicamycin-induced apoptosis in MDA-MB-231 cells. *J Pineal Res* 2015; **58**: 310-320 [PMID: 25711465 DOI: 10.1111/jpi.12217]

36 **Aparicio-Soto M**, Alarcón-de-la-Lastra C, Cárdeno A, Sánchez-Fidalgo S, Sanchez-Hidalgo M. Melatonin modulates microsomal PGE synthase 1 and NF-E2-related factor-2-regulated antioxidant enzyme expression in LPS-induced murine peritoneal macrophages. *Br J Pharmacol* 2014; **171**: 134-144 [PMID: 24116971 DOI: 10.1111/bph.12428]

P- Reviewer: Kleszczynski K, Zavodnik IB **S- Editor**: Yu J
L- Editor: Filipodia **E- Editor**: Liu WX

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v23.i6.999

World J Gastroenterol 2017 February 14; 23(6): 999-1009
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

Effect of toll-like receptor 3 agonist poly I:C on intestinal mucosa and epithelial barrier function in mouse models of acute colitis

Hong-Wei Zhao, Yue-Hong Yue, Hua Han, Xiu-Li Chen, Yong-Gang Lu, Ji-Min Zheng, Hong-Tao Hou, Xiao-Meng Lang, Li-Li He, Qi-Lu Hu, Zi-Qian Dun

Hong-Wei Zhao, Ji-Min Zheng, Hong-Tao Hou, Department of Gastroenterology, Hebei General Hospital, Shijiazhuang 050051, Hebei Province, China

Yue-Hong Yue, Department of Neurology, Hebei General Hospital, Shijiazhuang 050051, Hebei Province, China

Hua Han, Department of Obstetrics and Gynecology, Hebei General Hospital, Shijiazhuang 050051, Hebei Province, China

Xiu-Li Chen, Research Center, The Fifth Hospital of Shijiazhuang, Shijiazhuang 050021, Hebei Province, China

Yong-Gang Lu, Department of Clinical Laboratory, Hebei General Hospital, Shijiazhuang 050051, Hebei Province, China

Xiao-Meng Lang, Department of Spleen and Stomach, Hebei Province Hospital of Traditional Chinese Medicine, Shijiazhuang 500011, Hebei Province, China

Li-Li He, Department of Gerontology, Hebei General Hospital, Shijiazhuang 050051, Hebei Province, China

Qi-Lu Hu, Department of Oncology, Hebei General Hospital, Shijiazhuang 050051, Hebei Province, China

Zi-Qian Dun, Department of General Internal Medicine, The Fifth Hospital of Shijiazhuang, Shijiazhuang 050021, Hebei Province, China

Author contributions: Zhao HW, Yue YH, Han H, Chen XL and Lu YG participated in study design, experiment performance, data interpretation and manuscript revision and coordination; Zheng JM, Hou HT and Lang XM contributed to experiment performance, data collection and manuscript drafting; He LL, Hu QL and Dun ZQ worked on experiment performance and manuscript revision; all authors contributed significantly to this work.

Supported by the Scientific Research Foundation of Health Department of Hebei Province, No. 20160483.

Institutional review board statement: This work was approved by the Institutional Review Board of Hebei General Hospital.

Institutional animal care and use committee statement: All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC, Approval ID: I07-038-3).

Conflict-of-interest statement: The authors declare no conflict of interest.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Correspondence to: Hong-Wei Zhao, MD, Department of Gastroenterology, Hebei General Hospital, No.348 Hepingxi Road, Shijiazhuang 050051, Hebei Province, China. hongweizhao788@163.com
Telephone: +86-13303039680
Fax: +86-311-85988449

Received: August 17, 2016
Peer-review started: August 19, 2016
First decision: September 6, 2016
Revised: September 26, 2016
Accepted: October 30, 2016

Article in press: October 31, 2016
Published online: February 14, 2017

Abstract

AIM

To investigate potential effects of poly I:C on mucosal injury and epithelial barrier disruption in dextran sulfate sodium (DSS)-induced acute colitis.

METHODS

Thirty C57BL/6 mice were given either regular drinking water (control group) or 2% (w/v) DSS drinking water (model and poly I:C groups) *ad libitum* for 7 d. Poly I:C was administrated subcutaneously (20 μg/mouse) 2 h prior to DSS induction in mice of the poly I:C group. Severity of colitis was evaluated by disease activity index, body weight, colon length, histology and myeloperoxidase (MPO) activity, as well as the production of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin 17 (IL-17) and interferon-γ (IFN-γ). Intestinal permeability was analyzed by the fluorescein isothiocyanate labeled-dextran (FITC-D) method. Ultrastructural features of the colon tissue were observed under electron microscopy. Expressions of tight junction (TJ) proteins, including zo-1, occludin and claudin-1, were measured by immunohistochemistry/immunofluorescence, Western blot and real-time quantitative polymerase chain reaction (RT-qPCR).

RESULTS

DSS caused significant damage to the colon tissue in the model group. Administration of poly I:C dramatically protected against DSS-induced colitis, as demonstrated by less body weight loss, lower disease activity index score, longer colon length, colonic MPO activity, and improved macroscopic and histological scores. It also ameliorated DSS-induced ultrastructural changes of the colon epithelium, as observed under scanning electron microscopy, as well as FITC-D permeability. The mRNA and protein expressions of TJ protein, zo-1, occludin and claudin-1 were also found to be significantly enhanced in the poly I:C group, as determined by immunohistochemistry/immunofluorescence, Western blot and RT-qPCR. By contrast, poly I:C pretreatment markedly reversed the DSS-induced up-regulated expressions of the inflammatory cytokines TNF-α, IL-17 and IFN-γ.

CONCLUSION

Our study suggested that poly I:C may protect against DSS-induced colitis through maintaining integrity of the epithelial barrier and regulating innate immune responses, which may shed light on the therapeutic potential of poly I:C in human colitis.

Key words: Dextran sulfate sodium-induced acute colitis; Mucosal injury; Epithelial barrier disruption; Tight junction; Poly I:C

Core tip: Poly I:C, a toll-like receptor 3 agonist, has been previously reported to protect against acute colitis. The potential effects of poly I:C on mucosal injury and epithelial barrier disruption were investigated in mouse models of dextran sulfate sodium (DSS)-induced acute colitis. Poly I:C administration dramatically protected against DSS-induced colitis, with ameliorated ultrastructural changes of colon epithelium, intestinal permeability and tight junction protein expressions. Poly I:C may protect against DSS-induced colitis through maintaining integrity of the epithelial barrier and regulating innate immune responses.

Zhao HW, Yue YH, Han H, Chen XL, Lu YG, Zheng JM, Hou HT, Lang XM, He LL, Hu QL, Dun ZQ. Effect of toll-like receptor 3 agonist poly I:C on intestinal mucosa and epithelial barrier function in mouse models of acute colitis. *World J Gastroenterol* 2017; 23(6): 999-1009 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i6/999.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i6.999

INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are common chronic diseases that are characterized by abnormal mucosal immune response to luminal bacteria[1,2]. The innate and adaptive immune responses have been suggested to engage in the initiation of inflammation and relapse of disease activity, with increased intestinal levels of tumor necrosis factor-α (TNF-α), interleukin 17 (IL-17) and interferon-γ (IFN-γ)[3,4]. The increased intestinal inflammation may, in turn, lead to impaired mucosal barrier function and intestinal permeability, which allow subsequent translocation of microorganisms to the mucosal lymphatic tissue. The down-regulated expressions of junction complex proteins have been demonstrated in the intestinal mucosa of patients with IBD[5]. The impaired gut epithelial barrier function may cause persistent immune activation and, thereby, enhance inflammation of the intestinal mucosa[6].

Epithelial barrier function is determined by intestinal permeability, which is mainly maintained by tight junctions (TJs)[7]. As the most apical intercellular structure of the junctional complex in epithelial cells, TJs serve as the permeability barrier to paracellular transport of solutes[8,9]. Increased intestinal permeability has been reported to associate with the pathogenesis of IBD[10]. Therefore, maintenance of TJ and barrier function may be beneficial for patients with IBD[11-14].

Toll-like receptors (TLRs) are ancient microbial pattern recognition receptors that play an essential role in initiation of immune responses. Subcutaneous

administration of poly I:C, a synthetic TLR3 agonist, has been reported to dramatically protect against dextran sulfate sodium (DSS)-induced acute colitis[15]. However, another study also indicated that abnormal activation of TLR3 signaling by poly I:C broke down the mucosal homeostasis and caused mucosal damage in the small intestine[16]. TLR3 signaling may be involved in the process of epithelial destruction and mucosal injury[17]. However, the potential effects of TLR3 activation by poly I:C on mucosal injury and epithelial barrier disruption in DSS-induced acute colitis have not been well investigated up to now. Therefore, the present study aimed to investigate the potential role of poly I:C administration on the intestinal mucosal barrier function and intestinal permeability in the mouse model of DSS-induced acute colitis.

MATERIALS AND METHODS

Animals

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC, Approval ID: I07-038-3). Male C57BL/6 mice (8 wk, 18-22 g) were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) [License No.: SCXK (Beijing) 2006-0009]. Animals were housed under standard conditions in a barrier facility, according to the protocols of IACUC and Hebei Medical University Vivarium (GB 14925-2001).

Poly I:C reconstitution

Poly I:C was prepared as described previously[18]. Briefly, poly I:C (GE Healthcare, Piscataway, NJ, United States) was dissolved in sterile phosphate-buffered saline (PBS) at a concentration of 2 mg/mL and heated at 50 ℃ until solubilization. The solution was then slowly cooled down to room temperature for proper annealing. The same lot of poly I:C was employed throughout the study.

Acute colitis models

Acute colitis was induced by DSS (40000-50000 MW; Sigma, St. Louis, MO, United States) as described in our previous report[19]. Briefly, 30 C57BL/6 mice were randomly assigned to three groups: control group, model group, and poly I:C group (n = 10 per group). Mice in the poly I:C group were administrated poly I:C subcutaneously (20 μg/mouse) in 100 μL PBS at 2 h prior to DSS treatment. Mice in the control and model groups were given normal saline. Animals were then given either regular drinking water (control group) or 2% (w/v) DSS drinking water (model and poly I:C groups) *ad libitum* for 7 d, and resumed on water for the remainder of the experiments.

Disease activity assessment

Rachmilewitz disease activity index (DAI) was assessed by an investigator blinded to the protocol and according to the well-established scoring system[20]. The data of body weight (BW), stool consistency and occult blood (OB) in the stool were recorded. Loss in BW was scored as: 0: no weight loss, 1: 1%-5% weight loss from baseline, 2: 5%-10%, 3: 10%-20%, and 4: more than 20% weight loss. Stool consistency was scored as: 0: well-formed pellets, 2: pasty and semi-formed stools that do not adhere to the anus, and 4: liquid stools that adhere to the anus. OB was scored as: 0: no blood, 2: positive hemoccult, and 4: gross bleeding. These scores were added together and divided by 3 to result in the DAI ranging from 0 (healthy) to 4 (maximal activity of colitis).

Colonic injury and inflammation

The entire colon (from the cecum to anus) was removed and the lengths were measured as an inflammation marker. The degree of colonic damage was examined macroscopically using a 0-4 scale[19]: 0: normal colon tissue; 1: minimal colon wall thickening without congestion; 2: moderate colon wall thickening with congestion; 3: moderate colon wall thickening, rigidity and congestion; and 4: marked colon wall thickening, rigidity, and congestion. The extent of tissue damage was assessed microscopically using a semi-quantitative scoring system[21]. In detail, the proximal colon was removed, fixed in 10% formalin and embedded in paraffin. The sections of 4 μm thickness were stained with hematoxylin and eosin (HE) and scored using the following parameters: (1) severity of inflammation (0-3: none, slight, moderate, severe); (2) extent of injury (0-3: none, mucosal, mucosal and submucosal, transmural); and (3) crypt damage (0-4: none, basal 1/3 damaged, basal 2/3 damaged, only surface epithelium intact, entire crypt and epithelium lost). The total score was the sum of each parameter multiplying an equivalent reflecting the percentage of tissue involved (× 1: 0%-25%, × 2: 26%-50%, × 3: 51%-75%, × 4: 76%-100%).

Myeloperoxidase activity assay

Colons (100 mg wet weight) were isolated from each group and homogenized in 1 mL buffer (0.05% hexadecyltrimethylammonium bromide in 50 mmol/L phosphate buffer, pH 6.0). The resulting homogenates were centrifuged at 2000 g and 4 ℃. The supernatants were harvested and stored at -80 ℃ for Myeloperoxidase (MPO) activity assay. The samples (10 μL) were transferred to a 96-well plate and incubated with 3 μL odianisidine hydrochloride (20 mg/mL) in 290 μL 50 mmol/L phosphate buffer and 3 μL H_2O_2 (20 mmol/L). The reaction was stopped by adding 3 μL sodium azide (30%). Light absorbance at 460 nm was read. MPO activity was determined by the curve obtained from the standard MPO[22].

Immunohistochemistry/immunofluorescence

For immunohistochemistry staining, the sections were

Table 1 Primers used for real-time quantitative polymerase chain reaction analysis

Gene		Primers	Length, bp
zo-1	Forward	5′-TCATCCCAAATAAGAACAGAGC-3	198
	Reverse	5′-GAAGAACAACCCTTTCATAAGC-3′	
Occludin	Forward	5′-CTTTGGCTACGGAGGTGGCTAT-3′	86
	Reverse	5′-CTTTGGCTGCTCTTGGGTCTG-3′	
Claudin-1	Forward	5′-GCTGGGTTTCATCCTGGCTTCT-3′	110
	Reverse	5′-CCTGAGCGGTCACGATGTTGTC-3′	
IL-17	Forward	5′-TATCCCTCTGTGATCTGGGAAG-3′	161
	Reverse	5′ATCTTCTCGACCCTGAAAGTGA-3′	
IFN-γ	Reverse	5′-ATGAACGCTACACACTGCATCTT-3′	139
	Forward	5′-TTTCTTCCACATCTATGCCACTT3′	
TNF-α	Reverse	5′-GGTTCTGTCCCTTTCACTCACT-3′	169
	Forward	5′-GAGAAGAGGCTGAGACATAGGC-3′	
GAPDH	Reverse	5′-GAGACCTTCAACACCCCAGC-3′	263
	Forward	5′-ATGTCACGCACGATTTCCC-3′	

IL-17: Interleukin 17; IFN-γ: Interferon-γ; TNF-α: Tumor necrosis factor-α; *GAPDH:* Glyceraldehyde 3-phosphate dehydrogenase.

boiled 10 min in 10 mmol/L citrate (pH 6.0) for antigen retrieval. The slides were then incubated with mouse monoclonal antibodies against claudin-1, occludin (Santa Cruz Biotechnology, Dallas, TX, United States), TNF-α, and IL-17 (Sigma), and rabbit polyclonal antibodies against zo-1 (1:150; Invitrogen, Carlsbad, CA, United States) and IFN-γ (1:300; Santa Cruz Biotechnology), followed by peroxidase-conjugated secondary antibodies (1:1500). The signals were visualized by a diaminobenzidine peroxidase substrate kit (Vector Laboratories, Burlingame, CA, United States). For immunofluorescence staining, the sections were incubated with mouse monoclonal antibodies against claudin-1 and occludin (1:200; Santa Cruz Biotechnology) and rabbit polyclonal antibody against zo-1 (1:150; Invitrogen), and subsequently with FITC- or Cy3-conjugated secondary antibodies. Images were captured under a Leica DMIRE2 confocal laser scanning microscope.

In vivo permeability

In vivo permeability assay was performed to assess barrier function by using a FITC-labeled dextran method[23]. Briefly, food and water were withdrawn for 4 h and mice were then gavaged with permeability tracer (FITC-D, 60 mg/100 g body weight, MW 4000; Sigma). Serum was collected, and fluorescence intensity and FITC-dextran concentrations were determined. Permeability was calculated by linear regression of sample fluorescence.

Scanning electron microscopy

Specimens were fixed with 2.5% glutaraldehyde for 3 h at room temperature. The samples were then washed in acetone, critical point dried, and coated with gold for scanning electron microscopy (SEM).

Real-time quantitative polymerase chain reaction

Total RNA was extracted from colon tissues using Trizol reagent (Gibco-BRL Co., Grand Island, NY, United States) according to the manufacturer's protocol and quantified using a UV spectrophotometer. Total RNA of 5 μg was used for first-strand cDNA synthesis. Real-time polymerase chain reaction (PCR) was performed with Quantitect™ SYBR W Green PCR Mastermix (Qiagen, Hilden, Germany) using 2 μL of cDNA template. The amplification program was 95 ℃ for 5 min, 45 cycles of 95 ℃ for 1 min and 60 ℃ for 10 s. The primers used are summarized in Table 1. The relative quantitative analysis was performed by $2^{-\Delta\Delta Ct}$ method using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal control.

Western blot

Protein concentration was determined by using Coomassie brilliant blue G250. Protein samples (100 μg) were resolved on 8% SDS polyacrylamide gel, electrotransferred to nitrocellulose membrane, and subsequently blocked in 5% skim milk in PBS. The membranes were incubated overnight at 4 ℃ with rabbit polyclonal antibodies against zo-1 (1:100) and IFN-γ (1:200), mouse monoclonal antibodies against claudin-1 (1:200), occludin (1:200), TNF-α (1:200), and IL-17 (1:200), and rabbit anti-GAPDH monoclonal antibody (1:100). The secondary antibodies used were goat anti-rabbit or -mouse IgG (1:2000) and were incubated with membranes at room temperature for 2 h. Blots were visualized using enhanced chemiluminescence detection reagents (Santa Cruz Biotechnology) and quantified by Bandscan 5.0 software using GAPDH as the internal control.

Statistical analysis

Data were expressed as mean ± SD and analyzed with SPSS 13.0 software (SPSS, Inc., Chicago, IL, United States). Comparison between the groups was done by Student's *t*-test. Comparison among the groups was conducted by one-way ANOVA analysis followed by LSD post-hoc test. Statistical significance was considered at $P < 0.05$.

RESULTS

Poly I:C ameliorated DSS-induced acute colitis

DSS caused significant damage to the colon tissue, with the gut inflammation and weight loss noted in the model group during 1 to 8 d observation. Administration of poly I:C partially reversed the DSS-induced effects, with less BW loss (Figure 1A). The general condition of mice was evaluated using a DAI that scored the extent of BW loss, stool consistency, and OB. As shown in Figure 1B, DAI was significantly enhanced in the DSS-induced model group, and this effect was partially alleviated by poly I:C. The colitic parameters were further quantified after monitoring clinical development of colitis for 8 d. As shown in Figure 1 C and D, the colon length in the model group was significantly shorter than that of the control group ($P < 0.01$), while pretreatment with poly I:C significantly reduced the DSS-induced colon shortening ($P < 0.01$). Macroscopically, mice in the model group had significantly higher inflammation score than those in the control and poly I:C groups ($P < 0.01$; Figure 1E). Histopathological analysis in the model group showed extensive ulceration of the epithelial layer, edema and crypt damage of the bowel wall. The fibrosis of muscularis mucosae and infiltration of granulocytes and mononuclear cells into the mucosa were also observed (Figure 1G). The histology score was significantly higher in the model group than that in the control group ($P < 0.01$). In contrast, poly I:C pretreatment obviously reversed this DDS-induced effect, with comparatively lower histology score noted in the poly I:C group (Figure 1H).

Poly I:C attenuated DSS-induced paracellular permeability

MPO activity is shown in Figure 2A. Mice in the model group showed significantly higher MPO activity than that in the control group ($P < 0.01$), while the data were significantly lower in mice of the poly I:C group ($P < 0.01$). To investigate the effect of poly I:C on paracellular permeability, intestinal permeability to FITC-D was determined. The results showed the increased permeability to 4-kDa FITC-D induced by colitis, while mice in the poly I:C group showed a lower intestinal permeability than that of the model group ($P < 0.01$; Figure 2B). SEM observations of the colonic mucosa showed severe mucosal loss with typical histological inflammation feature in the mice of the model group (Figure 2C). Besides, enterocytes in the mice of the model group showed less glycocalyx and more irregular surface than the control group. By contrast, pretreatment with poly I:C obviously ameliorated the DSS-induced ultrastructural changes, with few histological lesions observed.

Poly I:C inhibited DSS-induced colonic inflammation

To determine the anti-inflammatory effect of poly I:C on the DSS-induced colitis, expressions of inflammatory markers, including IL-17, TNF-α and IFN-γ, were analyzed by real-time quantitative polymerase chain reaction (RT-qPCR), immunohistochemistry, and Western blot. RT-qPCR analysis showed the up-regulated expressions of IL-17, TNF-α and IFN-γ in the mice of the model group, while pretreatment with poly I:C caused the down-regulated expression of these inflammatory cytokines ($P < 0.01$; Figure 3A). These results were further confirmed by immunohistochemistry and Western blot analysis, which indicated the enhanced expressions of IL-17, TNF-α and IFN-γ in the model group. By contrast, poly I:C pretreatment markedly reversed the DSS-induced up-regulation of the inflammatory cytokines ($P < 0.01$; Figure 3B and C).

Poly I:C prevented DSS-induced TJ disruption

To investigate the protective effect of poly I:C on the DSS-induced disruption of TJ, the expression of TJ markers, including zo-1, occludin and claudin-1, were analyzed by immunohistochemistry, immunofluorescence, RT-qPCR, and Western blot. Immunohistochemistry and immunofluorescence assays showed that, in the control group, TJ proteins were expressed in the cytomembrane of epithelial cells, and most commonly in the spinous and granular layers, whereas expressions of these TJ proteins in the model group were significantly decreased. In the poly I:C group, however, the increased expression of TJ proteins were observed in the both cytomembrane and cytoplasm of spinous and granular layers in the mucosa (Figure 4A and B). Further quantitative analysis of TJ protein expressions by RT-qPCR and Western blot confirmed the down-regulated expression of TJ proteins in the model group when compared with the control group. Meanwhile, this effect was compromised by poly I:C pretreatment, with the comparatively higher expressed TJ markers ($P < 0.01$; Figure 4C and D).

DISCUSSION

Poly I:C is a ligand for TLR3 that is involved in the innate immune response to viral infection[24]. Administration of poly I:C in wild-type mice has been reported to dramatically protect against DSS-induced colitis, as demonstrated by parameter analysis of body weight, rectal bleeding, colonic MPO activity, histopathology, and *etc*[15]. However, another study also indicated that abnormal activation of TLR3 signaling by poly I:C broke down the mucosal homeostasis and caused intestinal mucosal damage[16]. Therefore, the potential role of poly I:C in the mucosal injury and epithelial barrier disruption was further investigated in DSS-induced acute colitis. The findings of our study showed that poly I:C administration obviously ameliorated the clinical symptoms of DSS-induced acute colitis, as demonstrated by less BW loss, lower DAI score, longer colon length, and

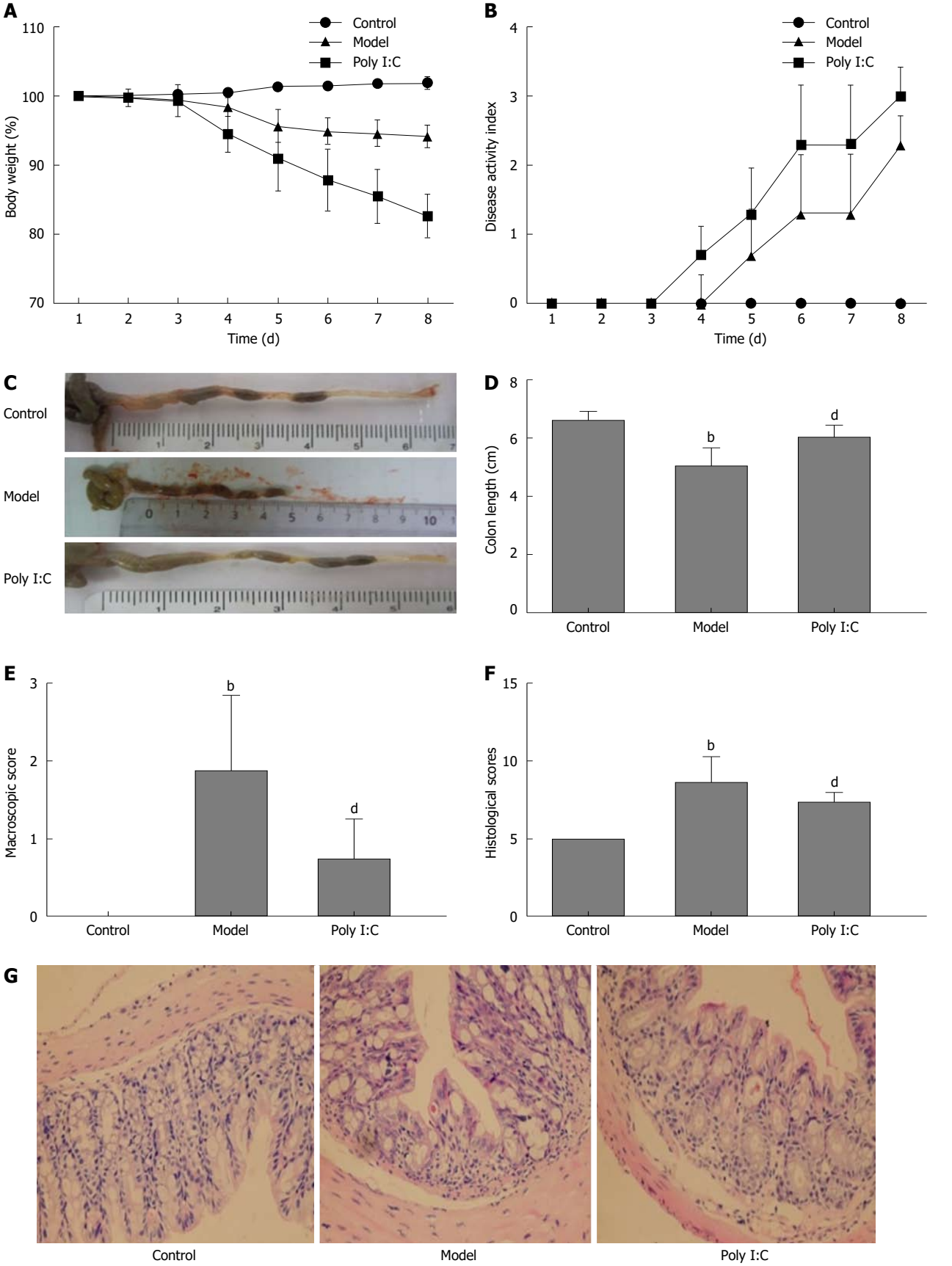

Figure 1 Effect of poly I:C administration on dextran sulfate sodium-induced acute colitis. A: The percentage of body weight change assessed daily during 1 to 8 d observation; B: Change in the disease activity index that was comprised of body weight loss, stool consistency and occult blood; C: Representative photographs of colon obtained after monitoring clinical development of colitis for 8 d; D: Quantitative analysis of the length of the colon; E: Macroscopic score inflammation assessed; F: Histological scores of the colon tissues; G: Representative photographs of HE staining of colon tissues (Magnifications, × 200). Data are expressed as mean ± SD. $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* model group.

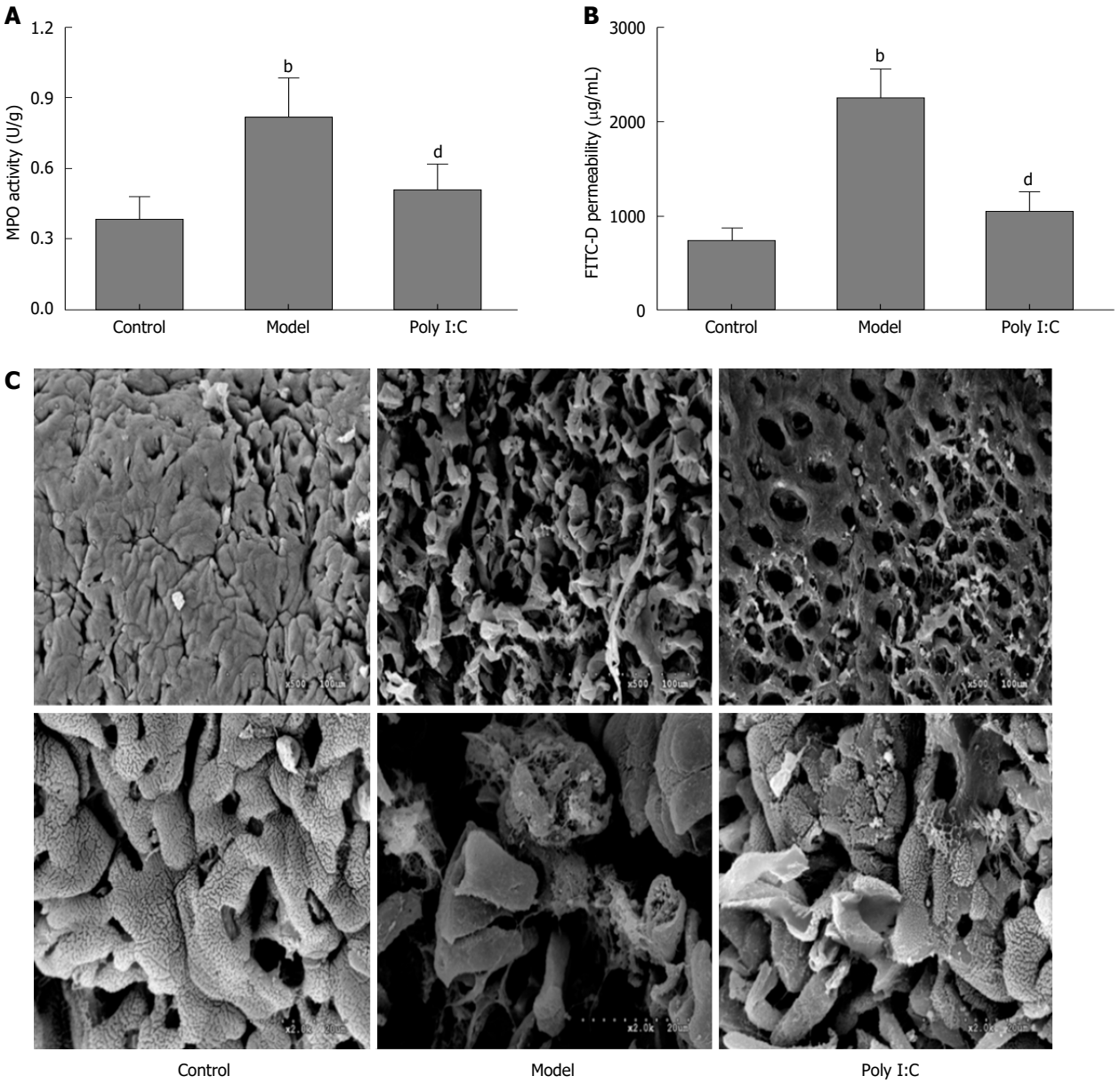

Figure 2 Effect of poly I:C on intestinal barrier in dextran sulfate sodium-induced acute colitis. A: MPO activity was measured; B: Quantification of serum FITC-D as a measure of intestinal barrier functions; C: The ultrastructural features of colon under scanning electron microscope. The colon mucosa in the control group was regular without histological lesion. In the model group, the mucosa showed severe loss with obvious histological lesions, including crypt distortion and abscesses. The lesions were significantly ameliorated by poly I:C pretreatment (Upper images: × 500; Lower images: × 2000). Data are expressed as mean ± SD. $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* model group. FITC-D: Fluorescein isothiocyanate labeled-dextran; MPO: Myeloperoxidase.

improved macroscopic and histological scores. These results were consistent with the findings reported by Vijay-Kumar *et al*[15], which indicated the protective effect of TLR3 activation by poly I:C on DSS-induced acute colitis.

The intestinal barrier has been reported to be defective and associated with paracellular leakiness in inflammatory bowel conditions of UC[25]. TJ protein is the most apical component responsible for restricting paracellular permeability of the intestinal mucosa[26]. Poly I:C administration has been demonstrated to protect against DSS-induced acute colitis. However, abnormal activation of TLR3 signaling by poly I:C has been reported to break down the mucosal homeostasis and cause mucosal damage in the small intestine[16]. TLR3 signaling may be involved in the process of epithelial destruction and mucosal injury[17]. These conflicting results urged us to study the effect of poly I:C on paracellular permeability and epithelial barrier function in DSS-induced acute colitis models.

Corroborating the findings of our previous study[19] and the study of others[15], the present study showed increased paracellular permeability and disrupted TJ induced by DSS, as assessed by FITC-D level, ultrastructure of colon mucosa, and expression and distribution of TJ proteins (zo-1, occludin, and claudin-1). However, unlike the results of our study, another study also indicated that colonic paracellular permeability

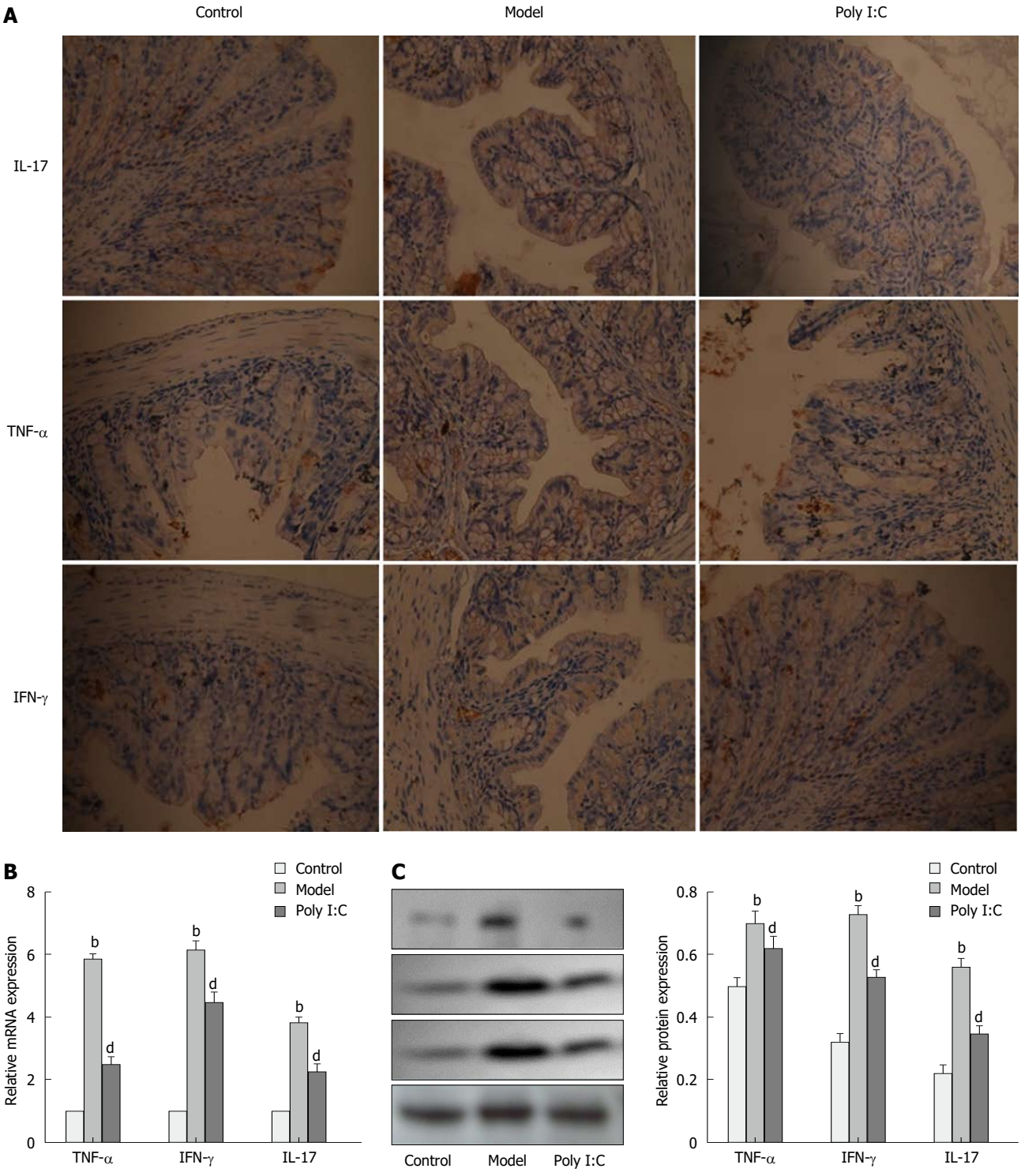

Figure 3 Effect of poly I:C on the expressions of inflammatory markers. A: Representative photographs of immunohistochemistry staining of IL-17, TNF-α and IFN-γ in colon tissues (Magnifications, × 200); B: Expressions of IL-17, TNF-α and IFN-γ at the mRNA level, as analyzed by RT-qPCR; C: Protein expressions of IL-17, TNF-α and IFN-γ as analyzed by Western Blot. Data are expressed as mean ± SD. $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* model group. RT-qPCR: Real-time quantitative polymerase chain reaction; IL-17: Interleukin-17; TNF-α: Tumor necrosis factor-α; IFN-γ: Interferon-γ.

was not significantly altered by DSS exposure, and DSS mice only showed a trend of increased expressions of claudin-1 and claudin-2[27]. This variation may be partially explained by the different methods used to establish the acute colitis models.

The potential effects of poly I:C administration were then analyzed. Paracellular permeability and TJ were found to be significantly ameliorated when mice were pretreated with poly I:C, as demonstrated by decreased FITC-D permeability, ameliorated ultrastructural changes, and enhanced expression of TJ proteins at the both mRNA and protein levels. The direct effect

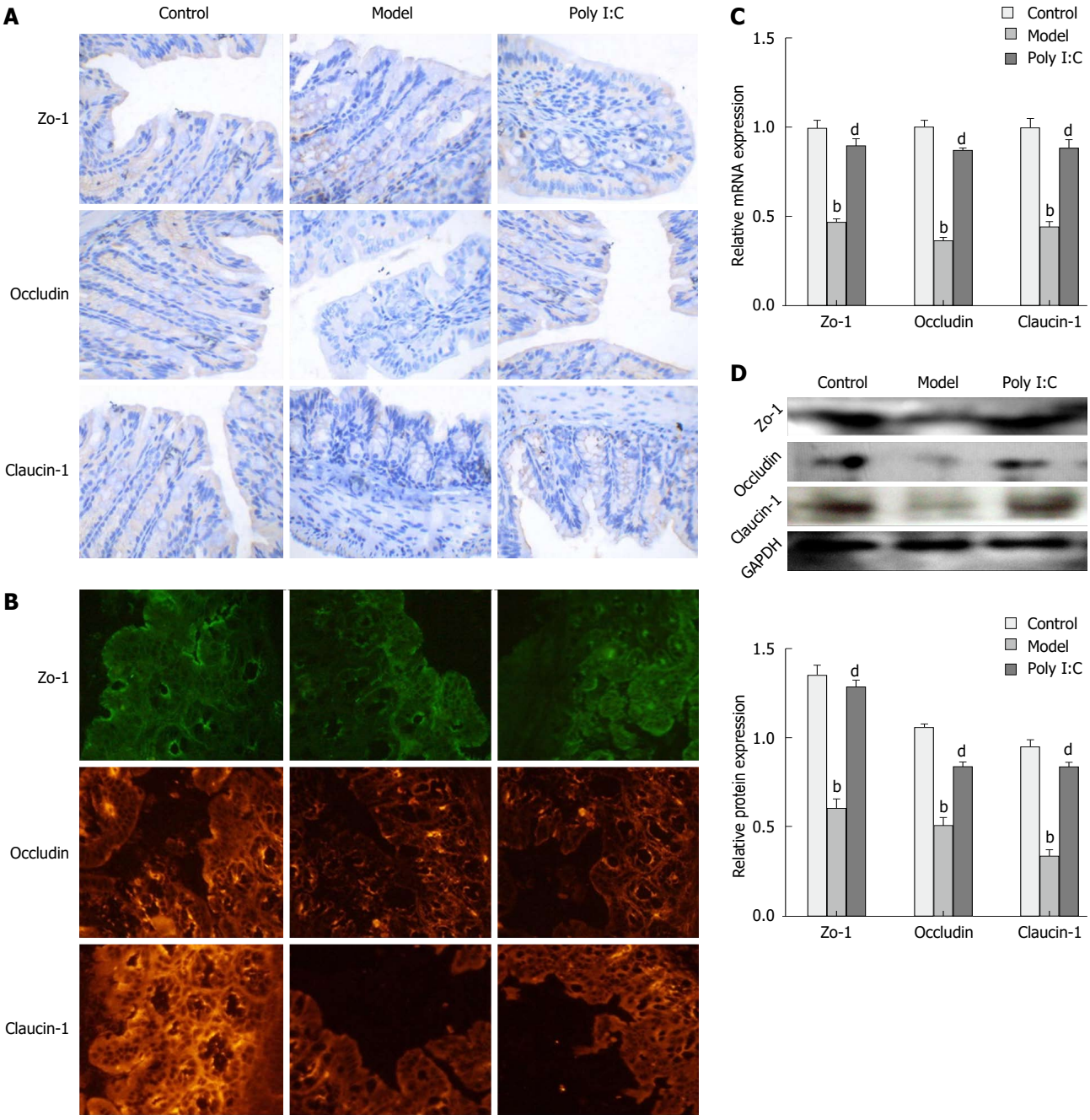

Figure 4 Effect of poly I:C on colonic epithelial junctions. A: Representative photographs of immunohistochemistry staining for zo-1, occludin and claudin-1 (Magnification: × 200); B: Representative photographs of immunofluorescent staining for zo-1 (green), occludin and claudin-1 (red); C: Expressions of zo-1, occludin and clandin-1 at the mRNA level, as analyzed by RT-qPCR; D: Protein expressions of zo-1, occludin and clandin-1 as analyzed by Western Blot. Data are expressed as mean ± SD. $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* model group. RT-qPCR: Real-time quantitative polymerase chain reaction.

of poly I:C on intestinal barrier function has been investigated *in vitro* by Moyano-Porcile *et al*[28], and indicated that acute exposure of rat ileum and colon tissues to poly I:C reduced colon permeability to macromolecules, while increasing ileum permeability to micromolecules. Intraperitoneal injection of poly I:C has been reported to cause severe mucosal injury of the small intestine, along with the increased levels of IL-15[16].

Activation of TLR-3 seems to cause adverse effects on various epithelial barriers, like the blood-brain barrier and nasal epithelial barriers[29,30]. However, to the best of our knowledge, this study is the first to investigate the effect of poly I:C on intestinal barrier function in DSS-induced acute colitis. Our results suggested that administration of poly I:C in DSS-induced colitis models may help to maintain the integrity of ultrastructure of the epithelial mucosa and junction complex, and therefore decrease paracellular permeability and recover epithelial barrier function.

TJ is known to be the rate-limiting step in transepithelial transport and the principal determinant of inte-

stinal permeability[31]. Increased intestinal permeability and disrupted TJ may cause bowel inflammation[32,33]. TNF-α is a proinflammatory cytokine that has long been established as a key player in the pathogenesis of IBD diseases. Proinflammatory cytokines such as TNF-α and IFN-γ have also been linked to the increased paracellular permeability[34-36]. Ligation of TLR3 by poly I:C has been suggested to be effective in IBD-associated gut inflammation[15]. This may shed light on the barrier protective effect of poly I:C as a likely candidate mechanism that contributes to therapeutic efficacy in IBD diseases. Therefore, the expressions of proinflammatory cytokines (IL-17, TNF-α, and IFN-γ) in colon epithelium were analyzed. Consistent with the result of the above mentioned study, our study showed the increased production of proinflammatory cytokines TNF-α, IL-17 and IFN-γ in mice of the DSS-induced model group. However, mice pretreated with poly I:C showed ameliorated DSS-induced high-expression of these proinflammatory cytokines. These results suggested that poly I:C may ameliorate inflammation in the colitis models.

In conclusion, the findings of our study showed evidence to suggest that poly I:C may protect against DSS-induced acute colitis through maintaining epithelial integrity and regulating the innate immune responses, which may shed light on the therapeutic potential of poly I:C in clinical colitis.

COMMENTS

Background

The toll-like receptor 3 (TLR3) agonist poly I:C has been reported to protect against acute colitis.

Research frontiers

TLR3 signaling may be involved in the process of epithelial destruction and mucosal injury. However, the potential effects of TLR3 activation by poly I:C on the mucosal injury and epithelial barrier disruption in dextran sulfate sodium (DSS)-induced acute colitis have not been well investigated up to now.

Innovations and breakthroughs

The potential roles of poly I:C administration in intestinal mucosal barrier function and intestinal permeability were investigated in a mouse model of DSS-induced acute colitis. Poly I:C administration dramatically protected against DSS-induced colitis. It also ameliorated DSS-induced ultrastructural changes of the colon epithelium, as well as fluorescein isothiocyanate labeled-dextran permeability and tight junction protein expressions.

Applications

Poly I:C may protect against DSS-induced colitis through maintaining integrity of the epithelial barrier and regulating innate immune responses, which may shed light on the therapeutic potential of poly I:C in human colitis.

Peer-review

In general, the present work is well done and the paper describes results of interest for the inflammatory bowel disease research community working with animal models of gut inflammation.

REFERENCES

1 **Song-Zhao GX**, Maloy KJ. Experimental mouse models of T cell-dependent inflammatory bowel disease. *Methods Mol Biol* 2014; **1193**: 199-211 [PMID: 25151008 DOI: 10.1007/978-1-4939-1212-418]

2 **Geem D**, Harusato A, Flannigan K, Denning TL. Harnessing regulatory T cells for the treatment of inflammatory bowel disease. *Inflamm Bowel Dis* 2015; **21**: 1409-1418 [PMID: 25793328 DOI: 10.1097/MIB.0000000000000343]

3 **Guéry L**, Hugues S. Th17 Cell Plasticity and Functions in Cancer Immunity. *Biomed Res Int* 2015; **2015**: 314620 [PMID: 26583099 DOI: 10.1155/2015/314620]

4 **Park JS**, Yi TG, Park JM, Han YM, Kim JH, Shin DH, Tak SJ, Lee K, Lee YS, Jeon MS, Hahm KB, Song SU, Park SH. Therapeutic effects of mouse bone marrow-derived clonal mesenchymal stem cells in a mouse model of inflammatory bowel disease. *J Clin Biochem Nutr* 2015; **57**: 192-203 [PMID: 26566304 DOI: 10.3164/jcbn.15-56]

5 **Aherne CM**, Saeedi B, Collins CB, Masterson JC, McNamee EN, Perrenoud L, Rapp CR, Curtis VF, Bayless A, Fletcher A, Glover LE, Evans CM, Jedlicka P, Furuta GT, de Zoeten EF, Colgan SP, Eltzschig HK. Epithelial-specific A2B adenosine receptor signaling protects the colonic epithelial barrier during acute colitis. *Mucosal Immunol* 2015; **8**: 1324-1338 [PMID: 25850656 DOI: 10.1038/mi.2015.22]

6 **Luo K**, Cao SS. Endoplasmic reticulum stress in intestinal epithelial cell function and inflammatory bowel disease. *Gastroenterol Res Pract* 2015; **2015**: 328791 [PMID: 25755668 DOI: 10.1155/2015/328791]

7 **Schulzke JD**, Bojarski C, Zeissig S, Heller F, Gitter AH, Fromm M. Disrupted barrier function through epithelial cell apoptosis. *Ann N Y Acad Sci* 2006; **1072**: 288-299 [PMID: 17057208 DOI: 10.1196/annals.1326.027]

8 **Borkowski AW**, Kuo IH, Bernard JJ, Yoshida T, Williams MR, Hung NJ, Yu BD, Beck LA, Gallo RL. Toll-like receptor 3 activation is required for normal skin barrier repair following UV damage. *J Invest Dermatol* 2015; **135**: 569-578 [PMID: 25118157 DOI: 10.1038/jid.2014.354]

9 **Clark PR**, Kim RK, Pober JS, Kluger MS. Tumor necrosis factor disrupts claudin-5 endothelial tight junction barriers in two distinct NF-κB-dependent phases. *PLoS One* 2015; **10**: e0120075 [PMID: 25816133 DOI: 10.1371/journal.pone.0120075]

10 **Luissint AC**, Bennett A, Nishio H, Hilgarth R, McCall I, Nusrat A, Parkos C. CLMP Expression is Increased in the Intestinal Epithelium Under Inflammatory Conditions and Regulates Intercellular Adhesion, Proliferation and Migration. *FASEB J* 2015; **29**: 282-289 [DOI: 10.1096/fj.1530-6860]

11 **Mielke L**, Preaudet A, Belz G, Putoczki T. Confocal laser endomicroscopy to monitor the colonic mucosa of mice. *J Immunol Methods* 2015; **421**: 81-88 [PMID: 25960174 DOI: 10.1016/j.jim.2015.04.012]

12 **Hummel S**, Veltman K, Cichon C, Sonnenborn U, Schmidt MA. Differential targeting of the E-Cadherin/β-Catenin complex by gram-positive probiotic lactobacilli improves epithelial barrier function. *Appl Environ Microbiol* 2012; **78**: 1140-1147 [PMID: 22179242 DOI: 10.1128/AEM.06983-11]

13 **McLamb BL**, Gibson AJ, Overman EL, Stahl C, Moeser AJ. Early weaning stress in pigs impairs innate mucosal immune responses to enterotoxigenic E. coli challenge and exacerbates intestinal injury and clinical disease. *PLoS One* 2013; **8**: e59838 [PMID: 23637741 DOI: 10.1371/journal.pone.0059838]

14 **Salvo Romero E**, Alonso Cotoner C, Pardo Camacho C, Casado Bedmar M, Vicario M. The intestinal barrier function and its involvement in digestive disease. *Rev Esp Enferm Dig* 2015; **107**: 686-696 [PMID: 26541659 DOI: 10.17235/reed.2015.3846/2015]

15 **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 [PMID: 17393379 DOI: 10.1002/ibd.20142]

16 **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 [PMID: 17372013 DOI: 10.4049/jimmunol.178.7.4548]

17 **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 [PMID: 17463084 DOI: 10.1073/pnas.0700822104]

18 **Vijay-Kumar M**, Gentsch JR, Kaiser WJ, Borregaard N, Offermann MK, Neish AS, Gewirtz AT. Protein kinase R mediates intestinal epithelial gene remodeling in response to double-stranded RNA and live rotavirus. *J Immunol* 2005; **174**: 6322-6331 [PMID: 15879132 DOI: 10.4049/jimmunol.174.10.6322]

19 **Zhao H**, Zhang H, Wu H, Li H, Liu L, Guo J, Li C, Shih DQ, Zhang X. Protective role of 1,25(OH)2 vitamin D3 in the mucosal injury and epithelial barrier disruption in DSS-induced acute colitis in mice. *BMC Gastroenterol* 2012; **12**: 57 [PMID: 22647055 DOI: 10.1186/1471-230X-12-57]

20 **Cooper HS**, Murthy SN, Shah RS, Sedergran DJ. Clinicopathologic study of dextran sulfate sodium experimental murine colitis. *Lab Invest* 1993; **69**: 238-249 [PMID: 8350599]

21 **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 [PMID: 15254593 DOI: 10.1172/JCI21233]

22 **Shimizu T**, Suzuki T, Yu HP, Yokoyama Y, Choudhry MA, Bland KI, Chaudry IH. The role of estrogen receptor subtypes on hepatic neutrophil accumulation following trauma-hemorrhage: direct modulation of CINC-1 production by Kupffer cells. *Cytokine* 2008; **43**: 88-92 [PMID: 18468914 DOI: 10.1016/j.cyto.2008.04.001]

23 **Nagy JA**, Herzberg KT, Masse EM, Zientara GP, Dvorak HF. Exchange of macromolecules between plasma and peritoneal cavity in ascites tumor-bearing, normal, and serotonin-injected mice. *Cancer Res* 1989; **49**: 5448-5458 [PMID: 2475250]

24 **Cunningham C**, Campion S, Teeling J, Felton L, Perry VH. The sickness behaviour and CNS inflammatory mediator profile induced by systemic challenge of mice with synthetic double-stranded RNA (poly I: C). *Brain Behav Immun* 2007; **21**: 490-502 [PMID: 17321719 DOI: 10.1016/j.bbi.2006.12.007]

25 **Vodovotz Y**, Constantine G, Faeder J, Mi Q, Rubin J, Bartels J, Sarkar J, Squires RH, Okonkwo DO, Gerlach J, Zamora R, Luckhart S, Ermentrout B, An G. Translational systems approaches to the biology of inflammation and healing. *Immunopharmacol Immunotoxicol* 2010; **32**: 181-195 [PMID: 20170421 DOI: 10.3109/08923970903369867]

26 **Wu HL**, Gao X, Jiang ZD, Duan ZT, Wang SK, He BS, Zhang ZY, Xie HG. Attenuated expression of the tight junction proteins is involved in clopidogrel-induced gastric injury through p38 MAPK activation. *Toxicology* 2013; **304**: 41-48 [PMID: 23220562 DOI: 10.1016/j.tox.2012.11.020]

27 **Carlsson AH**, Yakymenko O, Olivier I, Håkansson F, Postma E, Keita AV, Söderholm JD. Faecalibacterium prausnitzii supernatant improves intestinal barrier function in mice DSS colitis. *Scand J Gastroenterol* 2013; **48**: 1136-1144 [PMID: 23971882 DOI: 10.3109/00365521.2013.828773]

28 **Moyano-Porcile V**, Olavarría-Ramírez L, González-Arancibia C, Bravo JA, Julio-Pieper M. Short-term effects of Poly(I: C) on gut permeability. *Pharmacol Res* 2015; **101**: 130-136 [PMID: 26145280 DOI: 10.1016/j.phrs.2015.06.016]

29 **Ohkuni T**, Kojima T, Ogasawara N, Masaki T, Fuchimoto J, Kamekura R, Koizumi J, Ichimiya S, Murata M, Tanaka S, Himi T, Sawada N. Poly(I: C) reduces expression of JAM-A and induces secretion of IL-8 and TNF-α via distinct NF-κB pathways in human nasal epithelial cells. *Toxicol Appl Pharmacol* 2011; **250**: 29-38 [PMID: 20932985 DOI: 10.1016/j.taap.2010.09.023]

30 **Wang T**, Town T, Alexopoulou L, Anderson JF, Fikrig E, Flavell RA. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat Med* 2004; **10**: 1366-1373 [PMID: 15558055 DOI: 10.1038/nm1140]

31 **Chen Y**, Zhang HS, Fong GH, Xi QL, Wu GH, Bai CG, Ling ZQ, Fan L, Xu YM, Qin YQ, Yuan TL, Sun H, Fang J. PHD3 Stabilizes the Tight Junction Protein Occludin and Protects Intestinal Epithelial Barrier Function. *J Biol Chem* 2015; **290**: 20580-20589 [PMID: 26124271 DOI: 10.1074/jbc.M115.653584]

32 **Marchiando AM**, Graham WV, Turner JR. Epithelial barriers in homeostasis and disease. *Annu Rev Pathol* 2010; **5**: 119-144 [PMID: 20078218 DOI: 10.1146/annurev.pathol.4.110807.092135]

33 **Turner JR**. Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol* 2009; **9**: 799-809 [PMID: 19855405 DOI: 10.1038/nri2653]

34 **Overman EL**, Rivier JE, Moeser AJ. CRF induces intestinal epithelial barrier injury via the release of mast cell proteases and TNF-α. *PLoS One* 2012; **7**: e39935 [PMID: 22768175 DOI: 10.1371/journal.pone.0039935]

35 **Hering NA**, Fromm M, Schulzke JD. Determinants of colonic barrier function in inflammatory bowel disease and potential therapeutics. *J Physiol* 2012; **590**: 1035-1044 [PMID: 22219336 DOI: 10.1113/jphysiol.2011.224568]

36 **Gilbert S**, Zhang R, Denson L, Moriggl R, Steinbrecher K, Shroyer N, Lin J, Han X. Enterocyte STAT5 promotes mucosal wound healing via suppression of myosin light chain kinase-mediated loss of barrier function and inflammation. *EMBO Mol Med* 2012; **4**: 109-124 [PMID: 22228679 DOI: 10.1002/emmm.201100192]

P- Reviewer: Gazouli M, Sandvik AK **S- Editor**: Gong ZM
L- Editor: Filipodia **E- Editor**: Liu WX

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v23.i6.1010

World J Gastroenterol 2017 February 14; 23(6): 1010-1017
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

Molecular mechanisms of apoptosis in hepatocellular carcinoma cells induced by ethanol extracts of *Solanum lyratum* Thumb through the mitochondrial pathway

Xiao-Qiang Mo, Hong-Yu Wei, Gan-Rong Huang, Ling-Yuan Xu, Yu-Li Chen, Jiang Qi, Wei Xian, Yan-Chun Qin, Lian-Deng Wei, Li-Juan Zhao, Yan-Qiang Huang, Wei Xing, Hong-Qin Pu, Peng-Ya Wei, Chao-Gan Li, Qiu-Chun Liang

Xiao-Qiang Mo, Hong-Yu Wei, Gan-Rong Huang, Yu-Li Chen, Wei Xian, Yan-Chun Qin, Lian-Deng Wei, Li-Juan Zhao, Yan-Qiang Huang, Wei Xing, Hong-Qin Pu, Peng-Ya Wei, Chao-Gan Li, Qiu-Chun Liang, Breeding Base of Microbial Infection Key Laboratory in the West Guangxi, Youjiang Medical University for Nationalities, Baise 533000, Guangxi Zhuang Autonomous Region, China

Ling-Yuan Xu, Jiang Qi, Affiliated Hospital of Youjiang Medical University for Nationalities, Baise 533000, Guangxi Zhuang Autonomous Region, China

Author contributions: Mo XQ, Wei HY and Huang GR performed research and contributed equally to this work; Xu LY, Chen YL, Qi J, Xian W, Qin YC, Wei LD, Zhao LJ, Pu HQ, Wei PY, Li CG and Liang QC analyzed the data; Huang YQ and Xing W designed the research, analyzed the data and contributed equally to this work; all authors drafted the article and made critical revisions related to the intellectual content of the manuscript, and approved the final version of the article to be published.

Supported by the Guangxi Key Disciplines (Pathogen Biology) [2013] 16, Key Laboratory Cultivation Base of Universities in Guangxi (Guangxi Education Research [2014] 6); the Science and Technology Research Projects of Universities in Guangxi in 2014, No. YB2014307; the Guangxi Natural Science Fund Project, No. 2013GXNSFAA019249, No. 2014GXNSFBA118148; and the Scientific Research Project of The Department of Education of Guangxi Zhuang Autonomous Region, No. 200810LX327.

Institutional review board statement: This study was reviewed by the Youjiang Medical University Institutional Review Board.

Conflict-of-interest statement: We declare that there are no conflicts of interest to disclose.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Correspondence to: Dr. Yan-Qiang Huang, Professor, Breeding Base of Microbial Infection Key Laboratory in the West Guangxi, Youjiang Medical University for Nationalities, No. 98 Countryside Road, Baise 533000, Guangxi Zhuang Autonomous Region, China. hyq77615@163.com
Telephone: +86-776-2853272
Fax: +86-776-2853272

Received: September 25, 2016
Peer-review started: September 30, 2016
First decision: October 20, 2016
Revised: November 4, 2016
Accepted: November 23, 2016
Article in press: November 28, 2016
Published online: February 14, 2017

Abstract

AIM

To explore the induction effects and mechanism of *Solanum lyratum* Thumb (ST) on human hepatocellular

carcinoma SMMC-7721 cells through the mitochondrial pathway.

METHODS

The experiments were conducted on three groups: an experimental group (with ST ethanol extracts' concentration being 2.5, 5 and 10 mg/L), a negative control group (with only nutrient solution, 0 mg/L ST ethanol extracts), and a positive control group (2.5 mg/L DDP). The inhibition rate of cell proliferation was checked by using the methyl thiazolyl tetrazolium method, and cell apoptosis was tested by TUNEL method. Furthermore, RT-PCR was used to examine mRNA expression of Fas, FasL, caspase-8, caspase-3, p53 and Bcl-2 genes.

RESULTS

Compared with the negative control group, the inhibition and apoptosis rates of the experimental group with different concentrations of ST extracts on human hepatocellular carcinoma SMMC-7721 cells significantly increased ($P < 0.05$). Besides, the mRNA expression of FasL and Bcl-2 significantly decreased ($P < 0.05$) while the mRNA expression of Fas, caspase-8, caspase-3 and p53 increased significantly. When compared with the positive control group, the experimental groups with 5 mg/L ST ethanol extracts showed effects similar to the positive control group.

CONCLUSION

ST ethanol extracts induced the apoptosis of hepatocellular carcinoma SMMC-7721 cells through up-regulated Fas, caspase-8, caspse-3 and p53, and down-regulated FasL and Bcl-2 in the mitochondrial pathway.

Key words: *Solanum lyratum* Thumb; Hepatocellular carcinoma cell; Cell apoptosis; Mitochondrial pathway; Molecular mechanism

Core tip: Chinese herbal medicine has a very good effect on the tumor. *Solanum lyratum* Thumb (ST) belonging to Solanaceae, is generally used to treat tumors, so it is a commonly used anticancer drug. However, the effects and mechanism of ST on tumor cells are unclear. This experiment verified that ST can induce the apoptosis of hepatocellular carcinoma SMMC-7721 cells; moreover, the apoptosis mechanism was related to the expression of Fas, FasL, caspase-8, caspase-3, p53 and Bcl-2 in the mitochondrial pathway. This result provides powerful evidence of the improved apoptosis effects of ST on hepatocellular carcinoma cells.

Mo XQ, Wei HY, Huang GR, Xu LY, Chen YL, Qi J, Xian W, Qin YC, Wei LD, Zhao LJ, Huang YQ, Xing W, Pu HQ, Wei PY, Li CG, Liang QC. Molecular mechanisms of apoptosis in hepatocellular carcinoma cells induced by ethanol extracts of *Solanum lyratum* Thumb through the mitochondrial pathway. *World J Gastroenterol* 2017; 23(6): 1010-1017 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i6/1010.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i6.1010

INTRODUCTION

Solanum lyratum Thumb (ST), belonging to Solanaceae, is generally used to treat tumors[1-3], including liver, gastric, esophageal and bladder cancers, with exact curative effects, and it is a commonly used anticancer drug. However, the effects on tumor cells are unclear. The occurrence of tumors is closely related to the abnormality of cell differentiation and is a disordered cell apoptosis. Cell apoptosis is strictly controlled by multiple genes and factors. With the development of the technologies used in molecular biology and proteomics, cell apoptosis is gradually being understood, and some new regulatory genes have been found, with the result that the pathway of cell apoptosis is now better recognized. The mitochondrial pathway is currently recognized as one of the important methods of signal transmission in the process of cell apoptosis. Genes including Fas, FasL, caspase-8, caspase-3, p53 and Bcl-2 are involved in regulation of this pathway. Furthermore, the coordinated network regulation system formed by these genes promotes or inhibits cell apoptosis[4-7].

To date, there is no report on whether ST extracts can induce the apoptosis of hepatocellular carcinoma cells through the mitochondrial pathway or by what mechanism such apoptosis occurs. This research aimed to fill this gap in the current knowledge.

MATERIALS AND METHODS

Materials

Tumor cells: Human hepatocarcinoma SMMC-7721 cells were purchased from the Shanghai Institute of Cell Biology of Chinese Academy of Science, China.

Main reagents: ST was purchased from the biological medicine chain in Baise, Guangxi Province, China. RPMI 1640 cultural medium and fetal bovine serum were purchased from Gibco Company, United States. The detection kit for *in situ* cell apoptosis was sourced from Beijing Zhongshan Jinqiao Biotech Company, China. Methyl thiazolyl tetrazolium (MTT) was produced by Shanghai Pufei Biotech Co., Ltd, China. The polymerase chain reaction (PCR) primer was bought from Sangon Biotech Shanghai Co., Ltd, China. In addition, the Trizol Reagent Kit and the 2 × SYBRGreen qPCR Mix were purchased from Shanghai Invitrogen Company, China and Beijing Zhuangmeng Co., Ltd, China respectively. The RevertAid First Strand cDNA Synthesis Kit and DNase I were obtained from Fermentas, United States.

Table 1 Reverse transcription polymerase chain reaction primers

Gene name	Primer sequence	Product length, bp
Fas	Forward: 5′-GAATGCAAGGGACTGATAGC-3′ Reverse: 5′-TGGTTCGTGTGCAAGGCTC-3′	414
FasL	Forward: 5′-GGAATGGGAAGACACATATGGAACTGC-3′ Reverse: 5′-CATATCTGGCCAGTAGTGCAGTAATT-3′	239
Caspase-3	Forward: 5′-GCTATTGTGAGGCGGTTGT-3′ Reverse: 5′-CGTTTGGAGTCCCTTTGT-3′	270
Caspase-8	Forward: 5′-TCTGGAGCATCTGCTGTCTG-3′ Reverse: 5′-TTGACGTCTGTGGTCCGTCC-3′	427
p53	Forward: 5′-CTTTCAACTCTGTCTCCTTC-3′ Reverse: 5′-TGGGCAACCAGCCCTGTCGT-3′	180
Bcl-2	Forward: 5′-CAGCTGCACCTGACGCCCTT-3′ Reverse: 5′-GCCTCCGTTATCCTGGATCC-3′	231
β-actin	Forward: 5′-ACACTGTGCCCATCTACGAGG-3′ Reverse: 5′-AGGGGCCGGACTCGTCATACT-3′	621

Main instruments: The main instruments used included a carbon dioxide incubator (MCO-18AIC), a biosafety cabinet (BHC-1300 II A/B33), an automatic microplate spectrophotometer (Multiskan MK3), an inverted microscope (Cioc), and a BX51 microscope (Olympus). Furthermore, a table-top, high-speed freezing centrifuge (1-15PK), a microcentrifuge (Uni Force 6K) and a RT-PCR instrument (IQ5) were also used in this study.

Methods

Ethanol extracts of ST: After being smashed, ST of 50 g was immersed for 3 h at 40 ℃ in 75% ethanol and filtered. The immersion and filtration were conducted three times. Afterwards, the filter liquors were mixed and dried by using a rotary evaporator, thus obtaining ST extractum.

Setting ST of different concentrations: The ST extractum was dissolved using dimethylsulphoxide (DMSO) to prepare the drug solution with a concentration of 10 mg/mL, which was then diluted by RPMI 1640 cell culture solution to obtain ST extract solutions with concentrations of 0, 2.5, 5 and 10 mg/L.

Medicine intervention for tumor cells: Hepatoma carcinoma SMMC-7721 cells were cultured using the culture solution for 24 h and then the culture solution was discarded. Then, the prepared drug solution containing ST extracts was added to the cells. Moreover, a negative control group and a positive control group were set by adding the drug solution without ST extracts (0 mg/L) and 2.5 mg/L DDP, respectively. The cells were subjected to the effects of the drugs for 48 h.

MTT method: Hepatocellular carcinoma SMMC-7721 cell suspension of 200 μL was put into a culture plate with 96 holes at density of 1×10^4 mL^{-1} to be cultured for 24 h. Afterwards, the culture solution was removed and discarded, and 200 μL of the prepared ST solutions with different concentrations were added to the culture plate. Four holes were established for each concentration in the experimental group. The negative control group with culture solution (0 mg/L ST extracts) and the DDP positive control group were placed into the incubator to be cultured for 44 h, and then dosed with 20 μL MTT (5 mg/mL), followed by 4 h of continuous culture. After the supernatants were poured out and 150 μL DMSO was added, the solutions were shocked for 10 min. Finally, the wavelength of the enzyme labelling instrument was adjusted to 492 nm to detect the light absorption value (OD value) of the solutions (three replicates), thus allowing for calculation of the average inhibition rate. The formula for calculating the inhibition rate was (1 - OD value of the experimental group/OD value of the control groups) × 100%.

TUNEL method: The experiment was conducted according to the specification of the purchased detection kit for *in situ* cell apoptosis. Based on the analysis results under a visible light microscope, the apoptosis rate was calculated as the percentage of positive cells counted in randomly 10 high-power fields.

RT-PCR test: (1) Primer design: Primers were designed by using Primer Premier 5.0 software and checked in GenBank. The primers for Fas, FasL, caspase-8, caspase-3, p53, Bcl-2 and β-actin are shown in Table 1; (2) RNA extraction: after medical intervention for tumor cells using ST, total RNA was extracted in accordance with the specification of the purchased Trizol Reagent Kit; (3) reverse transcription: RNA of 5 μL, along with 1 μL random primer and 5 μL RNase-free ddH_2O was added into PCR tubes for warm-bath conditioning for 5 min at 70 ℃ and ice-bath treatment for 10 s, followed by centrifugation. Then, after being dosed with 4 μL buffer, 2.0 μL dNTP mix, 1.0 μL RNase inhibitor and 2.0 μL AMV reverse transcriptase, the tubes underwent warm-bath conditioning at 37 ℃ for 5 min, 42 ℃ for 60 min and 70 ℃ for 10 min, before termination of the reaction; (4) fluorescence quantitative PCR (qPCR) detection: cDNA samples were diluted 6-fold: the 20 μL reaction system contained 10 μL SYBRGreen qPCR master mix, 1 μL upstream primer

Table 2 Comparison of the inhibition rates of proliferation of hepatocellular carcinoma SMMC-7721 cells in the groups with different *Solanum lyratum* Thumb concentrations, $\bar{x} \pm s$

Group	Concentration, mg/L	*n*	OD value	Inhibition rate, %
Three ST groups with different concentrations	2.5	12	1.516 ± 0.205	18.22 ± 2.32
	5	12	1.242 ± 0.236	36.56 ± 2.51
	10	12	0.815 ± 0.276	55.98 ± 3.12
Negative control group	0	12	1.842 ± 0.183	-
Positive control group	2.5	12	1.135 ± 0.172	38.60 ± 1.78

The comparison between ST groups and the negative control group shows that $P < 0.05$, while that of ST groups and the positive control group indicates that $P > 0.05$. ST: *Solanum lyratum* Thumb.

Table 3 Comparison of apoptosis rates of hepatocellular carcinoma SMMC-7721 cells in different groups, $\bar{x} \pm s$

Group	Concentration, C/mg·L^{-1}	Apoptosis rate of SMMC-7721 cells, %
Three ST groups with different concentrations	2.5	10.75 ± 2.51
	5	17.31 ± 2.33
	10	30.21 ± 3.62
Negative control group	0	3.75 ± 1.23
Positive control group	2.5	17.36 ± 1.62

The comparison of the ST groups and the negative control group shows that $P < 0.05$, while that of ST groups with the positive control group reveals that $P > 0.05$. ST: *Solanum lyratum* Thumb.

(10 μmol/L), 1 μL downstream primer R (10 μmol/L), 7 μL ddH$_2$O and 1 μL template (cDNA). The heat cycle was conducted by pre-denaturation for 2 min at 95 ℃, denaturation for 10 s at 95 ℃, and annealing for 40 s at 60 ℃, for a total of 40 cycles; and (5) relative quantitative analysis method: The $2^{-\Delta\Delta CT}$ method was used for relative quantitative analysis of data. The calculation formulae were expressed as: ΔCT = CT target gene - CT reference gene, and ΔΔCT = ΔCT target gene - the average value of target gene ΔCT in the control groups. $2^{-\Delta\Delta CT}$ represents the relative expression of target genes.

Statistical analysis

Inhibition and apoptosis rate were analyzed by using SPSS17 software and data were represented by mean ± standard variance (± *s*). A homogeneity test of variance and one-way ANOVA were used for comparison among groups. As for further pairwise comparison, if the variance was homogeneous, the Student-Newman-Keuls method was used, while Games-Howell was used when the variance was inhomogeneous. Moreover, χ^2 was used for the comparison of the rates. If $P < 0.05$, the difference was deemed to have been statistically significant.

RESULTS

Inhibition rate of hepatocellular carcinoma cells

The inhibition rate of hepatocellular carcinoma SMMC-7721 cells in the experimental group of each ST concentration was significantly higher than that of the negative control group ($P < 0.05$), and the higher the ST concentration, the higher the inhibition rate. Furthermore, the inhibition rate of the group with 5 mg/L ST was equal to that of the positive control group (Table 2).

Apoptosis rate of hepatocellular carcinoma cells

The apoptosis rate of hepatocellular carcinoma SMMC-7721 cells in each ST group with different concentrations was significantly higher than that of the positive control group ($P < 0.05$). The higher the ST concentration, the higher the cell apoptosis rate. Moreover, the apoptosis rate of the group with 5 mg/L ST was identical to that of the positive control group (Table 3).

Influences of ST on the mRNA expressions of genes relating to hepatocellular carcinoma cells

After intervention of ST extracts in each concentration on the hepatocellular carcinoma SMMC-7721 cells, the mRNA expressions of FasL and Bcl-2 decreased markedly ($P < 0.05$) and the reductions were negatively correlated with the ST concentration. However, the mRNA expressions of Fas, caspase-8, caspase-3 and p53 increased and the increase showed positive correlation with the ST concentration. When the concentration was greater than, or equal to, 5 mg/L, the mRNA expressions increased ($P < 0.05$). Moreover, the mRNA expressions of these genes in 5 mg/L ST group were equivalent to those of the positive control group, as demonstrated in Figure 1 and Tables 4 and 5.

DISCUSSION

ST has functions including cooling and dehumidifying, detoxifying, detumescence and anticancer effects, and it can cure cold and fever, icteric hepatitis, gallstone disease, cholecystitis, nephritis and uterine erosion. Clinically, it has certain effects on the treatment of various types of cancers, especially for lung, liver, stomach and cervical cancers[8-10]. The experimental results show that the inhibition and apoptosis rates of hepatocellular carcinoma SMMC-7721 cells in ST groups with different concentrations were significantly higher than those of the negative control group and showed positive correlation with the ST concentration,

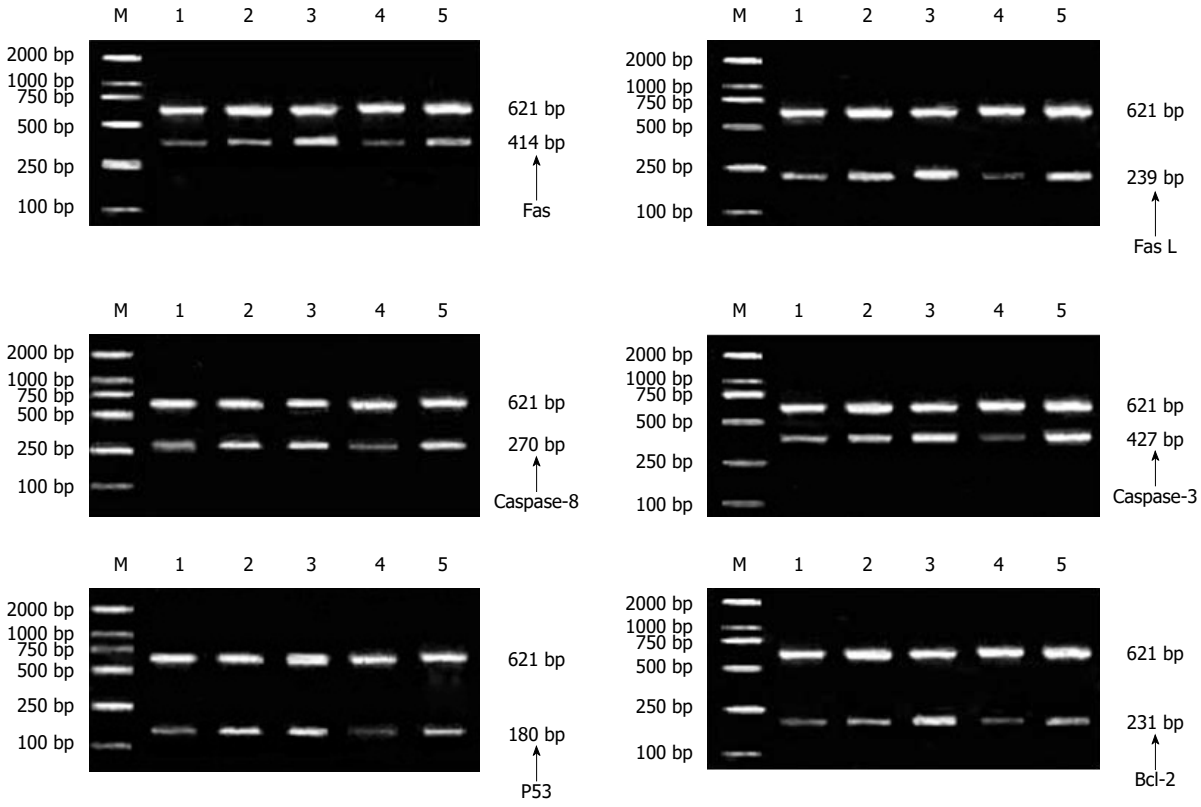

Figure 1 mRNA expressions of genes relating to hepatocellular carcinoma SMMC-7721 cells in each group tested by reverse transcription polymerase chain reaction. M: DNA maker; 1: 2.5 mg/L ST; 2: 5 mg/L ST; 3: 10 mg/L ST; 4: Negative control group (0 mg/L ST); 5: Positive control group (5 mg/L DDP). ST: *Solanum lyratum* Thumb.

Table 4 mRNA expressions of genes relating to hepatocellular carcinoma SMMC-7721 cells in each group

Group	Concentration, C/mg•L^{-1}	mRNA expression of genes						
		Fas	FasL	Caspase-8	Caspase-3	p53	Bcl-2	β-actin
Three ST groups with	2.5	52.47 ± 0.10	58.25 ± 0.51	30.57 ± 0.10	22.15 ± 0.02	34.17 ± 0.01	36.28 ± 0.14	17.53 ± 0.14
different concentrations	5	52.74 ± 0.08	56.81 ± 0.30	30.76 ± 0.03	22.33 ± 0.09	34.45 ± 0.20	35.40 ± 0.51	18.15 ± 0.41
	10	53.41 ± 0.64	55.94 ± 0.80	31.30 ± 0.24	23.01 ± 0.47	35.11 ± 0.69	33.54 ± 0.51	19.10 ± 0.91
Negative control group	0	52.32 ± 0.04	59.13 ± 0.07	30.44 ± 0.06	22.05 ± 0.03	34.11 ± 0.02	37.59 ± 0.33	17.30 ± 0.17
Positive control group	2.5	52.79 ± 0.11	56.69 ± 0.34	30.77 ± 0.03	22.35 ± 0.12	34.43 ± 0.20	35.53 ± 0.35	18.18 ± 0.41

ST: *Solanum lyratum* Thumb.

Table 5 Differences in mRNA expressions of genes relating to hepatocellular carcinoma SMMC-7721 cells in each group

Group	Concentration, C/mg•L^{-1}	$2^{-\Delta\Delta CT}$					
		Fas	FasL	Caspase-8	Caspase-3	p53	Bcl-2
Negative control group	0	1	1	1	1	1	1
Three ST groups with	2.5	1.06 ± 0.06	2.30 ± 0.93^a	1.08 ± 0.04	1.10 ± 0.12	1.13 ± 0.11	3.09 ± 0.59^a
different concentrations	5	1.34 ± 0.25^a	9.02 ± 0.32^b	1.47 ± 0.31^a	1.50 ± 0.27^a	1.43 ± 0.16^a	9.46 ± 0.58^b
	10	1.63 ± 0.14^a	31.34 ± 0.13^b	2.02 ± 0.71^a	1.82 ± 0.40^a	1.75 ± 0.25^a	47.29 ± 0.68^b
Positive control group	5	1.33 ± 0.17^a	10.02 ± 0.57^b	1.49 ± 0.30^a	1.50 ± 0.19^a	1.47 ± 0.11^a	8.92 ± 0.09^b

By comparing the ST group with the negative control group. $^aP < 0.05$, $^bP < 0.01$, *vs* control groups. ST: *Solanum lyratum* Thumb.

confirming that ST can inhibit the growth of hepatocellular carcinoma SMMC-7721 cells. However, the molecular mechanism of this anti-hepatoma behavior is not clear and requires further clarification.

At present, cell apoptosis has been studied and found to be a normal metabolic procedure; however, if the apoptosis process is disordered, a lot of diseases, tumors for instance, can appear. Cell apoptosis is a multi-factor and multi-pathway process regulated by a network, involving a series of proteins, such as

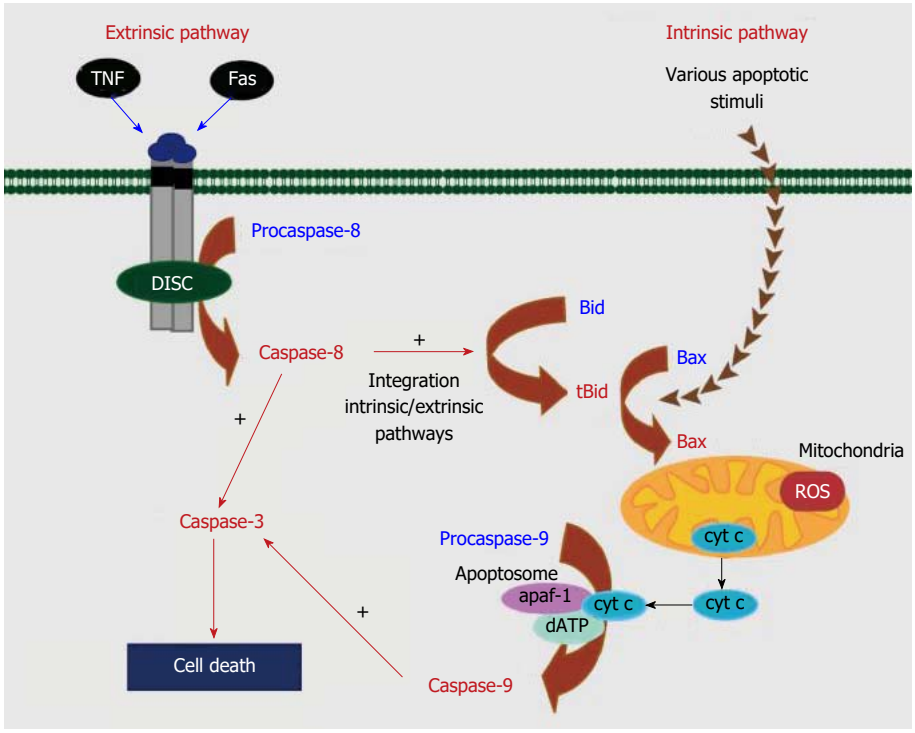

Figure 2 Intrinsic and extrinsic apoptotic pathways. TNF: Tumor necrosis factor; ROS: Reactive oxygen species.

caspase family proteins, Bcl-2 family proteins, p53 protein and survivin. Therefore, the exact mechanism of apoptosis is unclear, while the intrinsic and extrinsic pathways of apoptosis have been clarified[11]. As shown in Figure 2, the extrinsic pathway is triggered by death receptors such as Fas and tumor necrosis factor receptor (TNF-R) on the surface of cells[12,13]. While the intrinsic or mitochondrial pathway is induced by many stress conditions, chemical treatment reagents, and medicines.

Under physiological or pathological conditions, no matter whether the apoptosis is induced by DNA damage or other receptors relating to organelles, various receptors affect the intrinsic pathway of apoptosis[14,15]. In the intrinsic pathway of cell apoptosis, the mitochondria control cell activities and are important in regulating cell apoptosis. There are two main changes in mitochondria in the early stage of apoptosis. One is the decrease of mitochondrial internal transmembrane potential, and the other is the increase of mitochondrial external transmembrane permeability.

For the change in interaction of these two aspects, if the difference in the internal and external potentials of the mitochondrial membrane decreases, the mitochondrial transmembrane potential decreases and then the mitochondrial membrane permeability increases, resulting in the release of caspase activated cytochrome C and the activation of caspase protein. The activated caspase affects other protein substrates in the cells, leading to cascade reaction of apoptosis and finally cell apoptosis[16-18].

Caspase, as a cysteine-aspartic specific protease, can excise fragments containing aspartic acids. So far, at least 14 sub-types of caspase with similar molecular structure and high homology have been found. According to their functions, the sub-types can be divided into two categories, namely, initiator and effector caspases. Moreover, caspase plays an essential role in apoptosis. The initiator caspase acts on the inactive effector caspase, thereby activating the effector caspase.

Caspase-8, belonging to the initiator caspase subtype, is the initiator of the cascade reaction of cell apoptosis. It can self-activate and transmit apoptotic signals in the participation of other proteins and activate downstream effector caspase, thus forming a cascade amplification system to induce cell apoptosis. Caspase-3 (being subjected to the effector caspase) is the most important final excision enzyme and is also an important part of the killing mechanism of cytotoxic T lymphocyte (CTL) cells and can be activated by a variety of factors. As to the killing effects on CTL cells, caspase-3 can be activated by the Fas/FasL pathway and the B pathway of granzymes. When caspase-8, upstream of cells, is activated, the activated caspase-3 excises poly (ADP-ribose) polymerase (PARP) into two fragments, separating two zinc fingers binding with DNA in PARP from the C-terminal catalytic region to influence its normal function. As a result, the activity of Ca/Mg-dependent endonuclease is adversely affected by PARP increases, so that the DNA between nucleosomes is cracked, leading to cell apoptosis[19-21].

p53, as a tumor suppressor gene, slows down or monitors cell division and the integrity of the genome

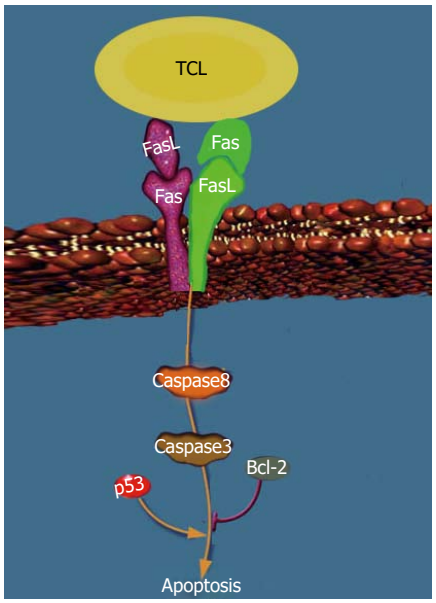

Figure 3 Mitochondrial apoptotic pathway of hepatocellular carcinoma cells induced by *Solanum lyratum* Thumb.

when checking DNA damage loci in the G1 phase. If there is damage, the p53 protein prevents DNA replication and provides sufficient time to repair the damaged DNA[22].

Bcl-2, as a kind of negative regulatory gene of cell apoptosis, can control the membrane potential by changing the redox state of mitochondrial thiols. Furthermore, it can also regulate the permeability of the mitochondrial membrane for some apoptosis protein precursors and locate apoptosis protein precursor Apaf-1 on the mitochondrial membrane to stop apoptosis[23].

Fas and FasL are membrane surface molecules, which regulate the apoptosis induced by toxicity in T-cell development[24]. FasL, with its high-level expression on cell surfaces, identifies Fas on the target cell surface after CTL cells recognize target cells. Then, by triggering the apoptosis process in the interior of target cells using Fas, the programmed cell death of target cells occurs[24,25].

It can be found from this experiment that, after the intervention of ST extracts with different concentrations on hepatocellular carcinoma SMMC-7721 cells, the inhibition and apoptosis rates of the cells increased significantly ($P < 0.05$). This indicated that ST inhibited hepatocellular carcinoma cells. The mRNA expressions of FasL and Bcl-2 were markedly reduced ($P < 0.05$), which showed that the negative feedback regulation protected CTL cells to certain extent. In addition, the mRNA expressions of Fas, caspase-8, caspase-3 and p53 increased. Compared with the positive control group, the effects of 5 mg/L ST extracts were equivalent to those of the positive control group, which suggested that these genes were positive regulatory genes for apoptosis of hepatocellular carcinoma cells induced by ST through the mitochondrial pathway. Therefore, ST regulated hepatocellular carcinoma SMMC-7721 cells by the combination of Fas/FasL on the surface of the cells with that on the surface of T cells. The mitochondrial pathway was then stimulated to transmit apoptosis signals, which activated the upstream caspase-8 and further the downstream caspase-3. In this way, a cascade amplification system was formed to induce apoptosis. In addition, the apoptosis process was affected by the synergistic action of tumor suppressor gene p53 and negative feedback gene Bcl-2 for apoptosis, jointly forming the regulation network of ST inducing apoptosis of hepatocellular carcinoma SMMC-7721 cells, as shown in Figure 3.

Although this experiment verified that ST can induce the apoptosis of hepatocellular carcinoma SMMC-7721 cells, the apoptosis mechanism was related to the expression of Fas, FasL, caspase-8, caspase-3, p53 and Bcl-2 in the mitochondrial pathway. This result provides powerful evidence of the improved apoptosis effects of ST on hepatocellular carcinoma cells; however, due to only a few genes being involved in apoptosis, the regulatory effects of other genes were unable to be determined here. Moreover, the apoptosis may be related to other proteins in the caspase family, so the molecular mechanism of the apoptosis induced by ST is not completely explained and needs to be further verified by other studies.

COMMENTS

Background

Solanum lyratum Thumb (ST) belonging to Solanaceae is generally used to treat tumors, and it is a commonly used anticancer drug. However, the effects and mechanism of ST on tumor cells are unclear.

Research frontiers

Precision treatment of Chinese herbal medicine is the focus of the current research and hot spots of investigations. This study verified the mechanism of Chinese herbal medicine by using molecular biological technology, providing powerful evidence of the improved apoptosis effects of *Solanum lyratum* Thumb on hepatocellular carcinoma cells.

Innovations and breakthroughs

The experiment verified that ST ethanol extracts up-regulated Fas and down-regulated FasL in the mitochondrial pathway, inducing the up-regulation for the expression of caspase-8 and caspse-3. In addition, ST ethanol extracts induced the apoptosis of hepatocellular carcinoma SMMC-7721 cells through feedback regulation by up-regulating the *p53* gene that inhibits cancers and down-regulating the *Bcl-2* gene.

Applications

These findings provide powerful evidence of the improved apoptosis effects of ST on hepatocellular carcinoma cells.

Peer-review

This study is well designed and the results are interesting. The authors try to explore the induction effects and mechanism of ST on human hepatocellular carcinoma SMMC-7721 cells through the mitochondrial pathway.

REFERENCES

1 **Liu SH**, Shen XH, Wei XF, Mao XH, Huang T. Immunomodulatory

activity of butanol extract from Solanum lyratum in tumor-bearing mice. *Immunopharmacol Immunotoxicol* 2011; **33**: 100-106 [PMID: 20482445 DOI: 10.3109/08923973]
2 **Guan Y**, Zhao H, Yan X, Meng J, Wang W. A study on anti-tumour effect of Solanum lyratum Thunb. extract in S_{180} tumour-bearing mice. *Afr J Tradit Complement Altern Med* 2013; **10**: 345-351 [PMID: 24311848]
3 **Nanjing University of Chinese Traditional Medicine**. Dictionary of Chinese Medicine. Shanghai: Shanghai Science and Technology Press, 2006
4 **Elkholi R**, Renault TT, Serasinghe MN, Chipuk JE. Putting the pieces together: How is the mitochondrial pathway of apoptosis regulated in cancer and chemotherapy? *Cancer Metab* 2014; **2**: 16 [PMID: 25621172 DOI: 10.1186/2049-3002-2-16]
5 **Loison F**, Zhu H, Karatepe K, Kasorn A, Liu P, Ye K, Zhou J, Cao S, Gong H, Jenne DE, Remold-O'Donnell E, Xu Y, Luo HR. Proteinase 3-dependent caspase-3 cleavage modulates neutrophil death and inflammation. *J Clin Invest* 2014; **124**: 4445-4458 [PMID: 25180606 DOI: 10.1172/JCI76246]
6 **Rahman MA**, Bishayee K, Huh SO. Angelica polymorpha Maxim Induces Apoptosis of Human SH-SY5Y Neuroblastoma Cells by Regulating an Intrinsic Caspase Pathway. *Mol Cells* 2016; **39**: 119-128 [PMID: 26674967 DOI: 10.14348/molcells.2016.2232]
7 **Neophytou CM**, Constantinou C, Papageorgis P, Constantinou AI. D-alpha-tocopheryl polyethylene glycol succinate (TPGS) induces cell cycle arrest and apoptosis selectively in Survivin-overexpressing breast cancer cells. *Biochem Pharmacol* 2014; **89**: 31-42 [PMID: 24560876 DOI: 10.1016/j.bcp.2014.02.003]
8 **Wei X**, Li ZG, Nong S, Huang XM. The influence of Solanum lyratum Thunb extract on apoptosis and the expression of fas/fasL genes in Hela cells. *Zhongyaocai* 2006; **29**: 1203-1206 [PMID: 17228663]
9 **Tu S**, Wan FS, Wei X, Nong S, Zhu WF, Liu ZQ. Solamum lyratum Thunbery Alkaloid Induces Human Lung Adenoearcinoma A549 Cells Apoptosis by Activating FAS-pathway. *Shizhen Guoyi Guoyao* 2013; **24**: 66-68
10 **Jia YR**, Tian XL, Liu K, Chen C, Wang XL, Zhang CC, Sun LX. Simultaneous determination of four alkaloids in Solanum lyratum Thunb by UPLC-MS/MS method. *Pharmazie* 2012; **67**: 111-115 [PMID: 22512079 DOI: 10.1691/ph.2012.1087]
11 **Marí M**, Morales A, Colell A, García-Ruiz C, Kaplowitz N, Fernández-Checa JC. Mitochondrial glutathione: features, regulation and role in disease. *Biochim Biophys Acta* 2013; **1830**: 3317-3328 [PMID: 23123815 DOI: 10.1016/j.bbagen.2012.10.018]
12 **Marí M**, Morales A, Colell A, García-Ruiz C, Kaplowitz N, Fernández-Checa JC. Mitochondrial glutathione: features, regulation and role in disease. *Biochim Biophys Acta* 2013; **1830**: 3317-3328 [PMID: 23123815 DOI: 10.1016/j.bbagen.2012.10.018]
13 **Saralamma VV**, Nagappan A, Hong GE, Lee HJ, Yumnam S, Raha S, Heo JD, Lee SJ, Lee WS, Kim EH, Kim GS. Poncirin Induces Apoptosis in AGS Human Gastric Cancer Cells through Extrinsic Apoptotic Pathway by up-Regulation of Fas Ligand. *Int J Mol Sci* 2015; **16**: 22676-22691 [PMID: 26393583 DOI: 10.3390/ijms160922676]
14 **Davidson MT**, Deitch EA, Lu Q, Haskó G, Abungu B, Németh ZH, Zaets SB, Gaspers LD, Thomas AP, Xu DZ. Trauma-hemorrhagic shock mesenteric lymph induces endothelial apoptosis that involves both caspase-dependent and caspase-independent mechanisms. *Ann Surg* 2004; **240**: 123-131 [PMID: 15213628 DOI: 10.1097/sla.0000129341.94219.cf]
15 **He YC**, Zhou FL, Shen Y, Liao DF, Cao D. Apoptotic death of cancer stem cells for cancer therapy. *Int J Mol Sci* 2014; **15**: 8335-8351 [PMID: 24823879 DOI: 10.3390/ijms15058335]
16 **Castedo M**, Ferri K, Roumier T, Métivier D, Zamzami N, Kroemer G. Quantitation of mitochondrial alterations associated with apoptosis. *J Immunol Methods* 2002; **265**: 39-47 [PMID: 12072177 DOI: 10.1016/S0022-1759(02)00069-8]
17 **Tian H**, Zhang DF, Zhang BF, Li HZ, Zhang Q, Li LT, Pei DS, Zheng JN. Melanoma differentiation associated gene-7/interleukin-24 induces caspase-3 denitrosylation to facilitate the activation of cancer cell apoptosis. *J Interferon Cytokine Res* 2015; **35**: 157-167 [PMID: 25347351 DOI: 10.1089/jir.2014.0061]
18 **Saito-Hakoda A**, Uruno A, Yokoyama A, Shimizu K, Parvin R, Kudo M, Saito-Ito T, Sato I, Kogure N, Suzuki D, Shimada H, Yoshikawa T, Fujiwara I, Kagechika H, Iwasaki Y, Kure S, Ito S, Sugawara A. Effects of RXR Agonists on Cell Proliferation/Apoptosis and ACTH Secretion/Pomc Expression. *PLoS One* 2015; **10**: e0141960 [PMID: 26714014 DOI: 10.1371/journal.pone.0141960]
19 **Ram DR**, Ilyukha V, Volkova T, Buzdin A, Tai A, Smirnova I, Poltorak A. Balance between short and long isoforms of cFLIP regulates Fas-mediated apoptosis in vivo. *Proc Natl Acad Sci USA* 2016; **113**: 1606-1611 [PMID: 26798068 DOI: 10.1073/pnas.1517562113]
20 **Kwon YH**, Bishayee K, Rahman A, Hong JS, Lim SS, Huh SO. Morus alba Accumulates Reactive Oxygen Species to Initiate Apoptosis via FOXO-Caspase 3-Dependent Pathway in Neuroblastoma Cells. *Mol Cells* 2015; **38**: 630-637 [PMID: 25921607 DOI: 10.14348/molcells.2015.0030]
21 **Hsiao PC**, Lee WJ, Yang SF, Tan P, Chen HY, Lee LM, Chang JL, Lai GM, Chow JM, Chien MH. Nobiletin suppresses the proliferation and induces apoptosis involving MAPKs and caspase-8/-9/-3 signals in human acute myeloid leukemia cells. *Tumour Biol* 2014; **35**: 11903-11911 [PMID: 25164609 DOI: 10.1007/s13277-014-2457-0]
22 **Lai YJ**, Lin CI, Wang CL, Chao JI. Expression of survivin and p53 modulates honokiol-induced apoptosis in colorectal cancer cells. *J Cell Biochem* 2014; **115**: 1888-1899 [PMID: 24905183 DOI: 10.1002/jcb.24858]
23 **Hyun HB**, Lee WS, Go SI, Nagappan A, Park C, Han MH, Hong SH, Kim G, Kim GY, Cheong J, Ryu CH, Shin SC, Choi YH. The flavonoid morin from Moraceae induces apoptosis by modulation of Bcl-2 family members and Fas receptor in HCT 116 cells. *Int J Oncol* 2015; **46**: 2670-2678 [PMID: 25892545 DOI: 10.3892/ijo.2015.2967]
24 **Chen YF**, Yang JS, Chang WS, Tsai SC, Peng SF, Zhou YR. Houttuynia cordata Thunb extract modulates G0/G1 arrest and Fas/CD95-mediated death receptor apoptotic cell death in human lung cancer A549 cells. *J Biomed Sci* 2013; **20**: 18 [PMID: 23506616 DOI: 10.1186/1423-0127-20-18]
25 **Choi HS**, Seo HS, Kim JH, Um JY, Shin YC, Ko SG. Ethanol extract of paeonia suffruticosa Andrews (PSE) induced AGS human gastric cancer cell apoptosis via fas-dependent apoptosis and MDM2-p53 pathways. *J Biomed Sci* 2012; **19**: 82 [PMID: 22963678 DOI: 10.1186/1423-0127-19-82]

P- Reviewer: Abdollahi M, Tokunaga Y **S- Editor**: Gong ZM
L- Editor: Filipodia **E- Editor**: Liu WX

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v23.i6.1018

World J Gastroenterol 2017 February 14; 23(6): 1018-1029
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Case Control Study

Cerebral magnetic resonance imaging in quiescent Crohn's disease patients with fatigue

Sanne van Erp, Ece Ercan, Perla Breedveld, Lianne Brakenhoff, Eidrees Ghariq, Sophie Schmid, Matthias van Osch, Mark van Buchem, Bart Emmer, Jeroen van der Grond, Ron Wolterbeek, Daniel Hommes, Herma Fidder, Nic van der Wee, Tom Huizinga, Désirée van der Heijde, Huub Middelkoop, Itamar Ronen, Andrea van der Meulen-de Jong

Sanne van Erp, Lianne Brakenhoff, Daniel Hommes, Andrea van der Meulen-de Jong, Department of Gastroenterology and Hepatology, Leiden University Medical Center, Leiden, Zuid-Holland 2333 ZA, the Netherlands

Ece Ercan, Perla Breedveld, Eidrees Ghariq, Sophie Schmid, Matthias van Osch, Itamar Ronen, C.J. Gorter Center, Leiden University Medical Center, Leiden, Zuid-Holland 2333 ZA, the Netherlands

Ece Ercan, Perla Breedveld, Eidrees Ghariq, Sophie Schmid, Matthias van Osch, Mark van Buchem, Bart Emmer, Jeroen van der Grond, Itamar Ronen, Department of Radiology, Leiden University Medical Center, Leiden, Zuid-Holland 2333 ZA, the Netherlands

Ron Wolterbeek, Department of Medical Statistics, Leiden University Medical Center, Leiden, Zuid-Holland 2333 ZA, the Netherlands

Daniel Hommes, Center for Inflammatory Bowel Diseases, University of California Los Angeles, Los Angeles, CA 90095, United States

Herma Fidder, Department of Gastroenterology and Hepatology, University Medical Center Utrecht, Utrecht 3584 CX, the Netherlands

Nic van der Wee, Department of Psychiatry, Leiden University Medical Center, Leiden, Zuid-Holland 2333 ZA, the Netherlands

Tom Huizinga, Désirée van der Heijde, Department of Rheumatology, Leiden University Medical Center, Leiden, Zuid-Holland 2333 ZA, the Netherlands

Huub Middelkoop, Department of Neuropsychology, Leiden University Medical Center, Leiden, Zuid-Holland 2333 ZA, the Netherlands

Author contributions: Van Erp S and Ercan E contributed equally to this work; Brakenhoff L, Hommes D, Middelkoop H, Ronen I and van der Meulen-de Jong A designed the research; Ercan E, Brakenhoff L, Hommes D, Fidder H, Huizinga T, van der Heijde D and van der Meulen-de Jong A treated patients and collected material and clinical data from patients; van Erp S, Ercan E, Breedveld P and Brakenhoff L performed the research; van Buchem M, Emmer B and van der Grond J contribed new analytic tools; van Erp S, Ercan E, Breedveld P, Brakenhoff L and Wolterbeek R analysed the data; van Erp S and Ercan E wrote the paper; all authors contributed to critical revision of the manuscript and final approval of the submitted manuscript.

Institutional review board statement: This study was approved by the institutional medical ethical committee of the LUMC.

Informed consent statement: All patients signed a written informed consent prior to study enrolment.

Conflict-of-interest statement: The authors have no conflicts of interest to disclose.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Correspondence to: Dr. Sanne van Erp, Department of Gastroenterology and Hepatology, Leiden University Medical Center, Albinusdreef 2, Leiden 2333 ZA, the Netherlands. s.j.h.van_erp@lumc.nl

Telephone: +31-71-5262915
Fax: +31-71-5248115

Received: August 26, 2016
Peer-review started: August 28, 2016
First decision: October 10, 2016
Revised: November 14, 2016
Accepted: December 8, 2016
Article in press: December 8, 2016
Published online: February 14, 2017

Abstract

AIM

To evaluate brain involvement in quiescent Crohn's disease (CD) patients with fatigue using quantitative magnetic resonance imaging (MRI).

METHODS

Multiple MRI techniques were used to assess cerebral changes in 20 quiescent CD patients with fatigue (defined with at least 6 points out of an 11-point numeric rating scale compared with 17 healthy age and gender matched controls without fatigue. Furthermore, mental status was assessed by cognitive functioning, based on the neuropsychological inventory including the different domains global cognitive functioning, memory and executive functioning and in addition mood and quality of life scores. Cognitive functioning and mood status were correlated with MRI findings in the both study groups.

RESULTS

Reduced glutamate + glutamine (Glx = Glu + Gln) concentrations (P = 0.02) and ratios to total creatine (P = 0.02) were found in CD patients compared with controls. Significant increased Cerebral Blood Flow (P = 0.05) was found in CD patients (53.08 ± 6.14 mL/100 g/min) compared with controls (47.60 ± 8.62 mL/100 g/min). CD patients encountered significantly more depressive symptoms (P < 0.001). Cognitive functioning scores related to memory (P = 0.007) and executive functioning (P = 0.02) were lower in CD patients and both scores showed correlation with depression and anxiety. No correlation was found subcortical volumes between CD patients and controls in the T_1-weighted analysis. In addition, no correlation was found between mental status and MRI findings.

CONCLUSION

This work shows evidence for perfusion, neurochemical and mental differences in the brain of CD patients with fatigue compared with healthy controls.

Key words: Magnetic resonance imaging; Systemic inflammation; Fatigue; Crohn's disease; Cognition

Core tip: The present study explores perfusion, neurochemical and mental differences in the brain of Crohn's disease (CD) patients compared with healthy controls. This implies that for a gastroenterologist it is important to focus, besides gastrointestinal symptoms due to inflammation, on the effects of systemic inflammation on the brain and mental status. Knowledge and understanding of these effects in CD patients may help health professionals to set up interventions to maintain CD remission and improve mental status by *e.g.* psychosocial interventions.

Van Erp S, Ercan E, Breedveld P, Brakenhoff L, Ghariq E, Schmid S, van Osch M, van Buchem M, Emmer B, van der Grond J, Wolterbeek R, Hommes D, Fidder H, van der Wee N, Huizinga T, van der Heijde D, Middelkoop H, Ronen I, van der Meulen-de Jong A. Cerebral magnetic resonance imaging in quiescent Crohn's disease patients with fatigue. *World J Gastroenterol* 2017; 23(6): 1018-1029 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i6/1018.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i6.1018

INTRODUCTION

Crohn's disease (CD) is a relapsing inflammatory bowel disease (IBD)[1], characterized by segmental transmural lesions that can affect any part of the gastrointestinal tract[2]. Besides gastrointestinal symptoms, fatigue is common in CD patients. In contrast to regular fatigue which affects nearly everyone, disease-related fatigue is more long lasting and may occur despite sufficient sleep and rest. Generally, fatigue lasting for more than 6 mo is considered chronic and is significantly more prevalent in IBD patients than in healthy controls[3]. Although fatigue is influenced by IBD disease activity, 40% of the patients with quiescent disease report fatigue as well and contributes negatively to the patients' health-related quality of life (QoL)[4,5].

The pathogenesis of CD is multifactorial and results from an impaired interaction between environment, commensal microbiota and the human immune system, leading to a chronic inflammatory status and eventually CD[6,7]. Furthermore, in both quiescent and active CD patients increased levels of circulating inflammatory cytokines, such as tumour necrosis factor-α (TNF-α) are reported[8-10]. Although quiescent CD patients report fewer clinical symptoms and score less on the clinical activity score compared with active CD patients, inflammatory cytokines are present[9,10]. TNF-α can be secreted by a large variety of cells[8] and can initiate a signalling stimulus to the brain parenchyma that will subsequently activate microglia. Activated microglia stimulates the production of monocyte chemo-attractant protein (MCP)-/CCP2, which recruits monocytes into the brain[9-11]. Moreover, this cerebral infiltration of monocytes plays an important role in driving inflammation in the brain[11-13].

Magnetic resonance imaging (MRI) is an imaging technique widely used to visualize the effect of several neurological diseases, such as Multiple Sclerosis (MS), Parkinson's Disease and Alzheimer disease, in the brain[14]. A variety of MRI methods can be employed to identify cerebral changes due to a specific disease. These methods include T_1-weighted imaging, magnetization transfer imaging (MTI), magnetic resonance spectroscopy (MRS), arterial spin labeling (ASL) and diffusion tensor imaging (DTI). T_1-weighted imaging provides high-resolution, high-contrast anatomical images of the brain and can be used to determine the volumes of the grey matter (GM), white matter (WM), cerebral spinal fluid (CSF) and subcortical structures[15]. Through voxel based morphometry (VBM), it is possible to visualize local changes in GM volumes[16]. MTI is a technique sensitive to brain tissue microstructural changes, stemming from changes in macromolecules such as myelin or cell membranes[17]. MRS measures the concentration of certain metabolites in living tissues and gives evidence for neurochemical changes[18]. ASL is a non-invasive tool for the quantification of regional cerebral blood flow (CBF)[19] and can reveal changes in tissue perfusion. DTI is sensitive to minute changes in tissue microstructure, such as changes in myelin integrity and axonal density in white matter fiber tracts, based on the random motion or diffusion of water molecules[20].

Previous MRI studies have shown that systemic inflammation contributes to cognitive decline, for example in relation to aging[21], but also to brain diseases including Alzheimer disease, MS and Parkinson's disease by promoting activation of the immune system[22-24]. Metabolic and cerebral perfusion changes have been found in the brain of patients with Rheumatoid Arthritis (RA), Systemic Sclerosis and Systemic Lupus Erythematosus (SLE)[11,12,25-31]. In addition, previous studies performed in patients with Chronic Fatigue Syndrome (CFS) found an association between fatigue complaints and metabolic changes in the brain as well[32-34]. In CFS patients, the mean ratio of choline (Cho) to creatine (Cr) in the occipital cortex was significantly higher than in controls, indicating an abnormality of phospholipid metabolism in the brain in CFS[32-33]. These findings suggest that systemic inflammation and fatigue complaints could have structural, neurochemical and functional correlates in the brain. So far, the link between systemic inflammation, disease-induced fatigue and changes in the brain have not been explored in CD patients. The aim of this exploratory study was to investigate to what extent systemic inflammation affects the brain of quiescent CD patients, by using a variety of MRI acquisition methods and neuropsychological examinations that assess cognition, mood and QoL. Furthermore, the correlation between MRI changes, clinical characteristics, including fatigue scores, and mental status was investigated.

MATERIALS AND METHODS

Study population and study design

In this case-control study 20 CD patients and 17 age and gender matched healthy controls were included. Since it is known from literature that there is an age associated decrease in brain volume, primarily caused by a decrease in neuronal size and partly due to a reduction in numbers of neurons caused by apoptosis[35], a correction was made for this confounder by matching the subjects.

Consecutive CD patients, fulfilling the inclusion criteria, were recruited through the IBD outpatient clinic of the department of Gastroenterology and Hepatology of the Leiden University Medical Center (LUMC), the Netherlands. The patients had endoscopic proven CD for at least 3 mo before inclusion, were in clinical remission and experienced fatigue. CD patients with anemia (Hb < 7.0 mmol/L), primary sclerosing cholangitis and routine MRI-contraindications (*e.g.,* instable metal implants or a pacemaker) were excluded. All medication deemed necessary by the gastroenterologist was allowed at study inclusion, except for anti-TNFα or corticosteroid use, since this medication could reduce systemic inflammation the most and thus influence clinical disease activity. Healthy controls were recruited *via* an advertisement in het LUMC and included in the study if they had no anamnestic brain abnormalities, nervous system disease or chronic inflammation in the body. A 1-d program was set up for all participants by the relevant medical specialists, including a gastroenterologist, radiologist, psychiatrist and neuropsychologist and all individuals were asked to complete several questionnaires at study inclusion about demographics, mental status and QoL. This study was approved by the institutional medical ethical committee of the LUMC and all patients signed a written informed consent prior to study enrolment.

Clinical characteristics

Disease activity: The clinical disease activity of the CD patients was measured with the Harvey-Bradshaw Index (HBI). The HBI consists of 12 criteria, which include general well-being, abdominal pain, daily number of liquid stools, abdominal mass and extra intestinal manifestations (arthralgia, uveitis, erythema nodosum, aphthous ulcers, pyoderma gangrenosum, anal fissure, new fistula and abscess). Patients with an HBI score of 4 or less were classified as having quiescent CD disease[36].

Fatigue: Fatigue was assessed with the Multidimensional Fatigue Index (MFI) and the Visual Analogue Scale (VAS). The MFI is a self-report measurement containing 20 questions consisting of 5 subscales covering different dimensions: general fatigue, physical fatigue, mental fatigue, reduced activity and reduced motivation. The questions are about the fatigue experienced by the subject in the 7 d prior to examination.

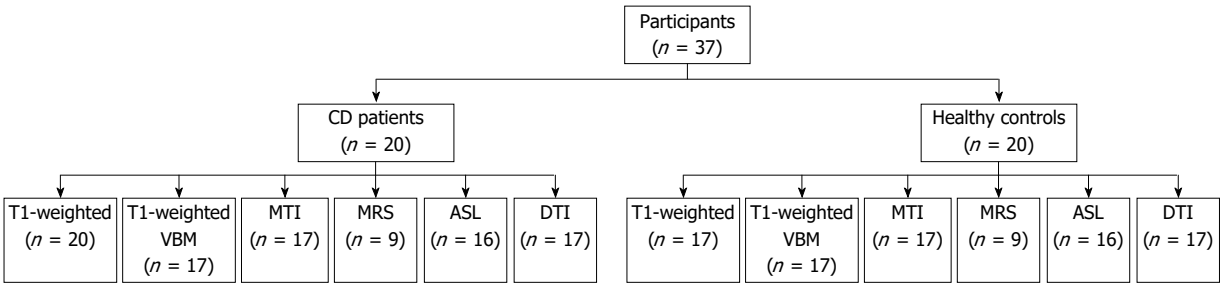

Figure 1 Flowchart of included participants in the magnetic resonance imaging analyses. CD: Crohn's disease; VBM: Voxel based morphometry; MTI: Magnetization transfer images; MRS: Magnetic resonance spectroscopy; ASL: Arterial spin labelling; DTI: Diffusion tensor imaging.

Scores range from 4 to 20, with higher scores indicating higher levels of fatigue[37]. The VAS consists of a 10 point self-rating scale that measures subjective experiences of fatigue. The participants had to indicate on a visual line how they were currently feeling. Six points or more indicated the presence and experience of fatigue in individuals[38].

MRI data acquisition

All study subjects underwent MRI of the brain, using a Philips Ingenia 3.0 Tesla MRI Scanner (Philips Medical Systems, Best, The Netherlands) equipped with a 12 channel head coil, and images were evaluated by an experienced neuroradiologist (MvB). The MRI protocol consisted of T_1-weighted imaging, MTI, MRS, ASL and DTI, and lasted for about 60 min. Since more CD patients were included and all patients and healthy controls were age-gender matched, in total 3 CD patients, who matched the least with the controls, got excluded from the voxel-based analysis of the T_1-weighted and DTI data. For the MRS and ASL analyses data of some CD subjects were either missing because of time limitations or excluded due to low quality, caused by subject motion. For the MRS analysis only 9 CD patients and 9 age and gender matched controls were included, and for the ASL analysis 16 CD patients and 16 age and gender matched controls were included (Figure 1). The MRI scan protocol consisted of (1) axial 3D T1-weighted images (FOV: 224 mm × 144 mm × 182 mm, resolution: 0.88 mm × 0.88 mm × 1.20 mm, TR/TE = 9.75/4.59 ms); (2) sagittal FLAIR images (FOV: 224 mm × 144 mm × 180 mm, resolution: 0.5 mm × 0.5 mm × 3.6 mm, TR/TE/TI = 10000/120/1650 ms); (3) axial DTI (FOV: 176 mm × 144 mm × 224 mm, resolution: 1.75 mm × 1.75 mm × 3.6 mm, TR/TE = 4317/55.33 ms, one volume with b = 0 s/mm^2 and 32 diffusion-weighted volumes with b = 800 s/mm^2); (4) axial MTI (FOV: 224 × 144 × 180, resolution: 0.88 mm × 0.88 mm × 7.2 mm, TR/TE = 100/10.95 ms, two volumes acquired one with and one without a radiofrequency saturation pulse); (5) ASL (FOV: 240 mm × 240 mm × 133 mm, resolution: 3.0 mm × 3.0 mm × 7.0 mm, TR/TE = 4000/15.19 ms, labeling duration = 1650 ms, post-labeling delay = 1525 ms, 35 label and control pairs and background suppression inversion pulses at 50 and 1150 ms); and (6) a single volume, stimulated echo acquisition mode (STEAM) ^{1}H MRS scan with a volume of interest (VOI) located in the left centrum semi ovale, containing mostly white matter as shown in Figure 2 (voxel size = 30 mm × 15 mm × 15 mm, TR/TE = 2000/14 ms, mixing time = 19 ms, sample size = 2048, number of averages = 96).

Post-processing and data analysis

T_1-weighted image analysis: Brain extraction tool (BET) of FMRIB Software Library (FSL) (http://www.fmrib.ox.ac.uk/fsl) was used to extract the brain tissue from T_1-weighted images[15]. FSL FMRIB's Automated Segmentation Tool (FAST)[39] was used to segment GM, WM and CSF tissues from the brain extracted T_1-weighted images. FSL FMRIB's Integrated Registration and Segmentation Tool was used to segment subcortical structures: nucleus accumbens, amygdala, caudate, hippocampus, globus pallidus, putamen and thalamus[40]. Following segmentation, the volumes of GM, WM and subcortical structures were calculated using FSL Maths. The volumes were normalized to subject intracranial volume by dividing the volumes with the total brain volume of the same subject. VBM in FSL was used to assess local GM differences between CD patients and controls[16,41].

MTI analysis: MTI were split into images with and without saturation. Both images, with and without saturation, were brain extracted with BET and the image without saturation was aligned to the image with saturation. After alignment, the magnetization transfer ratio (MTR) of the whole brain was calculated using FSL Maths. The MTR images were then registered to the T_1-weighted images from the same subject with FLIRT[42]. Subsequently, MTR images were multiplied with the binary GM and WM masks from the same subject, to create GM and WM MTR images. Tissue-specific histograms of MTR values from the GM and WM of patients and controls were created using an in house-developed MATLAB® program (Mathworks, Natick, MA, United States).

MRS analysis: The MRS analysis was performed

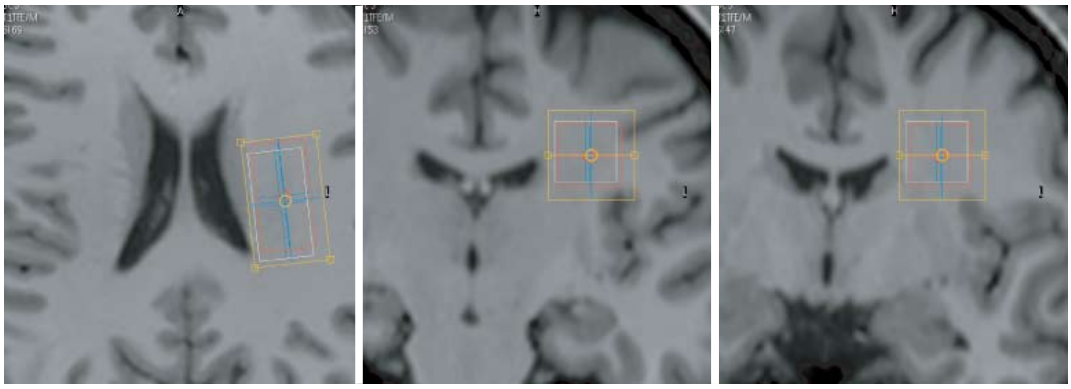

Figure 2 Planning of the ^{1}H-magnetic resonance spectroscopy volume of interest in the left centrum semi-ovale. Seen on axial (left) and on the coronal (right) T_1-weighted image slices. The effective volume of interest set at the tNAA frequency is shown (red rectangle) together with the shimming volume (yellow rectangle).

in MATLAB and LCmodel[43]. An in-house developed MATLAB code was used to calculate and correct for GM, WM and CSF tissue fraction (%) within the VOI for each subject separately. LCmodel was used for the calculation of the concentration and ratio to total creatine (tCr) of the metabolites N-acetyl-aspartate (NAA), creatine (Cr), glutamate (Glu), myo-inositol (Ins), glutamine (Gln), N-acetyl-aspartyl-glutamate (NAAG) and choline (Cho). Institutional units (IU) of concentration were expressed in mmol. Among these metabolites, NAA is a neuronal marker, NAAG is suggested to be related to excitatory neurotransmission, total Creatine (tCr), the sum of phosphocreatine and creatine, is a marker of energy metabolism. Cho is related to cell membrane turnover, Glu is an excitatory neurotransmitter predominantly found in neurons, Gln is a precursor for Glu and found mostly in astrocytes, and Ins is a possible astrocytic marker[44]. The mean ratios of NAA, Glu, Ins, Gln, NAAG and Cho to tCr were compared between the two study groups.

ASL analysis: The average GM Cerebral Blood Flow value was calculated in FSL[45]. The ASL label and control images were motion corrected by FSL MCFLIRT[46]. The perfusion maps were calculated for each subject by subtracting the label from the control images and averaging those images. Following that, perfusion maps from each subject were registered first linearly and then nonlinearly to GM volume segmented from the T_1-weighted image of the subject and subsequently they were first linearly and then nonlinearly registered to average brain template from the Montreal Neurological Institute (MNI). CBF of the GM is calculated by using a binary GM mask of the subject with a threshold of 60% GM probability and using the following equation:

$$CBF_{pCASL} = \frac{6000 \cdot \lambda \cdot \Delta M \cdot e^{(PLD/T_{1a})}}{SI_{PD} \cdot 2 \cdot \alpha_{pCASL} \cdot T_{1a} \cdot \alpha_{BSup} \cdot (1 - e^{-(\tau/T_{1a})})}$$

Where λ is the blood/brain partition coefficient in mL/g which was 0.9, ΔM is the signal intensity of the control image subtracted with the signal intensity of the label image, the post labelling delay was 1525 ms. T_1a is the longitudinal relaxation time of the blood was 1664 ms, SI_{PD} is the signal intensity of a proton density-weighted image and τ is the label duration which was 1650 ms. A_{pCASL} is the labelling efficiency, which was 0.85 and α_{BSup} was 0.83. A comparison of GM CBF was made between the patients and controls.

Diffusion tension images analysis: ExploreDTI software[47] was used for motion and distortion correction of the DTI images and for calculating the Fractional Anisotropy (FA) and Mean Diffusivity (MD) maps. FA and MD maps were used as an input to tract-based spatial statistics processing[48], which was carried out in FSL. The FA maps were first linearly registered with an affine transformation, subsequently non-linearly registered to the MNI space, and a mean FA skeleton was created. For each subject, the FA map was projected on the skeleton. Following that, randomisation was used to perform *t*-test based voxel-wise comparison of the FA skeletons between patients and controls. The same procedure was repeated for MD maps.

Assessment of cognitive performance

Cognition: Several neuropsychological assessments were conducted in both healthy controls and CD patients and evaluated by an experienced clinical neuropsychologist (HM). The examination took approximately one hour and included validated test methods in a fixed order. Since the cognitive functioning of patients with IBD has not been fully previously investigated, the focus was on a wide range of neuropsychological functions. Global cognitive functioning was assessed by the Minimal Mental State Examination (MMSE). The MMSE contained 11 questions, subdivided into 5 subdomains. All questions were scored individually and added to produce a total score ranging from 0 to 30, with higher scores indicating better cognitive functioning[49]. The memory domain was evaluated with the Digit Span Forward and Backward subtests of, respectively the revised Wechsler Adult Intelligence Scale (WAIS-R)[50] and the revised Wechsler Memory Scale (WMS-R). Higher scores reflected better memory performance[51].

Executive functioning was assessed by the Word Fluency Test (WFT)[52], Stroop-Color-Word test (SCWT)[53] given in three parts, and the Trail Making Test (TMT)[54] subdivided into two parts, whereby part A measured attention and performance speed, and part B measured mental flexibility and ability to shift attention. The TMT involved scanning, visuomotor tracking, divided attention and cognitive flexibility. The time used for each trial was noted, with more time used indicating lower performance. The SCWT was used to measure interference sensibility. One response (reading the word) should be inhibited in order to name the colour of the ink, which leads to a delay in reaction time. The number of correct responses within 45 seconds was counted[53]. Furthermore, the WAIS-R Digit symbol and Digit cancellation test was measured[50].

Mental status: Cognitive performance depends on the psychiatric status of the patient[55], and therefore the Hospital Anxiety Depression Scale (HADS) was included in the neuropsychological examination. The HADS was used to determine depressive symptoms and anxiety. HADS is a widely used measurement to identify emotional disorders in non-psychiatric patients. The scale includes 14 items, 7 items concerning anxiety and 7 concerning depression, each scored between 0 and 3. A score above 8 on each individual scale were considered as a possible case and a score above 10 as a probable case[56].

QoL: To determine the QoL, the Short Form-36 (SF-36) was used. The SF-36 is a generic questionnaire to assess self-reported QoL. This measurement includes in total 8 subscales covering physical and mental aspects of QoL. The score ranges from 0 to 100, with higher score indicating better QoL. The Dutch translation of the SF-36 was validated in both the general population and in CD patients[57].

Statistical analysis

Data analyses were performed using SPSS 20.0, IBM Corp, 2011, Armonk, NY, United States. Descriptive statistics were used for the patients' characteristics. All comparisons between the patient and control groups were performed with an independent *t*-test. A *P*-value $\leqslant 0.05$ was considered statistically significant. To correct for multiple testing, the level of significance was set at $P < 0.01$ (0.05/5) and $P < 0.006$ (0.05/8) for the fatigue (five MFI subscores) and QoL (eight SF-38 subscales) scores, respectively. Based on the individual cognitive tests corrected for education, Z-scores of the different cognitive domains were created by using the UNIANOVA test with an average mean ± SD. Correlations between the MRI outcomes, cognition and mood status were performed with the Pearson Correlation test.

RESULTS

Demographic characteristics

In this study, 20 CD patients and 17 healthy controls were age ($P = 0.46$) and gender matched ($P = 0.68$). All patients were in clinical remission at study inclusion (mean HBI = 2.16, SD = 1.12), with an average age of onset at 21.4 years and an IBD disease duration of 8.8 years. Based on the inclusion criteria, patients reported more fatigue complaints according to the MFI-20 ($P < 0.001$) and VAS fatigue score ($P < 0.001$) compared with the control subjects. Furthermore, the education level of the healthy controls was significantly higher than that of the CD patients. Since this variable might influence mental status scores, a correction was made. An overview of the clinical characteristics of the individuals is presented in Table 1.

MRI analysis

Volumetric data: The comparison of the subcortical volumes between the CD patients and controls in the analysis of the T_1weighted images did not show significant differences between the two subject groups. The volume differences in the right amygdala ($P = 0.08$) and nucleus accumbens ($P = 0.08$) just missed significance (Table 2). VBM analysis showed a lower GM content in the superior frontal gyrus in CD patients compared with healthy controls ($P < 0.05$) (Figure 3).

MTI data: No significant differences were observed in the mean MTR values or in the MTR histogram peak heights of the CD patients compared with healthy controls.

MRS data: Lower glutamate + glutamine (Glx = Glu + Gln) concentrations (4.85 ± 0.78 mmol *vs* 5.96 ± 0.98 mmol, $P = 0.02$) and ratios to tCr (0.92 ± 0.13 *vs* 1.10 ± 0.14, $P = 0.02$) were found in the patient population compared with control subjects (Table 3).

ASL data: Average GM CBF of the CD patients (53.1 ± 6.1 mL/100 g/min) was significantly higher than the GM CBF of the control group (47.6 ± 8.6 mL/100 g/min) ($P = 0.05$).

DTI data: No differences were observed across white matter in the FA and MD values between CD patients and controls.

Mental status

Neuropsychological examination and cognitive scores were corrected for educational level (Table 4). Generally, a difference close to significance between patients and controls was found in several individual cognitive test scores. Compared with controls, CD patients had a lower Stroop interference index ($P = 0.06$), a reduced total score of the WAIS-R Digit

Table 1 Demographic characteristics

	CD patients (*n* = 20)	Controls (*n* = 17)	*P* value
Age (yr) at inclusion, mean ± SD	30.1 ± 6.2	28.5 (6.7)	0.460
Female, *n* (%)	17 (85.0)	13 (76.5)	0.680
HBI score, mean ± SD[1]	2.2 ± 1.1	-	-
Age of IBD onset (yr), mean ± SD	21.4 ± 5.7	-	-
IBD disease duration (yr), mean ± SD	8.8 ± 7.2	-	-
Smoker, *n* (%)	11 (55.0)	4.0 (23.5)	0.400
VAS, mean ± SD	7.4 (1.3)	3.4 (2.3)	< 0.001
MFI, mean ± SD	66.1 (13.3)	36.4 (10.3)	< 0.001
General Fatigue	16.4 (2.8)	8.9 (3.3)	< 0.001
Physical Fatigue	14.4 (3.0)	6.2 (2.2)	< 0.001
Mental Fatigue	12.8 (4.1)	7.2 (2.9)	< 0.001
Reduced Activity	10.7 (3.5)	6.9 (2.7)	< 0.001
Reduced Motivation	12.0 (3.6)	7.1 (2.7)	< 0.001
Education level, *n* (%)			0.001
Low[a]	4 (20)	-	
Intermediate[b]	10 (50)	2 (11.8)	
High[c]	6 (30)	15 (88.2)	
Montreal classification			
Location CD, *n* (%)			
L1 ileal	3 (15.0)	-	-
L2 colonic	2 (10.0)	-	-
L3 ileocolonic	15 (75.0)	-	-
L4 upper	-	-	-
L1-3 + L4	-	-	-
Behaviour CD, *n* (%)			
B1 non-stricturing/penetrating	15 (75.0)	-	-
B2 stricturing	3 (15.0)	-	-
B3 penetrating	2 (10.0)	-	-
+ Perianal disease	3 (15.0)	-	-
Medication use, *n* (%)			
Immunosuppressive drugs (Aza/6MP)	12 (60.0)	-	-
None	8 (40.0)	-	-

[1]HBI missing of 1 Crohn's disease (CD) patient. [a]Low: primary education (elementary school) and lower secondary education (preparatory secondary education); [b]Intermediate: higher secondary education (higher general continued education, pre-university secondary education) and postsecondary education (intermediate vocational education); [c]High: tertiary education (higher professional education, university). To correct for multiple testing, the level of significance was set at $P < 0.01$ for the MFI score. HBI: Harvey Bradshaw Index; VAS: Visual Analogue Scale; MFI: Multidimensional fatigue index.

Symbol test ($P = 0.06$) and were slower in completing trial A of the TMT test ($P = 0.08$). When the individual tests were transformed into a Z-score based on the different cognitive domains, significant reduced Z-scores of the memory domain ($P = 0.007$) and executive functioning domain ($P = 0.02$) were found in the patient population compared with the healthy controls (Table 5). CD patients experienced more depressive symptoms ($P < 0.001$), were more anxious ($P = 0.002$) and reported a significantly lower QoL.

Correlation of MRI findings with clinical characteristics and mental status

No correlations were found between mental status, including depression and anxiety, and MRI findings. Depressive symptoms were correlated with reduced scores of global cognitive functioning ($r = -0.5$, $P = 0.003$), memory ($r = -0.34$, $P = 0.04$) and executive functioning ($r = 0.35$, $P = 0.04$). Additionally, CD patients reported in the present study increased symptoms of anxiety and this was significantly correlated with reduced global cognitive functioning ($r = -0.36$, $P = 0.03$) and memory scores ($r = -0.32$, $P = 0.05$). No further correlations between cognitive scores, disease activity, disease duration and MRI findings were found in this study.

Table 2 Group mean subcortical structure volumes as percentage of the total brain volume in Crohn's disease patients and controls

	CD patients (*n* = 20)	Controls (*n* = 17)	*P* value
Left Accumbens	0.04 ± 0.01	0.04 ± 0.01	0.56
Left Amygdala	0.09 ± 0.01	0.09 ± 0.01	0.61
Left Caudate	0.24 ± 0.02	0.24 ± 0.02	0.94
Left Hippocampus	0.27 ± 0.02	0.27 ± 0.03	0.94
Left Pallidus	0.13 ± 0.01	0.12 ± 0.01	0.32
Left Putamen	0.33 ± 0.02	0.32 ± 0.03	0.24
Left Thalamus	0.54 ± 0.02	0.54 ± 0.03	0.94
Right Accumbens	0.04 ± 0.00	0.03 ± 0.01	0.08
Right Amygdala	0.08 ± 0.01	0.09 ± 0.01	0.08
Right Caudate	0.25 ± 0.03	0.25 ± 0.02	0.76
Right Hippocampus	0.26 ± 0.02	0.27 ± 0.03	0.22
Right Pallidus	0.12 ± 0.01	0.13 ± 0.01	0.30
Right Putamen	0.31 ± 0.08	0.32 ± 0.02	0.56
Right Thalamus	0.53 ± 0.02	0.52 ± 0.03	0.48

Mean in % ± SD. CD: Crohn's disease.

Table 3 Mean metabolite ratio to total creatine

	CD patients (*n* = 9)	Controls (*n* = 9)	*P* value
Ratio Glu:tCr	0.76 ± 0.12	0.84 ± 0.10	0.19
Ratio Cho:tCr	0.29 ± 0.02	0.29 ± 0.04	0.81
Ratio Ins:tCr	0.66 ± 0.08	0.70 ± 0.10	0.38
Ratio NAA:tCr	1.31 ± 0.12	1.27 ± 0.09	0.44
Ratio NAA + NAAG:tCr	1.59 ± 0.18	1.56 ± 0.13	0.69
Ratio Glu + Gln:tCr	0.92 ± 0.13	1.10 ± 0.14	0.02

Mean metabolite ratio to tCr in mmol ± SD. CD: Crohn's disease; tCr: Total Creatine; Glu: Glutamate; Cho: Choline; Ins: Insulin; NAA: N-Acetyl Aspartate; NAAG: N-Acetyl Aspartate Glutamate; Gln: Glutamine.

DISCUSSION

Several MRI techniques were used in this study in a cross-sectional manner to examine the differences in brain morphology, neurochemistry and perfusion between CD patients with fatigue and healthy controls without fatigue. The most important findings reported in this study are the significant differences in perfusion, neurochemistry and mental status (*e.g.*, cognition, mood and QoL) between patients and controls. Lower levels of Glx concentration and their ratio to tCr were observed and an increased CBF was found in the patient population compared with control subjects. CD patients scored lower on several individual cognitive

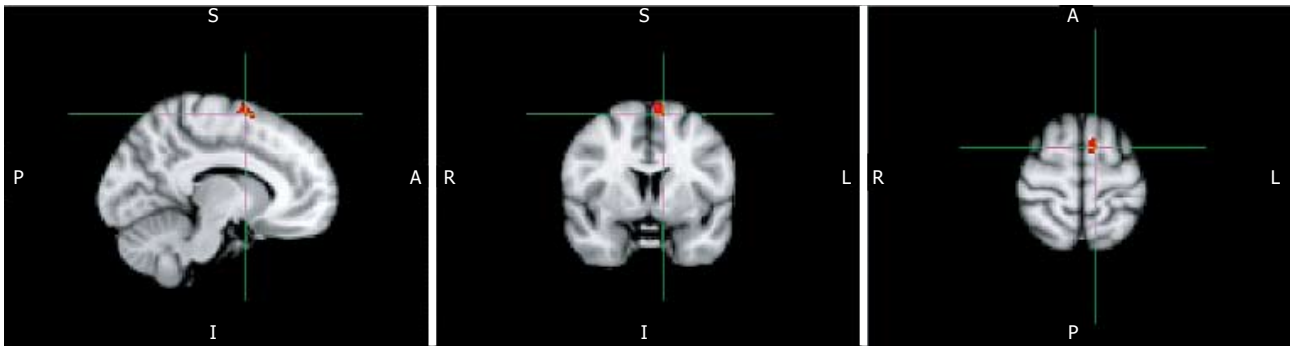

Figure 3 FMRIB Software Library voxel based morphometry analysis. Voxel based morphometry results shown on MNI152 standard space. The red colour indicates the voxels with significantly reduced grey matter volume in CD patients compared with healthy controls (with a *P*-value < 0.05, corrected for multiple comparison). The red voxels correspond to the left superior frontal gyrus.

test scores, with a trend towards significance, and scored significantly lower on the memory and the executive functioning domain compared with the healthy controls. Also, the patient population had a significantly lower QoL and mood status.

The present study observed with MRS a significantly reduced Glx concentration as well as a lower ratio of Glx to tCr in the CD group. Glutamate is the predominant excitatory neurotransmitter in the brain and is involved in different brain functions including memory and mood status. Receptors are mainly present in the hippocampus[58,59]. Glutamine is important in energy metabolism of the brain and previous studies reported that a reduced level of glutamine is associated with brain diseases such as Alzheimer[60,61]. Increasing evidence shows that major depression disorder is associated with altered function of the major excitatory and inhibitory neurotransmitters such as glutamate and GABA[62,63]. The present study did not find correlations between depressive symptoms and the reduced Glx concentration and ratio to tCr.

These reduced MRS results found in the present pilot study in CD patients are not in accordance with the findings of previous research performed in other inflammatory diseases such as RA and SLE[11,64]. RA and SLE patients were shown to have increased choline and myo-inisotol levels, indicating inflammation in the form of monocyte infiltration since this is a marker of cell membrane turnover[65,66]. In addition, in SLE patients only decreased NAA signals were reported, indicating neuronal loss[67-69], while an increased NAA ratio was found in our CD patient population. This contradiction may be due to the fact that RA and SLE are systemic inflammatory diseases, but not comparable with the systemic inflammation in IBD.

CBF values can reveal changes in tissue perfusion and are an indication for cerebral metabolism changes[70]. In the present study, significant higher CBF values were found in the patient population. Increased CBF is thought to be a compensatory mechanism in response to ischemia or injury, which could be the case in the CD patients due to inflammation[71,72]. Our findings are in line with the results of Wang *et al*[31] who described in their cohort that SLE patients had higher CBF values compared with healthy controls.

The volumetric results in this study extend on earlier findings in IBD patients. The reduced GM content of the superior frontal gyrus demonstrated in this study is in agreement with results presented by Agostini *et al*[73]. The superior frontal gyrus is involved the self-awareness, and important in processing information[74,75]. It has been suggested that the observed decrease in local GM volume could have many causes, including a decrease in cell size, neural or glial cell apoptosis or changes in blood flow[72]. It is not clear whether this local volume reduction is directly linked to systemic inflammation, but it may represent the anatomical substrate for the development of cognitive and emotional disturbances[73,76]. Similar significant positive correlations have been found between the GM volume in aging and measures of short-term memory[77].

Besides MRI findings, neuropsychological findings were assessed in this study. Previously, no evidence has been obtained on the association of the intrinsic disease process and cognitive dysfunction in IBD patients. It is probable that concurrent mood disorders, in particular depression, affect the cognitive performance of IBD patients in memory and executive functioning tasks[55]. This may be the case in the current cohort, since depressive symptoms were correlated with reduced neuropsychological scores in the three different domains: cognitive functioning, memory and executive functioning. However, Berrill *et al*[78] suggested that intellectual deficits existed in IBD patients compared to controls and remained significant after the correction for educational level and mood disorders.

Previous studies have shown a link between systemic inflammation and reduced brain volumes, possibly resulting in cognitive deficits. Zonis *et al*[79] suggested that chronic intestinal inflammation alters hippocampal neurogenesis and thus might underlie the behavioural manifestations in patients with IBD. In another study, SLE patients with cognitive deficits appeared to have reduced temporal lobe structures (hippocampus and amygdala) compared to SLE patients without cognitive

Table 4 Mental status

	CD patients (n = 20)	Controls (n = 17)	P value
Global cognitive functioning			
MMSE (total score), mean ± SD	28.9 (1.62)	29.65 (0.49)	0.87
Memory			
Verbal			
WMS memory quotient, mean ± SD[3]	109.2 (10.5)	115.7 (8.7)	0.72
Non verbal			
WMS visual reproduction (total score), mean ± SD[3]	11.6 (2.7)	12.9 (2.2)	0.75
WAIS-R Digit Span forward, mean ± SD	5.4 (1.0)	6.5 (1.3)	0.15
WAIS-R Digit Span backward, mean ± SD	4.5 (1.0)	5.2 (0.9)	0.15
Executive functioning			
WFT, mean ± SD[1]			
No. of good answers	42.7 (7.8)	48.2 (9.8)	0.29
No. of perseverative errors	0.28 (0.5)	0.47 (0.8)	0.58
Stroop Color-Word test, mean ± SD			
Stroop 1 time (s)	43.9 (7.2)	39.2 (8.6)	0.41
Stroop 1 No. of errors	0.2 (0.4)	0.1 (0.3)	0.98
Stroop 2 time (s)	56.6 (8.3)	53.8 (6.9)	0.62
Stroop 2 No. of errors	0.3 (0.8)	0 (0.0)	0.21
Stroop 3 time (s)	88.3 (14.7)	76.6 (8.8)	0.22
Stroop 3 No. of errors	0.6 (1.5)	0.13 (0.3)	0.20
Stroop interference index	50.1 (7.8)	56.1 (5.5)	0.06
TMT, mean ± SD			
Part A time (s)	30.3 (11.8)	22.1 (8.5)	0.08
Part A no. of errors	0.1 (0.2)	0.1 (0.2)	0.56
Part B time (s)	61.8 (29.2)	50.1 (17.4)	0.72
Part B no. of errors	0.1 (0.2)	0.2 (0.5)	0.41
WAIS-R Digit Symbol, mean ± SD[2]			
Total score	59.7 (8.4)	71.0 (6.2)	0.06
No. of errors	0 (0.0)	0.1 (0.3)	0.63
Digit cancellation test, mean ± SD[4]			
Total score	436.2 (88.1)	498.6 (82.9)	0.16
No. of good answers (%)	57.3 (29.5)	78.9 (20.9)	0.16
HADS, mean ± SD	13.1 (7.3)	4.8 (2.9)	< 0.001
Anxiety	7.5 (3.8)	3.7 (2.7)	0.002
Depression	6.1 (4.0)	0.9 (1.1)	< 0.001
SF-36			
Physical functioning	72.9 ± 20.2	96.6 ± 3.4	< 0.001
Social functioning	52.0 ± 29.0	90.7 ± 9.8	< 0.001
Role physical problem	71.3 ± 37.4	2.9 ± 8.3	< 0.001
Role emotional problem	40.4 ± 46.1	2.0 ± 8.1	0.002
Bodily pain	35.3 ± 20.7	5.6 ± 12.6	< 0.001
General health perception	65.8 ± 18.8	82.0 ± 15.2	< 0.001
Mental health	63.6 ± 16.0	80.4 ± 10.9	0.001
Vitality	30.1 ± 18.3	72.9 ± 14.3	< 0.001

[1]Missing in 2 CD patients; [2]Missing 1 CD patient and 1 healthy control; [3]Missing in 2 healthy controls; [4]Missing in 5 patients and 3 healthy controls. To correct for multiple testing, the level of significance was set at $P < 0.006$ for the SF-36 score. MMSE: Mini Mental State Examination; WMS: Wechsler Memory Scale; WAIS-R: Wechsler Adult Intelligence Scale-Revised; WFT: Word Fluency Test; TMT: Trial Making test; HADS: Hospital Anxiety Depression Scale; CD: Crohn's disease.

deficits[80]. In the present study, we did not find these correlations.

Some limitations of this study need to be revealed. Although this study is an exploratory study, the population size was limited. In this pilot study we have compared the most extreme cases; quiescent CD patients with fatigue *vs* healthy controls without fatigue. In this design, we have found significant differences between the groups and now further research is required. In addition, the significant difference in the fatigue score between patients and controls is not a finding of the study, but part of the design. As a consequence, it cannot be definitely concluded whether the differences in MRI measures are caused by CD per se or represent only patients with combined CD and fatigue. However, fatigue is a subjective measurement and was evaluated as such. It is hard to draw major conclusions from these questionnaires, since some healthy controls reported a high fatigue score as well due to other circumstances than IBD. In some MRI analyses, subjects got excluded due to the quality of the data. MRS data with high Cramer-Rao lower bounds, suggesting unreliable metabolite quantification, were excluded from data analysis. This could have been influenced by the patients' motion or bad shimming.

In conclusion, our findings support the hypothesis that systemic inflammation influences the brain and effects cognitive functioning and mood. This is a first step in the gathering of data and understanding of brain involvement in CD patients. This study implies that for a health professional, it is important to focus in CD patients not only on symptoms related to the gastrointestinal tract, but also on the effects of inflammation on the brain. Understanding these affects in CD patients may help health professionals to set up interventions to maintain CD remission by the use of medication and to improve mood status and QoL by *e.g.*, psychosocial interventions.

Table 5 Z-scores of the different domains of cognitive functioning

	CD patients (n = 20)	Controls (n = 17)	P value
Global cognitive functioning[1], mean ± SD	28.9 (1.6)	29.7 (0.5)	0.870
Memory[2], mean ± SD	1.1 (2.9)	1.3 (2.3)	0.007
Executive functioning[3], mean ± SD	2.5 (7.7)	2.9 (4.2)	0.020

[1]The global cognitive functioning domain includes the Minimal Mental State Examination; [2]The memory domain includes the Wechsler Adult Intelligence Scale and the revised Wechsler Memory Scale; [3]The executive functioning domain includes the Word Fluency Test, Stroop-Color-Word test and Trail Making Test. CD: Crohn's disease.

COMMENTS

Background

Both active and quiescent Crohn's disease (CD) is a chronic inflammatory status in which levels of circulating inflammatory cytokines, such as tumour necrosis factor-α are reported in the body. These cytokines may play a role in driving inflammation in the brain by activating microglia and the recruitment of monocytes.

Research frontiers

Metabolic and cerebral perfusion changes have been found in the brain of patients with other systemic diseases including rheumatoid arthritis, systemic

sclerosis and systemic lupus erythematosus. In addition, previous studies found an association with fatigue and metabolic brain changes. Thus it is of interest, whether systemic inflammation and fatigue complaints influence the brain in CD patients as well.

Innovations and breakthroughs

The present data support the hypothesis that systemic inflammation influences the brain and affects cognitive functioning and mood status in quiescent CD patients with fatigue. This is a first step in understanding brain involvement in CD patients.

Applications

This study implies that for a health professional, it is important to focus in CD patients also on the effects of inflammation on the brain. Understanding these affects in CD patients may help health professionals to set up interventions to maintain CD remission by the use of medication and to improve mood status and QoL by *e.g.,* psychosocial interventions.

Peer-review

Since this is an exploratory study, the authors have compared the most extreme cases; quiescent CD patients with fatigue *vs* healthy controls without fatigue. In this design, the authors have found significant differences between the groups and now further research is required.

REFERENCES

1 **Baumgart DC**, Sandborn WJ. Crohn's disease. *Lancet* 2012; **380**: 1590-1605 [PMID: 22914295 DOI: 10.1016/S0140-6736(12)60026-9]

2 **Salem M**, Ammitzboell M, Nys K, Seidelin JB, Nielsen OH. ATG16L1: A multifunctional susceptibility factor in Crohn disease. *Autophagy* 2015; **11**: 585-594 [PMID: 25906181 DOI: 10.1080/15548627.2015.1017187]

3 **Jelsness-Jørgensen LP**, Bernklev T, Henriksen M, Torp R, Moum BA. Chronic fatigue is more prevalent in patients with inflammatory bowel disease than in healthy controls. *Inflamm Bowel Dis* 2011; **17**: 1564-1572 [PMID: 21674713 DOI: 10.1002/ibd.21530]

4 **Romberg-Camps MJ**, Bol Y, Dagnelie PC, Hesselink-van de Kruijs MA, Kester AD, Engels LG, van Deursen C, Hameeteman WH, Pierik M, Wolters F, Russel MG, Stockbrügger RW. Fatigue and health-related quality of life in inflammatory bowel disease: results from a population-based study in the Netherlands: the IBD-South Limburg cohort. *Inflamm Bowel Dis* 2010; **16**: 2137-2147 [PMID: 20848468 DOI: 10.1002/ibd.21285]

5 **Høivik ML**, Bernklev T, Solberg IC, Cvancarova M, Lygren I, Jahnsen J, Moum B. Patients with Crohn's disease experience reduced general health and vitality in the chronic stage: ten-year results from the IBSEN study. *J Crohns Colitis* 2012; **6**: 441-453 [PMID: 22398064 DOI: 10.1016/j.crohns.2011.10.001]

6 **Chen ML**, Sundrud MS. Cytokine Networks and T-Cell Subsets in Inflammatory Bowel Diseases. *Inflamm Bowel Dis* 2016; **22**: 1157-1167 [PMID: 26863267 DOI: 10.1097/MIB.0000000000000714]

7 **Loddo I**, Romano C. Inflammatory Bowel Disease: Genetics, Epigenetics, and Pathogenesis. *Front Immunol* 2015; **6**: 551 [PMID: 26579126 DOI: 10.3389/fimmu.2015.00551]

8 **Nadeau S**, Rivest S. Effects of circulating tumor necrosis factor on the neuronal activity and expression of the genes encoding the tumor necrosis factor receptors (p55 and p75) in the rat brain: a view from the blood-brain barrier. *Neuroscience* 1999; **93**: 1449-1464 [PMID: 10501470]

9 **Hagel AF**, de Rossi T, Konturek PC, Albrecht H, Walker S, Hahn EG, Raithel M. Plasma histamine and tumour necrosis factor-alpha levels in Crohn's disease and ulcerative colitis at various stages of disease. *J Physiol Pharmacol* 2015; **66**: 549-556 [PMID: 26348079]

10 **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 [PMID: 15667502]

11 **Emmer BJ**, van der Bijl AE, Huizinga TW, Breedveld FC, Steens SC, Th Bosma GP, van Buchem MA, van der Grond J. Brain involvement in rheumatoid arthritis: a magnetic resonance spectroscopy study. *Arthritis Rheum* 2009; **60**: 3190-3195 [PMID: 19877035 DOI: 10.1002/art.24932]

12 **O'Callaghan JP**, Sriram K, Miller DB. Defining "neuroinflammation". *Ann N Y Acad Sci* 2008; **1139**: 318-330 [PMID: 18991877 DOI: 10.1196/annals.1432.032]

13 **D'Mello C**, Le T, Swain MG. Cerebral microglia recruit monocytes into the brain in response to tumor necrosis factoralpha signaling during peripheral organ inflammation. *J Neurosci* 2009; **29**: 2089-2102 [PMID: 19228962 DOI: 10.1523/JNEUROSCI. 3567-08.2009]

14 **Hollingworth W**, Todd CJ, Bell MI, Arafat Q, Girling S, Karia KR, Dixon AK. The diagnostic and therapeutic impact of MRI: an observational multi-centre study. *Clin Radiol* 2000; **55**: 825-831 [PMID: 11069736 DOI: 10.1053/crad.2000.0546]

15 **Smith SM**. Fast robust automated brain extraction. *Hum Brain Mapp* 2002; **17**: 143-155 [PMID: 12391568 DOI: 10.1002/hbm.10062]

16 **Good CD**, Johnsrude IS, Ashburner J, Henson RN, Friston KJ, Frackowiak RS. A voxel-based morphometric study of ageing in 465 normal adult human brains. *Neuroimage* 2001; **14**: 21-36 [PMID: 11525331 DOI: 10.1006/nimg.2001.0786]

17 **Grossman RI**, Gomori JM, Ramer KN, Lexa FJ, Schnall MD. Magnetization transfer: theory and clinical applications in neuroradiology. *Radiographics* 1994; **14**: 279-290 [PMID: 8190954 DOI: 10.1148/radiographics.14.2.8190954]

18 **Rosen Y**, Lenkinski RE. Recent advances in magnetic resonance neurospectroscopy. *Neurotherapeutics* 2007; **4**: 330-345 [PMID: 17599700 DOI: 10.1016/j.nurt.2007.04.009]

19 **Detre JA**, Leigh JS, Williams DS, Koretsky AP. Perfusion imaging. *Magn Reson Med* 1992; **23**: 37-45 [PMID: 1734182]

20 **Schaefer PW**, Grant PE, Gonzalez RG. Diffusion-weighted MR imaging of the brain. *Radiology* 2000; **217**: 331-345 [PMID: 11058626 DOI: 10.1148/radiology.217.2.r00nv24331]

21 **Schmidt R**, Schmidt H, Curb JD, Masaki K, White LR, Launer LJ. Early inflammation and dementia: a 25-year follow-up of the Honolulu-Asia Aging Study. *Ann Neurol* 2002; **52**: 168-174 [PMID: 12210786 DOI: 10.1002/ana.10265]

22 **Holmes C**, Cunningham C, Zotova E, Woolford J, Dean C, Kerr S, Culliford D, Perry VH. Systemic inflammation and disease progression in Alzheimer disease. *Neurology* 2009; **73**: 768-774 [PMID: 19738171 DOI: 10.1212/WNL.0b013e3181b6bb95]

23 **Moreno B**, Jukes JP, Vergara-Irigaray N, Errea O, Villoslada P, Perry VH, Newman TA. Systemic inflammation induces axon injury during brain inflammation. *Ann Neurol* 2011; **70**: 932-942 [PMID: 22190366 DOI: 10.1002/ana.22550]

24 **Mulak A**, Bonaz B. Brain-gut-microbiota axis in Parkinson's disease. *World J Gastroenterol* 2015; **21**: 10609-10620 [PMID: 26457021 DOI: 10.3748/wjg.v21.i37.10609]

25 **Hamed SA**, Selim ZI, Elattar AM, Elserogy YM, Ahmed EA, Mohamed HO. Assessment of biocorrelates for brain involvement in female patients with rheumatoid arthritis. *Clin Rheumatol* 2012; **31**: 123-132 [PMID: 21695659 DOI: 10.1007/s10067-011-1795-1]

26 **Soares DP**, Law M. Magnetic resonance spectroscopy of the brain: review of metabolites and clinical applications. *Clin Radiol* 2009; **64**: 12-21 [PMID: 19070693 DOI: 10.1016/j.crad.2008.07.002]

27 **Cutolo M**, Nobili F, Sulli A, Pizzorni C, Briata M, Faelli F, Vitali P, Mariani G, Copello F, Seriolo B, Barone C, Rodriguez G. Evidence of cerebral hypoperfusion in scleroderma patients. *Rheumatology* (Oxford) 2000; **39**: 1366-1373 [PMID: 11136880]

28 **Emmer BJ**, Steens SC, Steup-Beekman GM, van der Grond J, Admiraal-Behloul F, Olofsen H, Bosma GP, Ouwendijk WJ, Huizinga TW, van Buchem MA. Detection of change in CNS involvement in neuropsychiatric SLE: a magnetization transfer study. *J Magn Reson Imaging* 2006; **24**: 812-816 [PMID:

16941632 DOI: 10.1002/jmri.20706]
29 **Luyendijk J**, Steens SC, Ouwendijk WJ, Steup-Beekman GM, Bollen EL, van der Grond J, Huizinga TW, Emmer BJ, van Buchem MA. Neuropsychiatric systemic lupus erythematosus: lessons learned from magnetic resonance imaging. *Arthritis Rheum* 2011; **63**: 722-732 [PMID: 21360502 DOI: 10.1002/art.30157]
30 **Emmer BJ**, Veer IM, Steup-Beekman GM, Huizinga TW, van der Grond J, van Buchem MA. Tract-based spatial statistics on diffusion tensor imaging in systemic lupus erythematosus reveals localized involvement of white matter tracts. *Arthritis Rheum* 2010; **62**: 3716-3721 [PMID: 20722009 DOI: 10.1002/art.27717]
31 **Wang PI**, Cagnoli PC, McCune WJ, Schmidt-Wilcke T, Lowe SE, Graft CC, Gebarski SS, Chenevert TL, Khalatbari S, Myles JD, Watcharotone K, Cronin P, Sundgren PC. Perfusion-weighted MR imaging in cerebral lupus erythematosus. *Acad Radiol* 2012; **19**: 965-970 [PMID: 22608862 DOI: 10.1016/j.acra.2012.03.023]
32 **Puri BK**, Counsell SJ, Zaman R, Main J, Collins AG, Hajnal JV, Davey NJ. Relative increase in choline in the occipital cortex in chronic fatigue syndrome. *Acta Psychiatr Scand* 2002; **106**: 224-226 [PMID: 12197861]
33 **Chaudhuri A**, Condon BR, Gow JW, Brennan D, Hadley DM. Proton magnetic resonance spectroscopy of basal ganglia in chronic fatigue syndrome. *Neuroreport* 2003; **14**: 225-228 [PMID: 12598734 DOI: 10.1097/01.wnr.0000054960.21656.64]
34 **Puri BK**, Holmes J, Hamilton G. Eicosapentaenoic acid-rich essential fatty acid supplementation in chronic fatigue syndrome associated with symptom remission and structural brain changes. *Int J Clin Pract* 2004; **58**: 297-299 [PMID: 15117099]
35 **Sandu AL**, Staff RT, McNeil CJ, Mustafa N, Ahearn T, Whalley LJ, Murray AD. Structural brain complexity and cognitive decline in late life--a longitudinal study in the Aberdeen 1936 Birth Cohort. *Neuroimage* 2014; **100**: 558-563 [PMID: 24993896 DOI: 10.1016/j.neuroimage.2014.06.054]
36 **Harvey RF**, Bradshaw JM. A simple index of Crohn's-disease activity. *Lancet* 1980; **1**: 514 [PMID: 6102236]
37 **Smets EM**, Garssen B, Bonke B, De Haes JC. The Multidimensional Fatigue Inventory (MFI) psychometric qualities of an instrument to assess fatigue. *J Psychosom Res* 1995; **39**: 315-325 [PMID: 7636775]
38 **Chalder T**, Berelowitz G, Pawlikowska T, Watts L, Wessely S, Wright D, Wallace EP. Development of a fatigue scale. *J Psychosom Res* 1993; **37**: 147-153 [PMID: 8463991]
39 **Zhang Y**, Brady M, Smith S. Segmentation of brain MR images through a hidden Markov random field model and the expectation-maximization algorithm. *IEEE Trans Med Imaging* 2001; **20**: 45-57 [PMID: 11293691 DOI: 10.1109/42.906424]
40 **Patenaude B**, Smith SM, Kennedy DN, Jenkinson M. A Bayesian model of shape and appearance for subcortical brain segmentation. *Neuroimage* 2011; **56**: 907-922 [PMID: 21352927 DOI: 10.1016/j.neuroimage.2011.02.046]
41 **Smith SM**, Jenkinson M, Woolrich MW, Beckmann CF, Behrens TE, Johansen-Berg H, Bannister PR, De Luca M, Drobnjak I, Flitney DE, Niazy RK, Saunders J, Vickers J, Zhang Y, De Stefano N, Brady JM, Matthews PM. Advances in functional and structural MR image analysis and implementation as FSL. *Neuroimage* 2004; **23** Suppl 1: S208-S219 [PMID: 15501092 DOI: 10.1016/j.neuroimage.2004.07.051]
42 **Jenkinson M**, Smith S. A global optimisation method for robust affine registration of brain images. *Med Image Anal* 2001; **5**: 143-156 [PMID: 11516708]
43 **Provencher SW**. Estimation of metabolite concentrations from localized in vivo proton NMR spectra. *Magn Reson Med* 1993; **30**: 672-679 [PMID: 8139448]
44 **Choi JK**, Dedeoglu A, Jenkins BG. Application of MRS to mouse models of neurodegenerative illness. *NMR Biomed* 2007; **20**: 216-237 [PMID: 17451183 DOI: 10.1002/nbm.1145]
45 **Alsop DC**, Detre JA, Golay X, Günther M, Hendrikse J, Hernandez-Garcia L, Lu H, MacIntosh BJ, Parkes LM, Smits M, van Osch MJ, Wang DJ, Wong EC, Zaharchuk G. Recommended implementation of arterial spin-labeled perfusion MRI for clinical applications: A consensus of the ISMRM perfusion study group and the European consortium for ASL in dementia. *Magn Reson Med* 2015; **73**: 102-116 [PMID: 24715426 DOI: 10.1002/mrm.25197]
46 **Jenkinson M**, Bannister P, Brady M, Smith S. Improved optimization for the robust and accurate linear registration and motion correction of brain images. *Neuroimage* 2002; **17**: 825-841 [PMID: 12377157]
47 **Leemans A**, Jeurissen B, Sijbers J, Jones DK. ExploreDTI: A graphical toolbox for processing, analyzing, and visualizing diffusion MR data. *Annual Meeting of Proc.intl.soc.mag.reson.med* 2009: 3537
48 **Smith SM**, Jenkinson M, Johansen-Berg H, Rueckert D, Nichols TE, Mackay CE, Watkins KE, Ciccarelli O, Cader MZ, Matthews PM, Behrens TE. Tract-based spatial statistics: voxelwise analysis of multi-subject diffusion data. *Neuroimage* 2006; **31**: 1487-1505 [PMID: 16624579 DOI: 10.1016/j.neuroimage.2006.02.024]
49 **Folstein MF**, Folstein SE, McHugh PR. "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. *J Psychiatr Res* 1975; **12**: 189-198 [PMID: 1202204]
50 **Wechsler D**. Wechsler Adult Intelligence Scale, Revised. The Psychological Corporation: 1981
51 **Wechsler D.** Wechsler Memory Scale, Revised. The Pyschological Corporation: 1987
52 **Pendleton MG**, Heaton RK, Lehman RA, Hulihan D. Diagnostic utility of the Thurstone Word Fluency Test in neuropsychological evaluations. *J Clin Neuropsychol* 1982; **4**: 307-317 [PMID: 7174838]
53 **Golden CJ**. Identification of brain disorders by the Stroop Color and Word Test. *J Clin Psychol* 1976; **32**: 654-658 [PMID: 956433]
54 **Reitan RM,** Wolfson D. The Halstead-Reitan Neuropsychological Test Battery: Theory and Clinical Interpretation. 2nd ed. Neuropsychology Press 1993
55 **Castaneda AE**, Tuulio-Henriksson A, Aronen ET, Marttunen M, Kolho KL. Cognitive functioning and depressive symptoms in adolescents with inflammatory bowel disease. *World J Gastroenterol* 2013; **19**: 1611-1617 [PMID: 23538788 DOI: 10.3748/wjg.v19.i10.1611]
56 **Brennan C**, Worrall-Davies A, McMillan D, Gilbody S, House A. The Hospital Anxiety and Depression Scale: a diagnostic meta-analysis of case-finding ability. *J Psychosom Res* 2010; **69**: 371-378 [PMID: 20846538 DOI: 10.1016/j.jpsychores.2010.04.006]
57 **Ware JE**, Sherbourne CD. The MOS 36-item short-form health survey (SF-36). I. Conceptual framework and item selection. *Med Care* 1992; **30**: 473-483 [PMID: 1593914]
58 **Riedel G**, Platt B, Micheau J. Glutamate receptor function in learning and memory. *Behav Brain Res* 2003; **140**: 1-47 [PMID: 12644276]
59 **Bianchin M**, Da Silva RC, Schmitz PK, Medina JH, Izquierdo I. Memory of inhibitory avoidance in the rat is regulated by glutamate metabotropic receptors in the hippocampus. *Behav Pharmacol* 1994; **5**: 356-359 [PMID: 11224286]
60 **Antuono PG**, Jones JL, Wang Y, Li SJ. Decreased glutamate + glutamine in Alzheimer's disease detected in vivo with (1)H-MRS at 0.5 T. *Neurology* 2001; **56**: 737-742 [PMID: 11274307]
61 **Gunnersen D**, Haley B. Detection of glutamine synthetase in the cerebrospinal fluid of Alzheimer diseased patients: a potential diagnostic biochemical marker. *Proc Natl Acad Sci USA* 1992; **89**: 11949-11953 [PMID: 1361232]
62 **Hasler G**, van der Veen JW, Tumonis T, Meyers N, Shen J, Drevets WC. Reduced prefrontal glutamate/glutamine and gamma-aminobutyric acid levels in major depression determined using proton magnetic resonance spectroscopy. *Arch Gen Psychiatry* 2007; **64**: 193-200 [PMID: 17283286]
63 **Bernstein HG**, Meyer-Lotz G, Dobrowolny H, Bannier J, Steiner J, Walter M, Bogerts B. Reduced density of glutamine synthetase immunoreactive astrocytes in different cortical areas in major depression but not in bipolar I disorder. *Front Cell Neurosci* 2015; **9**: 273 [PMID: 26321908 DOI: 10.3389/fncel.2015.00273]
64 **Brooks WM**, Jung RE, Ford CC, Greinel EJ, Sibbitt WL. Relationship between neurometabolite derangement and neurocog-

nitive dysfunction in systemic lupus erythematosus. *J Rheumatol* 1999; **26**: 81-85 [PMID: 9918245]
65 **Brenner RE**, Munro PM, Williams SC, Bell JD, Barker GJ, Hawkins CP, Landon DN, McDonald WI. The proton NMR spectrum in acute EAE: the significance of the change in the Cho: Cr ratio. *Magn Reson Med* 1993; **29**: 737-745 [PMID: 8350716]
66 **Bitsch A**, Bruhn H, Vougioukas V, Stringaris A, Lassmann H, Frahm J, Brück W. Inflammatory CNS demyelination: histopathologic correlation with in vivo quantitative proton MR spectroscopy. *AJNR Am J Neuroradiol* 1999; **20**: 1619-1627 [PMID: 10543631]
67 **Bjartmar C**, Kidd G, Mörk S, Rudick R, Trapp BD. Neurological disability correlates with spinal cord axonal loss and reduced N-acetyl aspartate in chronic multiple sclerosis patients. *Ann Neurol* 2000; **48**: 893-901 [PMID: 11117546]
68 **Demougeot C**, Garnier P, Mossiat C, Bertrand N, Giroud M, Beley A, Marie C. N-Acetylaspartate, a marker of both cellular dysfunction and neuronal loss: its relevance to studies of acute brain injury. *J Neurochem* 2001; **77**: 408-415 [PMID: 11299303]
69 **Adalsteinsson E**, Sullivan EV, Kleinhans N, Spielman DM, Pfefferbaum A. Longitudinal decline of the neuronal marker N-acetyl aspartate in Alzheimer's disease. *Lancet* 2000; **355**: 1696-1697 [PMID: 10905250]
70 **Wang Z**, Das SR, Xie SX, Arnold SE, Detre JA, Wolk DA. Arterial spin labeled MRI in prodromal Alzheimer's disease: A multi-site study. *Neuroimage Clin* 2013; **2**: 630-636 [PMID: 24179814 DOI: 10.1016/j.nicl.2013.04.014]
71 **Smith M**. Perioperative uses of transcranial perfusion monitoring. *Neurosurg Clin N Am* 2008; **19**: 489-502, vii [PMID: 18790384 DOI: 10.1016/j.nec.2008.07.008]
72 **Alsop DC**, Casement M, de Bazelaire C, Fong T, Press DZ. Hippocampal hyperperfusion in Alzheimer's disease. *Neuroimage* 2008; **42**: 1267-1274 [PMID: 18602481 DOI: 10.1016/j. neuroimage.2008.06.006]
73 **Agostini A**, Benuzzi F, Filippini N, Bertani A, Scarcelli A, Farinelli V, Marchetta C, Calabrese C, Rizzello F, Gionchetti P, Ercolani M, Campieri M, Nichelli P. New insights into the brain involvement in patients with Crohn's disease: a voxel-based morphometry study. *Neurogastroenterol Motil* 2013; **25**: 147-e82 [PMID: 22998431 DOI: 10.1111/nmo.12017]
74 **Goldberg II**, Harel M, Malach R. When the brain loses its self: prefrontal inactivation during sensorimotor processing. *Neuron* 2006; **50**: 329-339 [PMID: 16630842 DOI: 10.1016/j.neuron.2006.03.015]
75 **Miller AK**, Alston RL, Corsellis JA. Variation with age in the volumes of grey and white matter in the cerebral hemispheres of man: measurements with an image analyser. *Neuropathol Appl Neurobiol* 1980; **6**: 119-132 [PMID: 7374914]
76 **Zikou AK**, Kosmidou M, Astrakas LG, Tzarouchi LC, Tsianos E, Argyropoulou MI. Brain involvement in patients with inflammatory bowel disease: a voxel-based morphometry and diffusion tensor imaging study. *Eur Radiol* 2014; **24**: 2499-2506 [PMID: 25001084 DOI: 10.1007/s00330-014-3242-6]
77 **Taki Y**, Kinomura S, Sato K, Goto R, Wu K, Kawashima R, Fukuda H. Correlation between gray/white matter volume and cognition in healthy elderly people. *Brain Cogn* 2011; **75**: 170-176 [PMID: 21131121 DOI: 10.1016/j.bandc.2010.11.008]
78 **Berrill JW**, Gallacher J, Hood K, Green JT, Matthews SB, Campbell AK, Smith A. An observational study of cognitive function in patients with irritable bowel syndrome and inflammatory bowel disease. *Neurogastroenterol Motil* 2013; **25**: 918-e704 [PMID: 23981191 DOI: 10.1111/nmo.12219]
79 **Zonis S**, Pechnick RN, Ljubimov VA, Mahgerefteh M, Wawrowsky K, Michelsen KS, Chesnokova V. Chronic intestinal inflammation alters hippocampal neurogenesis. *J Neuroinflammation* 2015; **12**: 65 [PMID: 25889852 DOI: 10.1186/s12974-015-0281-0]
80 **Zimmermann N**, Corrêa DG, Kubo TA, Netto TM, Pereira DB, Fonseca RP, Gasparetto EL. Global Cognitive Impairment in Systemic Lupus Erythematosus Patients: A Structural MRI Study. *Clin Neuroradiol* 2015; Epub ahead of print [PMID: 25967601 DOI: 10.1007/s00062-015-0397-8]

P- Reviewer: Chow J, Koch TR, Lakatos PL, Prakash N
S- Editor: Qi Y **L- Editor**: A **E- Editor**: Liu WX

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v23.i6.1030

World J Gastroenterol 2017 February 14; 23(6): 1030-1037
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Retrospective Cohort Study

Is a split-dose regimen of 2 L polyethylene glycol plus ascorbic acid tolerable for colonoscopy in an early morning visit to a comprehensive medical check-up?

Ji Yeon Seo, Changhyun Lee, Eun Hyo Jin, Mi Hyun Yun, Joo Hyun Lim, Hae Yeon Kang, Jong In Yang, Su Jin Chung, Sun Young Yang, Joo Sung Kim

Ji Yeon Seo, Changhyun Lee, Eun Hyo Jin, Joo Hyun Lim, Hae Yeon Kang, Jong In Yang, Su Jin Chung, Sun Young Yang, Joo Sung Kim, Department of Internal Medicine and Healthcare Research Institute, Healthcare System Gangnam Center, Seoul National University Hospital, Seoul 06236, South Korea

Mi Hyun Yun, Department of Nursing, Healthcare System Gangnam Center, Seoul National University Hospital, Seoul 06236, South Korea

Author contributions: Seo JY, Lee C, Chung SJ and Kim JS designed the study; Seo JY, Lee C, Jin EH, Yun MH, Lim JH, Kang HY, Yang JI and Yang SY performed the research; Seo JY analyzed the data; Seo JY and Lee C wrote the paper; and Chung SJ, Yang SY and Kim JS critically revised the manuscript for important intellectual content.

Institutional review board statement: This study was approved by the Ethical Committee at SNUH (Seoul National University Hospital, IRB No. H-1601-007-729).

Informed consent statement: Informed consent was waived by Ethical Committee at SNUH.

Conflict-of-interest statement: The authors have no conflict of interest.

Data sharing statement: The dataset is available from the corresponding author at mdchlee@gmail.com.

Manuscript source: Unsolicited manuscript

Correspondence to: Changhyun Lee, MD, Department of Internal Medicine and Healthcare Research Institute, Healthcare System Gangnam Center, Seoul National University Hospital, 737 Yeoksam-dong, Gangnam-Gu, Seoul 06236, South Korea. mdchlee@gmail.com
Telephone: +82-2-21125578
Fax: +82-2-21125635

Received: December 1, 2016
Peer-review started: December 6, 2016
First decision: December 20, 2016
Revised: December 27, 2016
Accepted: January 17, 2017
Article in press: January 17, 2017
Published online: February 14, 2017

Abstract

AIM

To evaluate the effectiveness and tolerability of a split-dose 2 L polyethylene glycol (PEG)/ascorbic acid (AA) regimen for healthy examinees who visited for comprehensive medical check-up in the early morning.

METHODS

From February 2015 to March 2015, examinees of average risk who were scheduled for a colonoscopy in the morning were retrospectively enrolled.

RESULTS

The 189 examinees were divided into split-dose and non-split-dose groups. The adequacy of bowel preparation for the split-dose group *vs* the non-split-dose group was 96.8% *vs* 85.2%, respectively, $P < 0.001$, and the compliance of the last meal restriction

was 74.6% *vs* 58.2%, respectively, $P < 0.001$. The sleep disturbance ($P < 0.001$) was more prevalent in the split-dose group, however the willingness to repeat the same preparation method ($P = 0.243$) was not different in both groups. The split-dose regimen was the most important factor influencing adequate bowel preparation in multivariate analysis (HR = 10.89, 95%CI: 6.53-18.17, $P < 0.001$).

CONCLUSION

A split-dose regimen of 2 L PEG/AA for an early morning colonoscopy was more effective and showed better compliance for diet restriction without any difference in satisfaction and discomfort. Introducing a split-dose regimen of 2 L PEG/AA to morning colonoscopy examinees is effective and tolerable in a comprehensive medical check-up setting.

Key words: Compliance; Early morning colonoscopy; Effectiveness; Split-dose regimen; Tolerability

Core tip: A split-dose regimen of 2 L polyethylene glycol plus ascorbic acid is not widely used in comprehensive medical check-up, because this is considered intolerable for an early morning visit. We performed a retrospective cohort study to evaluate the effectiveness and tolerability of split-dose regimen for early morning visitors. A split-dose regimen for an early morning colonoscopy was more effective in bowel cleansing and showed better compliance for diet restriction compared with non-split-dose regimen without any difference in satisfaction and discomfort. Therefore, introducing a split-dose regimen to morning colonoscopy examinees is effective and tolerable even in comprehensive medical check-up settings.

Seo JY, Lee C, Jin EH, Yun MH, Lim JH, Kang HY, Yang JI, Chung SJ, Yang SY, Kim JS. Is a split-dose regimen of 2 L polyethylene glycol plus ascorbic acid tolerable for colonoscopy in an early morning visit to a comprehensive medical check-up? *World J Gastroenterol* 2017; 23(6): 1030-1037 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i6/1030.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i6.1030

INTRODUCTION

Bowel preparation is one of the most important factors for a complete colonoscopy. When bowel cleanliness is adequate, the adenoma detection rate increases[1-3], and the possibility of missed lesions decreases[4,5]. The polyethylene glycol (PEG) solution, an isosmotic non-absorbable polymer, is generally used for bowel preparation[6]. Recently, a low volume (2 L) of PEG with ascorbic acid (AA) was found to be preferred in Korea because 2 L PEG with AA (PEG/AA) was not inferior to 4 L PEG in bowel cleansing, despite the smaller volume[7-10]. High dose AA is not absorbed in intestine and promotes osmotic diarrhea. So the addition of high dose AA to PEG reduces the solution volume and improves taste[8].

It has already been reported that a split-dose regimen is more effective than a non-split-dose regimen for various drug preparations[11-13]. However, the compliance and tolerability of a split-dose regimen has always been a matter of concern[12,14]. In a split-dose regimen, it is uncomfortable to wake up at dawn to prepare for the colonoscopy scheduled in the early morning because patients should finish the laxative at least 2 h prior to the colonoscopy.

In case of comprehensive medical check-up in Korea, the colonoscopy appointment varied according to other examinations that were scheduled, but all patients visited the center early in the morning from 7 am to 9 am. Therefore, their bowel preparation had to be completed before 5 am to 7 am. We can easily imagine that this leads to poor compliance and dissatisfaction. Nevertheless, no study has directly compared the effectiveness of split-dose cleansing and non-split-dose cleansing using 2 L PEG/AA. Is a split-dose regimen still effective and tolerable in these healthy examinees?

This study aimed to compare the effectiveness, compliance and satisfaction of a split-dose regimen *vs* a non-split-dose regimen in healthy examinees who visited a heath check-up center in the early morning.

MATERIALS AND METHODS

Study population and ethical considerations

Examinees were retrospectively enrolled in the Seoul National University Hospital (SNUH) Healthcare System Gangnam Center from February 2015 to March 2015. Healthy examinees of average risk who visited our center from 7 am to 9 am and received a colonoscopy in the morning (8 am to 12 pm) were enrolled. The exclusion criteria for patients were as follows: younger than 18 years of age, had inflammatory bowel diseases or familial adenomatous polyposis, had previously received colorectal resection, did not receive a complete colonoscopy, or refused to participate. This study was approved by the Ethical Committee at SNUH (IRB No. H-1601-007-729).

Bowel preparation regimen

A non-split-dose regimen with 2 L PEG/AA has been used in the Gangnam Center because of the concern of patients' poor compliance and dissatisfaction with the split-dose regimen. According to the recent bowel cleansing guidelines[6,15] as part of a quality assurance program for colonoscopies, the split-dose regimen was introduced in March 2015. In this regimen, patients had to ingest 2 liters of PEG/AA solution (Coolprep®, Taejoon Pharm, Seoul, Korea) and 1 liter of water. Detailed regimens for 2 L PEG/AA in

Table 1 Bowel-cleansing regimens applied in this study

	Non-split-dose regimen	Split-dose regimen
Low-residue diet	3 d	3 d
Type of last meal	Rice porridge	Rice porridge
Time of last meal	Lunch (12:00 pm)	Dinner (6:00 pm)
Bowel cleansing product (day before examination)	PEG/AA 1 L + water 0.5 L (6:00 pm-7:30 pm) PEG/AA 1 L + water 0.5 L (8:30 pm-10:00 pm)	PEG/AA 1 L + water 0.5 L (9:00 pm-10:30 pm)
Bowel cleansing product (day of the examination)	-	PEG/AA 1 L + water 0.5 L (4:00 am-5:30 am)

AA: Ascorbic acid; PEG: Polyethylene glycol.

both groups are shown in Table 1. In the non-split-dose group, all of the bowel cleansing product and additional water was administered from 6:00 pm to 10:00 pm on the day before the colonoscopy. In the split-dose group, half of the product was administered the day before the colonoscopy, and the rest of the product was administered in the early morning (4:00 am to 5:30 am) on the day of the colonoscopy.

All patients received written instructions and phone calls from nurses about diet control and the method of bowel cleansing 1 wk prior to the colonoscopy.

Assessment of compliance, tolerability and safety

The compliance of patients was checked by nurses on an individual basis. Patients were questioned how long they had a low-residue diet, what food they ate for the last meal, the time when they had the last meal and the amount of PEG/AA and water they ingested.

The tolerability, dissatisfaction or discomfort experienced by the patients was evaluated using questionnaires before the colonoscopy. In terms of satisfaction, patients checked uncomfortable symptoms they had during bowel cleansing, such as nausea and/or vomiting, bloating, excessive diarrhea, anal pain, abdominal pain and/or discomfort, dizziness, fecal incontinence, sleep disturbance, large amount of fluid to intake, chilling and headache. Discomfort of bowel cleansing was assessed by using a visual analogue scale (on a scale of 1 to 10, with 10 being the worst). The willingness to repeat the same bowel cleansing regimen was also assessed.

For safety purposes, serious side effects that needed medical management such as dehydration and allergic reaction were monitored.

Colonoscopy and scoring of the bowel preparation

All procedures were performed by 17 expert endoscopists who had each performed more than 2000 colonoscopies. The effectiveness of bowel preparation was graded according to the Aronchick Bowel Preparation Scale[16]. The cleanliness of the total bowel was scored as one of five grades as follows: excellent, good, fair, poor, inadequate. In some patients, the colonoscopy was withdrawn before complete intubation because of huge amounts of solid materials in the left colon. The bowel preparation in these patients was also graded "inadequate". Degrees of bowel preparation that were deemed fair or better (fair, good and excellent) were considered 'adequate bowel preparation' in this study.

Statistical analysis

Continuous variables were expressed as the mean ± SD. Differences of continuous variables were analyzed by the independent-samples *t*-test. Categorical variables were expressed as a number (percent). These variables were analyzed using the χ^2 test and Fisher's exact test. The associations of the interval between when the bowel preparation was completed and the start of the colonoscopy and the quality of the bowel preparation were calculated according to linear regression and one-way analysis of variance. To evaluate the important factors associated with adequate bowel preparation, the logistic regression method was used. Variables with *P* values less than 0.05 in the univariate analysis were included in the multivariate analysis. *P* values < 0.05 were considered statistically significant. Statistical analyses for this study were conducted using SPSS for Windows 12.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Clinical characteristics of patients

The number of patients enrolled in this study was 378. Among them, 189 patients were in the non-split-dose group, and the other 189 patients were in the split-dose group. Demographics and clinical characteristics of the patients are shown in Table 2. No differences were observed in the proportion of males (58.7% in the non-split-dose group *vs* 60.3% in the split-dose group, *P* = 0.753), the mean age of participants (53.4 ± 10.9 *vs* 51.8 ± 9.3, respectively, *P* = 0.110) and the proportion of patients receiving the colonoscopy for screening purposes (22.8% *vs* 25.4%, respectively, *P* = 0.547). The mean frequency of bowel movements per week and the proportion of patients who were taking drugs for constipation were not different between the two groups.

Effectiveness of the bowel preparation method

The effectiveness of the bowel preparation according to the different regimens is displayed in Table 3. A

Table 2 Demographics and clinical characteristics of patients *n* (%)

	Non-spilt-dose regimen *n* = 189	Split-dose regimen *n* = 189	*P* value
Gender			0.753
Male	111 (58.7)	114 (60.3)	
Female	78 (41.3)	75 (39.7)	
Age (yr)	53.4 ± 10.9	51.8 ± 9.3	0.110
Previous examination			0.547
Screening	43 (22.8)	48 (25.4)	
Surveillance	146 (77.2)	141 (74.6)	
Bowel movement (wk^{-1})	7.1 ± 3.3	7.1 ± 3.5	0.738
Medications for constipation			0.869
Yes	5 (2.6)	4 (2.1)	
No	169 (89.4)	172 (91.0)	

Table 3 Difference of bowel preparation score between two groups *n* (%)

	Non-spilt-dose regimen *n* = 189	Split-dose regimen *n* = 189	*P* value
			< 0.001
Adequate			
Score 1 (Excellent)	7 (3.7)	33 (17.5)	
Score 2 (Good)	47 (24.9)	119 (63.0)	
Score 3 (Fair)	107 (56.6)	31 (16.4)	
Inadequate			
Score 4 (Poor)	20 (10.6)	5 (2.6)	
Score 5 (Inadequate)	8 (4.2)	1 (0.5)	

change in the bowel preparation regimen showed drastic improvement in bowel cleanliness ($P < 0.001$). When we numerically calculated the score of the bowel preparation, the mean score of the bowel preparation was 2.8 ± 0.8 in the non-split-dose group and 2.0 ± 0.7 in the split-dose group ($P < 0.001$). Adequate bowel preparation (excellent, good and fair) reached significance in the split-dose regimen group (85.2% in the non-split-dose group *vs* 96.8% in the split-dose group, $P < 0.001$). Only 1 patient experienced inadequate bowel preparation (received a score of 5) in the split-dose group. Only 1 (0.5%) patient in the split-dose group and 4 (2.1%) patients in the non-split-dose group received the additional bowel preparation method for the repeated colonoscopy.

Compliance of patients

The compliance of patients according to different preparation regimens was analyzed based on the nurses' medical records of the patients (Table 4). To achieve good compliance for bowel preparation, written instructions and phone calls were offered. Most examinees carefully read the instructions (97.4% in the non-split-dose group *vs* 98.9% in the split-dose group, $P = 0.429$), and the patients also received explanations from phone calls (87.8% *vs* 89.4%, respectively, $P = 0.134$). As a result, most patients understood the significance of bowel cleansing prior to a complete colonoscopy (97.9% *vs* 94.2%, respectively, $P = 0.131$).

Table 4 Compliance of bowel preparation according to different preparation regimens

	Non-spilt-dose regimen *n* = 189	Split-dose regimen *n* = 189	*P* value
Low-residue diet			0.542
< 3 d	54 (28.6%)	49 (25.9%)	
⩾ 3 d	134 (70.9%)	140 (74.1%)	
Type of last meal			< 0.001
Rice porridge	110 (58.2%)	141 (74.6%)	
Other low residue diet[1]	48 (25.4%)	25 (13.2%)	
Normal or high residue diet	19 (10.1%)	4 (2.1%)	
Time of last meal			< 0.001
As recommended or earlier	138 (73.0%)	176 (93.1%)	
After recommendation	50 (26.5%)	11 (5.8%)	
Dose of PEG/AA intake			0.736
2 L	185 (97.9%)	184 (97.4%)	
< 2 L	4 (2.1%)	5 (2.6%)	
Dose of water intake			0.081
⩾ 1 L	185 (97.9%)	189 (100%)	
< 1 L	3 (1.6%)	0 (0%)	

[1]Other low residue diet includes fish, egg, bread, potato, *etc*. AA: Ascorbic acid; PEG: Polyethylene glycol.

When we divided the bowel preparation into a "diet control" part and an ingesting "PEG/AA" part, the compliance of the ingestion of PEG/AA and water was higher than 97% in both groups. However, the compliance of the "diet control" part was not satisfactory. Patients who ingested a low-residue diet for more than 3 d had a compliance of only 70.9% and 74.1% in the non-split-dose group and split-dose group, respectively, $P = 0.542$. More patients in the split-dose group followed the restriction of the last meal. The percentage of patients who had rice porridge was much higher in the split-dose group compared to the non-split-dose group (74.6% *vs* 58.2%, respectively, $P < 0.001$). Only 73.0% followed the time limit of the last meal in the non-split-dose group compared to 93.1% in the split-dose group.

Tolerability and safety of the patients

The data relating to satisfaction and discomfort of the patients were collected and analyzed from questionnaire surveys (Table 5). According to the visual analogue scale of the discomfort index, no significant difference in discomfort between the two groups (5.2 ± 2.7 *vs* 4.8 ± 2.8, $P = 0.257$) was observed. Regardless of their discomfort, most patients in both groups answered that they were inclined to repeat the same regimen the next time (69.8% *vs* 70.4%, $P = 0.243$). In terms of adverse events, the most common causes of discomfort during bowel preparation were poor taste, nausea and/or vomiting and bloating. For the split-dose group, sleep disturbance was the 2nd most common complaint (40.9%), which was higher

Table 5 Subjective discomfort of patients according to different preparation regimens *n* (%)

	Non-split-dose regimen *n* = 189	Split-dose regimen *n* = 189	*P* value
Discomfort score (0-10)[1]	5.2 ± 2.7	4.8 ± 2.8	0.257
Willingness to repeat same regimen			
Yes	132 (69.8)	133 (70.4)	0.243
No	47 (24.9)	52 (27.5)	
Adverse events			
Poor taste	53 (50.5)	74 (54.0)	0.585
Nausea/vomiting	39 (37.1)	53 (38.7)	0.806
Bloating	38 (36.2)	54 (39.4)	0.608
Excessive diarrhea	27 (25.7)	27 (19.7)	0.266
Anal pain	20 (19.0)	14 (10.2)	0.050
Abdominal pain/discomfort	13 (12.4)	20 (14.6)	0.618
Dizziness	9 (8.6)	9 (6.6)	0.556
Fecal incontinence	8 (7.6)	7 (5.1)	0.422
Sleep disturbance	6 (5.7)	56 (40.9)	< 0.001
Bulky fluid	4 (3.8)	0 (0)	0.021
Chilling	3 (2.9)	1 (0.7)	0.198
Headache	1 (1.0)	0 (0)	0.252

[1]Patients graded subjective discomfort from 0 (tolerable) to 10 (extremely distressed).

Table 6 Factors associated with adequate bowel preparation

	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age	0.99 (0.97-1.01)	0.524		
Gender (female)	1.26 (0.83-1.91)	0.273		
Bowel movement (wk^{-1})	1.00 (0.94-1.06)	0.989		
Low-residue diet (⩾ 3 d)	1.55 (1.00-2.39)	0.048	1.49 (0.88-2.53)	0.139
Time of last meal[1]	3.47 (1.92-6.29)	< 0.001	1.61 (0.78-3.30)	0.195
Type of last meal				
Normal or high residue	1.00 (reference)		1.00 (reference)	
Rice porridge only	5.09 (1.83-14.12)	0.002	2.26 (0.70-7.30)	0.174
Other low residue[2]	2.86 (0.94-8.69)	0.064	2.53 (0.72-8.93)	0.150
Preparation regimen (split-dose)	10.63 (6.57-17.19)	< 0.001	10.89 (6.53-18.17)	< 0.001
Intake of PEG/AA (dose)	2.08 (0.20-22.06)	0.543		
Intake of water (dose)	0.036 (0.00-12.36)	0.265		

[1]Whether patients followed recommendations; lunch for non-spilt-dose regimen, dinner for split-dose regimen; [2]Other low residue diet includes fish, egg, bread, potato, *etc*. AA: Ascorbic acid; PEG: Polyethylene glycol.

than the non-split-dose group (5.7%, $P < 0.001$). Additionally, the complaint of the bulky fluid was more common in the non-split-dose group than the split-dose group (3.8% *vs* 0%, respectively, $P = 0.021$).

During bowel preparation, none of the patients experienced serious side effects. Only one case of urticaria was reported in the non-split-dose regimen group. The patient was a 75-year-old female who had generalized urticaria after ingesting half of the PEG/AA solution. She was fully recovered after receiving an oral antihistamine.

Factors associated with adequate bowel preparation

Important factors leading to adequate bowel preparation are shown in Table 6. In the univariate analysis, the factors that were significantly related to adequate bowel preparation were as follows: ⩾ 3 d of a low residue diet ($P = 0.048$), time of the last meal was as instructed ($P < 0.001$), rice porridge for the last meal ($P = 0.002$), and split-dose regimen ($P < 0.001$). Using these factors in the multivariate analysis, the split-dose regimen was the only significant factor related to adequate bowel preparation (adjusted HR = 10.89, 95%CI, 6.53-18.17, $P < 0.001$).

In the split-dose regimen group, the interval between the completion of the bowel preparation and the colonoscopy was shorter than that of the non-split-dose regimen group. The average intervals were 728.3 ± 91.0 min for the non-split-dose regimen group and 291.5 ± 65.0 min for the split-dose regimen group ($P < 0.001$). The linear regression of the continuous variables demonstrated that the quality of bowel preparation improved when the interval decreased between the completion of the bowel preparation and the start of the colonoscopy ($\beta = 0.002$, $r = 0.462$, $P < 0.001$). When we divided patients into their bowel preparation scores, we observed a meaningful difference in the interval time between

Table 7 Correlation of bowel cleanliness and interval between finishing bowel preparation and colonoscopy

Bowel preparation score	Inadequate *n* = 4	Poor *n* = 25	Fair *n* = 138	Good *n* = 165	Excellent *n* = 40	*P* value
Interval[1]	661.3 ± 91.3	667.3 ± 205.6	631.0 ± 201.3	413.7 ± 210.2	364.5 ± 167.8	< 0.001
T[2]	a	a	a	b	b	TukeyB

[1]Interval between finish of bowel preparation and start of colonoscopy (min). Statistical significances were tested by One-way analysis of variances among groups; [2]The same letters indicate non-significant difference between groups based on Tukey's multiple comparison test.

the patients who received good/excellent scores and the patients who received fair/poor/inadequate scores (one-way analysis of variance, $P < 0.001$; Table 7).

DISCUSSION

In this study, we observed that the effectiveness of bowel cleansing was markedly improved in the split-dose regimen group as previously described[6]. We also analyzed the importance of the time interval between the completion of the bowel preparation and the starting time of the colonoscopy. Patients who had a time interval less than 7 h showed a better outcome than that of patients with more than a 7-h interval. This result is in accordance with previous reports that bowel cleansing was better when the colonoscopy was performed within 8 h after ingesting the last fluid than after 8 h of the final ingestion[11] and that the degree of bowel preparation worsens with time[17].

Our results showed that the compliance of diet control was much better in the split-dose group than the non-split-dose group, particularly in regard to the type and time of the last meal. This result could be interpreted to mean that having an early dinner before the day of the colonoscopy was acceptable and tolerable in the split-dose regimen group. Additionally, it would be difficult to restrict food at lunch during working hours in the non-split-dose group, and having a low residue diet for dinner would be much easier. This study is the first of its kind that showed better compliance of diet control in the split-dose group compared to that of the non-split-dose group; the diet control regimen for the split-dose group was more tolerable and effective for bowel preparation.

However, keeping a low-residue diet for 3 d was not followed well in both groups; therefore, the proper number of days for diet restriction has not yet been determined. Some studies have focused on a liberal diet for better compliance, but the results were not satisfactory[18-20]. Various high-residue foods such as kimchi are a considerable part of the Korean diet, and these fiber materials are difficult to remove during a colonoscopy. Nevertheless, even though our patients knew the necessity of bowel preparation and conformed to ingesting whole PEG/AA, their compliance to a low-residue diet was still very poor. The usefulness of education about bowel preparation has been already reported[21,22]. Continuous education and promotion of diet control will be important, and further studies are required to reduce the days of diet control.

When we reviewed the patients who received an additional bowel preparation regimen during the study period, the only patient who failed the bowel preparation in the split-dose group was a 45-year-old man. He did not follow the diet control as instructed; he had high-residue side dishes for the last meal the day before the colonoscopy and a low-residue diet for only 1 d. When he followed the instructions of the split-dose regimen completely for the repeat colonoscopy, his bowel preparation was excellent. Furthermore, 4 patients needed additional bowel preparation in the non-split-dose group. In spite of ingesting additional PEG solution or perfectly following instructions of the non-split-dose regimen, the best score they received for the repeat colonoscopy was fair.

Despite the concerns of complaints in the split-dose group, no differences in tolerability and satisfaction were observed between the two groups. However, a significant difference in the details of complaints was observed. Examinees of the split-dose group experienced more sleep disturbance than the non-split-dose group. In contrast, examinees of the non-split-dose group complained about the bulky fluid because they had to ingest a large amount of fluid within a few hours. Interpreting our data, very little difference was observed in general satisfaction according to the type of regimen, and only in the different subtype of discomfort.

This study has inevitable limitations because of its non-randomized design and retrospective nature. The examinees who completed the survey during the study period were enrolled in this study, and the possibility of selection bias was present. Despite its shortcomings, the strength of this study is related to the following advantages: (1) this is the first study that compared effectiveness, compliance and tolerability of split-dose and non-split-dose regimens using low volume PEG for early morning visitors to a comprehensive medical check-up. Therefore, this study required a special condition that all examinees should complete their bowel preparation before their visit for the comprehensive medical check-up; (2) we presented the compliance of the bowel cleansing regimens step-by-step and detailed the complaints and practical dissatisfaction of the examinees; and (3) finally, we demonstrated that diet control in the split-dose group was more tolerable than the non-split-dose group.

In conclusion, a split-dose-regimen of 2 L PEG/AA

for an early morning colonoscopy was more effective and showed better compliance for diet restriction without any differences in satisfaction and discomfort. Therefore, it is reasonable to introduce a split-dose regimen for the early morning colonoscopy examinees undergoing comprehensive medical check-up considering its remarkable effectiveness and compliance.

COMMENTS

Background

Bowel preparation is one of the most important factors for a complete colonoscopy. It has already been reported that a split-dose regimen is more effective than a non-split-dose regimen for various drug preparations. However, a split-dose regimen of 2 L polyethylene glycol (PEG) plus ascorbic acid (AA) is not widely used in comprehensive medical check-up in Korea because the split-dose regimen is considered intolerable for an early morning visit.

Research frontiers

In a split-dose regimen, it is uncomfortable to wake up at dawn to prepare for the colonoscopy scheduled in the early morning because patients should finish the laxative at least 2 h prior to the colonoscopy. This study aimed to evaluate the effectiveness and tolerability of a split-dose 2 L PEG/AA regimen for healthy examinees who visited for comprehensive medical check-up in the early morning.

Innovations and breakthroughs

In this study, the authors found that effectiveness of bowel cleansing was markedly improved in the split-dose regimen group. Compliance of diet control was much better in the split-dose group than the non-split-dose group, particularly in regard to the type and time of the last meal. Despite the concerns of complaints in the split-dose group, no differences in tolerability and satisfaction were observed between the two groups. However, a significant difference in the details of complaints was observed. Examinees of the split-dose group experienced more sleep disturbance than the non-split-dose group. In contrast, examinees of the non-split-dose group complained about the bulky fluid because they had to ingest a large amount of fluid within a few hours.

Applications

It is reasonable to introduce a split-dose regimen for the early morning colonoscopy examinees undergoing comprehensive medical check-up considering its remarkable effectiveness and compliance.

Terminology

Split-dose regimen is a method of bowel preparation for colonoscopy that examinees take half of bowel cleansing dose at night on the day before the colonoscopy and the other half in the early morning on the day of colonoscopy.

Peer-review

This is a good study in which the authors analyzed the effectiveness and compliance of split-dose regimen of 2L PEG/AA for an early morning colonoscopy. This result is an important ground to introduce a split-dose regimen to morning colonoscopy examinees in a comprehensive medical check-up.

REFERENCES

1 **Gurudu SR**, Ramirez FC, Harrison ME, Leighton JA, Crowell MD. Increased adenoma detection rate with system-wide implementation of a split-dose preparation for colonoscopy. *Gastrointest Endosc* 2012; **76**: 603-608.e1 [PMID: 22732876 DOI: 10.1016/j.gie.2012.04.456]

2 **Froehlich F**, Wietlisbach V, Gonvers JJ, Burnand B, Vader JP. Impact of colonic cleansing on quality and diagnostic yield of colonoscopy: the European Panel of Appropriateness of Gastrointestinal Endoscopy European multicenter study. *Gastrointest Endosc* 2005; **61**: 378-384 [PMID: 15758907]

3 **Sherer EA**, Imler TD, Imperiale TF. The effect of colonoscopy preparation quality on adenoma detection rates. *Gastrointest Endosc* 2012; **75**: 545-553 [PMID: 22138085 DOI: 10.1016/j.gie.2011.09.022]

4 **Lebwohl B**, Kastrinos F, Glick M, Rosenbaum AJ, Wang T, Neugut AI. The impact of suboptimal bowel preparation on adenoma miss rates and the factors associated with early repeat colonoscopy. *Gastrointest Endosc* 2011; **73**: 1207-1214 [PMID: 21481857 DOI: 10.1016/j.gie.2011.01.051]

5 **Chokshi RV**, Hovis CE, Hollander T, Early DS, Wang JS. Prevalence of missed adenomas in patients with inadequate bowel preparation on screening colonoscopy. *Gastrointest Endosc* 2012; **75**: 1197-1203 [PMID: 22381531 DOI: 10.1016/j.gie.2012.01.005]

6 **Johnson DA**, Barkun AN, Cohen LB, Dominitz JA, Kaltenbach T, Martel M, Robertson DJ, Boland CR, Giardello FM, Lieberman DA, Levin TR, Rex DK. Optimizing adequacy of bowel cleansing for colonoscopy: recommendations from the US multi-society task force on colorectal cancer. *Gastroenterology* 2014; **147**: 903-924 [PMID: 25239068 DOI: 10.1053/j.gastro.2014.07.002]

7 **Bitoun A**, Ponchon T, Barthet M, Coffin B, Dugué C, Halphen M. Results of a prospective randomised multicentre controlled trial comparing a new 2-L ascorbic acid plus polyethylene glycol and electrolyte solution vs. sodium phosphate solution in patients undergoing elective colonoscopy. *Aliment Pharmacol Ther* 2006; **24**: 1631-1642 [PMID: 17094774 DOI: 10.1111/j.1365-2036.2006.03167.x]

8 **Ell C**, Fischbach W, Bronisch HJ, Dertinger S, Layer P, Rünzi M, Schneider T, Kachel G, Grüger J, Köllinger M, Nagell W, Goerg KJ, Wanitschke R, Gruss HJ. Randomized trial of low-volume PEG solution versus standard PEG + electrolytes for bowel cleansing before colonoscopy. *Am J Gastroenterol* 2008; **103**: 883-893 [PMID: 18190651 DOI: 10.1111/j.1572-0241.2007.01708.x]

9 **Moon CM**, Park DI, Choe YG, Yang DH, Yu YH, Eun CS, Han DS. Randomized trial of 2-L polyethylene glycol + ascorbic acid versus 4-L polyethylene glycol as bowel cleansing for colonoscopy in an optimal setting. *J Gastroenterol Hepatol* 2014; **29**: 1223-1228 [PMID: 24955451 DOI: 10.1111/jgh.12521]

10 **Ponchon T**, Boustière C, Heresbach D, Hagege H, Tarrerias AL, Halphen M. A low-volume polyethylene glycol plus ascorbate solution for bowel cleansing prior to colonoscopy: the NORMO randomised clinical trial. *Dig Liver Dis* 2013; **45**: 820-826 [PMID: 23769755 DOI: 10.1016/j.dld.2013.04.009]

11 **Marmo R**, Rotondano G, Riccio G, Marone A, Bianco MA, Stroppa I, Caruso A, Pandolfo N, Sansone S, Gregorio E, D'Alvano G, Procaccio N, Capo P, Marmo C, Cipolletta L. Effective bowel cleansing before colonoscopy: a randomized study of split-dosage versus non-split dosage regimens of high-volume versus low-volume polyethylene glycol solutions. *Gastrointest Endosc* 2010; **72**: 313-320 [PMID: 20561621 DOI: 10.1016/j.gie.2010.02.048]

12 **Kilgore TW**, Abdinoor AA, Szary NM, Schowengerdt SW, Yust JB, Choudhary A, Matteson ML, Puli SR, Marshall JB, Bechtold ML. Bowel preparation with split-dose polyethylene glycol before colonoscopy: a meta-analysis of randomized controlled trials. *Gastrointest Endosc* 2011; **73**: 1240-1245 [PMID: 21628016 DOI: 10.1016/j.gie.2011.02.007]

13 **Cohen LB**. Split dosing of bowel preparations for colonoscopy: an analysis of its efficacy, safety, and tolerability. *Gastrointest Endosc* 2010; **72**: 406-412 [PMID: 20579994 DOI: 10.1016/j.gie.2010.04.001]

14 **Enestvedt BK**, Tofani C, Laine LA, Tierney A, Fennerty MB. 4-Liter split-dose polyethylene glycol is superior to other bowel preparations, based on systematic review and meta-analysis. *Clin Gastroenterol Hepatol* 2012; **10**: 1225-1231 [PMID: 22940741 DOI: 10.1016/j.cgh.2012.08.029]

15 **Hassan C**, Bretthauer M, Kaminski MF, Polkowski M, Rembacken B, Saunders B, Benamouzig R, Holme O, Green S, Kuiper T, Marmo R, Omar M, Petruzziello L, Spada C, Zullo A, Dumonceau JM. Bowel preparation for colonoscopy: European Society of

Gastrointestinal Endoscopy (ESGE) guideline. *Endoscopy* 2013; **45**: 142-150 [PMID: 23335011 DOI: 10.1055/s-0032-1326186]

16 **Rostom A**, Jolicoeur E. Validation of a new scale for the assessment of bowel preparation quality. *Gastrointest Endosc* 2004; **59**: 482-486 [PMID: 15044882]

17 **Siddiqui AA**, Yang K, Spechler SJ, Cryer B, Davila R, Cipher D, Harford WV. Duration of the interval between the completion of bowel preparation and the start of colonoscopy predicts bowel-preparation quality. *Gastrointest Endosc* 2009; **69**: 700-706 [PMID: 19251013 DOI: 10.1016/j.gie.2008.09.047]

18 **Wu KL**, Rayner CK, Chuah SK, Chiu KW, Lu CC, Chiu YC. Impact of low-residue diet on bowel preparation for colonoscopy. *Dis Colon Rectum* 2011; **54**: 107-112 [PMID: 21160321 DOI: 10.1007/DCR.0b013e3181fb1e52]

19 **Aoun E**, Abdul-Baki H, Azar C, Mourad F, Barada K, Berro Z, Tarchichi M, Sharara AI. A randomized single-blind trial of split-dose PEG-electrolyte solution without dietary restriction compared with whole dose PEG-electrolyte solution with dietary restriction for colonoscopy preparation. *Gastrointest Endosc* 2005; **62**: 213-218 [PMID: 16046981 DOI: 10.1016/S0016-5107(05) 00371-8]

20 **Sipe BW**, Fischer M, Baluyut AR, Bishop RH, Born LJ, Daugherty DF, Lybik MJ, Shatara TJ, Scheidler MD, Wilson SA, Rex DK. A low-residue diet improved patient satisfaction with split-dose oral sulfate solution without impairing colonic preparation. *Gastrointest Endosc* 2013; **77**: 932-936 [PMID: 23531424 DOI: 10.1016/j.gie.2013.01.046]

21 **Abuksis G**, Mor M, Segal N, Shemesh I, Morad I, Plaut S, Weiss E, Sulkes J, Fraser G, Niv Y. A patient education program is cost-effective for preventing failure of endoscopic procedures in a gastroenterology department. *Am J Gastroenterol* 2001; **96**: 1786-1790 [PMID: 11419830 DOI: 10.1111/j.1572-0241.2001.03872.x]

22 **Spiegel BM**, Talley J, Shekelle P, Agarwal N, Snyder B, Bolus R, Kurzbard N, Chan M, Ho A, Kaneshiro M, Cordasco K, Cohen H. Development and validation of a novel patient educational booklet to enhance colonoscopy preparation. *Am J Gastroenterol* 2011; **106**: 875-883 [PMID: 21483463 DOI: 10.1038/ajg.2011.75]

P- Reviewer: Leitman IM, Roy PV **S- Editor**: Yu J
L- Editor: A **E- Editor**: Liu WX

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v23.i6.1038

World J Gastroenterol 2017 February 14; 23(6): 1038-1043
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Retrospective Study

Can patients determine the level of their dysphagia?

Hafiz Hamad Ashraf, Joanne Palmer, Harry Richard Dalton, Carolyn Waters, Thomas Luff, Madeline Strugnell, Iain Alexander Murray

Hafiz Hamad Ashraf, Harry Richard Dalton, Carolyn Waters, Iain Alexander Murray, Departments of Gastroenterology, Royal Cornwall Hospital NHS Trust, Truro TR1 3LJ, United Kingdom

Joanne Palmer, Research and Development, Royal Cornwall Hospital NHS Trust, Truro TR1 3LJ, United Kingdom

Thomas Luff, Madeline Strugnell, Clinical Imaging, Royal Cornwall Hospital NHS Trust, Truro TR1 3LJ, United Kingdom

Author contributions: Murray IA conceived the idea; Ashraf HH and Murray IA designed the research; all authors were involved in data acquisition except Palmer J who analysed the data; Murray IA, Dalton HR and Ashraf HH wrote the paper and all authors have reviewed and contributed to the final version.

Institutional review board statement: The Research, Development and Innovation sponsorship team, Royal Cornwall Hospitals NHS Trust reviewed the study and decided that it did not require formal ethics approval as it was within the remit of audit.

Informed consent statement: The Research, Development and Innovation sponsorship team, Royal Cornwall Hospitals NHS Trust reviewed the study and decided that it did not require formal ethics approval as it was within the remit of audit and did not require formal patient consent as it involved a retrospective review of anonymised data.

Conflict-of-interest statement: Dalton HR has received travel and accommodation costs and consultancy fees from GlaxoSmithKline, Wantai and Roche, travel accommodation and lecture fees from Merck, Gilead and GFE Blut and travel and accommodation fees from the Falk and Gates Foundations. None of the other authors has any conflict of interest to disclose.

Data sharing statement: Statistical code and dataset available from the corresponding author at iain.murray8@nhs.net. Consent was not obtained but the presented data are anonymised and risk of identification is low.

Manuscript source: Invited manuscript

Correspondence to: Iain Alexander Murray, BSc, MRCP, DM, Consultant Gastroenterologist, Departments of Gastroenterology, Royal Cornwall Hospital NHS Trust, 2 Penventinnie Ln, Treliske, Truro TR1 3LJ, United Kingdom. iain.murray8@nhs.net
Telephone: +44-1872-252717
Fax: +44-1872-252794

Received: October 11, 2016
Peer-review started: October 12, 2016
First decision: November 21, 2016
Revised: January 4, 2017
Accepted: January 18, 2017
Article in press: January 18, 2017
Published online: February 14, 2017

Abstract

AIM

To determine if patients can localise dysphagia level determined endoscopically or radiologically and association of gender, age, level and pathology.

METHODS

Retrospective review of consecutive patients presenting to dysphagia hotline between March 2004 and March 2015 was carried out. Demographics, clinical history and investigation findings were recorded including patient perception of obstruction level (pharyngeal, mid sternal or low sternal) was documented and the actual level of obstruction found on endoscopic or radiological examination (if any) was noted. All patients with evidence of obstruction including oesophageal

carcinoma, peptic stricture, Schatzki ring, oesophageal pouch and cricopharyngeal hypertrophy were included in the study who had given a perceived level of dysphagia. The upper GI endoscopy reports (barium study where upper GI endoscopy was not performed) were reviewed to confirm the distance of obstructing lesion from central incisors. A previously described anatomical classification of oesophagus was used to define the level of obstruction to be upper, middle or lower oesophagus and this was compared with patient perceived level.

RESULTS

Three thousand six hundred and sixty-eight patients were included, 42.0% of who were female, mean age 70.7 ± 12.8 years old. Of those with obstructing lesions, 726 gave a perceived level of dysphagia: 37.2% had oesophageal cancer, 36.0% peptic stricture, 13.1% pharyngeal pouches, 10.3% Schatzki rings and 3.3% achalasia. Twenty-seven point five percent of patients reported pharyngeal level (upper) dysphagia, 36.9% mid sternal dysphagia and 25.9% lower sternal dysphagia (9.5% reported multiple levels). The level of obstructing lesion seen on diagnostic testing was upper (17.2%), mid (19.4%) or lower (62.9%) or combined (0.3%). When patients localised their level of dysphagia to a single level, the kappa statistic was 0.245 (P < 0.001), indicating fair agreement. 48% of patients reporting a single level of dysphagia were accurate in localising the obstructing pathology. With respect to pathology, patients with pharyngeal pouches were most accurate localising their level of dysphagia (P < 0.001). With respect to level of dysphagia, those with pharyngeal level lesions were best able to identify the level of dysphagia accurately (P < 0.001). No association (P > 0.05) was found between gender, patient age or clinical symptoms with their ability to detect the level of dysphagia.

CONCLUSION

Patient perceived level of dysphagia is unreliable in determining actual level of obstructing pathology and should not be used to tailor investigations.

Key words: Deglutition disorders; Oesophageal stenosis; Oesophageal neoplasm; Gastroscopy; Fluoroscopy; Patient perception; Pharyngeal pouch

Core tip: Patient perception of the level of their dysphagia is only accurate in 48% of patients. It is most accurate for those with pharyngeal pouches and for those with pharyngeal or upper oesophageal pathology which might help guide initial investigations, *e.g.*, to barium swallow. No other patient features or history helps determine patient accuracy. Endoscopists and radiologists should be aware of the importance of carefully examining the whole oesophagus to avoid missing pathology irrespective of a patient's perceived level of dysphagia.

Ashraf HH, Palmer J, Dalton HR, Waters C, Luff T, Strugnell M, Murray IA. Can patients determine the level of their dysphagia? *World J Gastroenterol* 2017; 23(6): 1038-1043 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i6/1038.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i6.1038

INTRODUCTION

For more than 40 years, it has been established that a careful history can establish the diagnosis in up to 80% of patients presenting with dysphagia[1-3]. Indeed we and others have demonstrated that aspects of history and patient demographics can be highly predictive of specific diagnoses such as oesophageal cancer, peptic stricture, pharyngeal pouch and eosinophilic oesophagitis[4-7].

However studies which have explored whether patients are accurately able to localise the site of the cause of their dysphagia have been inconclusive. Some have found overall poor correlation between patient localisation and actual site of pathology[2,8,9] whilst others have found it to be good[10,11]. While some studies suggest that localisation of proximal pathology is more accurate[10,11] others have shown the converse[8]. Only the study by Roeder[8] has attempted to determine if the underlying pathology was related to the patient's ability to accurately localise the level of dysphagia and that was primarily to investigate whether diffuse localisation was associated with an underlying motility disorder (which was the case in only 9%).

Whether a patient is able to accurately localise the level of their dysphagia is important. If they can accurately localise the level of dysphagia, then a focussed examination might be feasible, reducing cost, time and, for barium examinations, radiation exposure. If they are unable to accurately localise the level of dysphagia it is equally important that radiologist or endoscopist is aware of this or they may fail to focus on the whole oesophagus and miss important pathology[12].

We reviewed the final outcome of 3668 consecutive patients presenting with dysphagia between March 2004 and March 2015. Patient localisation of the level of their dysphagia was compared to that found at endoscopy or barium swallow examination in patients with an obstructing oesophageal lesion. We explored whether the nature of the pathology, patient characteristics or clinical features were associated with the patients' ability to localise the level of dysphagia.

MATERIALS AND METHODS

Case ascertainment

We reviewed the final outcome of those patients presenting to our nurse led telephone triage dysphagia hotline[13] between March 2004 and March

Table 1 Diagnoses in patients with discrete pathology investigated for dysphagia between 2004 and 2015

Diagnosis	*n* (%)
Oesophageal cancer	270 (37.2)
Peptic stricture	261 (36.0)
Cricopharyngeal hypertrophy/pharyngeal pouch	95 (13.1)
Schatzki ring	75 (10.3)
Achalasia	24 (3.3)
Pharyngeal pouch and oesophageal cancer	1 (0.1)

2015. Firstly those patient with an obstructing lesion including oesophageal carcinoma, peptic stricture, Schatzki ring, oesophageal pouch and cricopharyngeal hypertrophy were identified. The patient group studied were those with an obstructing oesophageal lesion and patient-derived data regarding localisation levels. Patient data was contemporaneously recorded but reviewed retrospectively and included: patient demographics, patient perception of their level of dysphagia as upper (described as level of throat), middle (mid chest) or lower (bottom of chest); and associated symptoms.

Investigations and level of dysphagia

We next determined where those with an obstructing lesion who had given a defined level of obstruction had undergone upper gastrointestinal endoscopy only, barium swallow only or both. The level of dysphagia was taken as that recorded by the endoscopist (either as distance from the incisors or as upper, middle or lower oesophagus). When endoscopy was unsuccessful, refused or not performed for another reason then the level of dysphagia was taken as that recorded at barium swallow. If the radiologist had not recorded a level of dysphagia, the films were reviewed by a radiology registrar and consultant to agree a level. The final level was then recorded as per the subdivision of the oesophagus suggested by the National Cancer Institute[14], namely. Upper: from thoracic inlet to level of tracheal bifurcation; 18-23 cm from incisors. Middle: from tracheal bifurcation to midway to gastro-oesophageal junction; 24-32 cm from incisors. Lower: from midway between tracheal bifurcation and gastro-oesophageal junction to gastro-oesophageal junction; 32-40 cm from incisors.

Statistical analysis

The Kappa statistic was used to define if the patient localisation and diagnostic localisation matched. Categorical variables were explored using Pearson's χ^2 test to look at the association of the clinical and patient characteristic factors, such as diagnosis, the level of pathology and history of symptoms (duration, regurgitation, reflux, odynophagia, chest pain, diet change and weight change) and gender. Following test for assumption of normality, age was explored using Independent *t*-test to determine if there was a mean difference in age of people who could and could not determine the localisation of their obstruction. Missing data was handled using complete case analysis.

Data was analysed using IBM SPSS Statistics V22.0, and were two tailed tests where statistical significance was accepted if $P < 0.05$[15].

The statistical methods of this study were reviewed by Stig B Laursen of Odense University Hospital, Odense, Denmark.

The study was reviewed by the Research, Development and Innovation sponsorship team, Royal Cornwall Hospitals NHS Trust at the Royal Cornwall Hospital who ruled that formal ethics approval was not required as it fell within the remit of audit/service evaluation.

RESULTS

Patients and diagnoses

A total of 3668 consecutive patients were seen by the dysphagia hotline during the study period. Of these, 807 had an obstructing lesion of who 726 had given a perceived level of obstruction. Of these, 42.0% were female, mean age 70.7 ± 12.8 years old. The final diagnoses are shown in Table 1.

Six hundred and forty-one (88.3%) had undergone upper gastrointestinal endoscopy alone, 73 (10.0%) a barium swallow only and 12 (1.6%) had undergone both. Twenty-seven point five percent of patients reported pharyngeal level (upper) dysphagia, 36.9% mid sternal dysphagia and 25.9% lower sternal dysphagia. The remaining 9.5% reported less well defined dysphagia, giving more than one level of perceived dysphagia, most commonly pharyngeal and mid sternal dysphagia (7.4%). The levels of obstructing lesions seen on diagnostic testing were upper (17.2%), mid (19.4%), lower (62.9%) or combined (0.3%).

Symptoms were described as progressive (42.5%), intermittent (56.8%), dysphagia to solids (98.9%) or dysphagia to liquids (17.4%), associated with regurgitation (63.6%) or reflux (51.5%). Odynophagia was described in 23.8%, chest pain 25.1%, diet change due to dysphagia in 48.2% and weight loss of 2 kg or more in 36.9%.

Association between patient localisation of disease and actual localisation

Where patients described multiple levels of dysphagia there can be no agreement between patient described levels of dysphagia and actual levels. Comparing only when patients were able to accurately pinpoint their level of dysphagia (single localisation point), gave a kappa statistic of 0.245 ($P < 0.001$), indicating a fair agreement[16] between patient localisation and actual level of lesion. Of those patients indicating a single level of dysphagia, 48.0% of patient had an absolute match between perceived and actual level of dysphagia.

Table 2 Accuracy in perceiving correct level of dysphagia correlated to final diagnosis

Diagnosis	Perceived single level (*n*)	Perceived diffuse level (*n*)	Absolute match
Achalasia	23	1	34.8%
Oesophageal cancer	243	27	47.3%
Peptic stricture	235	26	38.7%
Pharyngeal pouch	88	7	84.1%
Schatzki ring	66	9	40.9%

One patient perceived the obstruction as pharyngeal and had both pharyngeal (pouch) and distal (cancer) obstruction. Their data is not included in this table.

Table 3 Effect of level of obstructing lesion on patient ability to accurately localise the cause of their dysphagia

Level of obstructing lesion	Total (*n*)	Perceived as pharyngeal	Perceived as mid sternal	Perceived as lower sternal	Correct level identified
Upper	115	94	18	3	81.7%
Middle	130	39	64	27	49.2%
Lower	410	66	186	158	38.5%
Total	655	199	268	188	48.2%

Six hundred and fifty-five patients with a single obstructing oesophageal lesion with the test derived level shown in column 1 and the patient perceived level in columns 3-5. When a patient perceived their dysphagia correctly this is shown in bold. Some patients perceived more than one level of dysphagia (diffuse) but their data is not shown here for clarity ($n = 71$). Pearson χ^2 test gave $P < 0.001$.

Table 4 Associated symptoms and their ability to improve patient localisation of dysphagia *n* (%)

Symptom	Number reporting symptom	Number with symptom correctly localising dysphagia	*P* value
Regurgitation/choking	416/647 (67.4)	195/416 (46.9)	0.478
Reflux	339/649 (52.2)	152/339 (44.8)	0.059
Odynophagia	158/651 (24.3)	72/158 (45.6)	0.415
Chest pain	162/649 (25.0)	71/162 (43.8)	0.180
Change in diet	315/651 (48.4)	151/315 (47.9)	0.883
Weight gain	47/635 (7.9)	20/47 (42.6)	0.422
Weight loss	244/611 (39.9)	116/244 (47.5)	0.816

Effect of nature of obstructing lesion on localisation of dysphagia

Patients with pharyngeal pouches were most likely to accurately localise their level of dysphagia. Regarding patients perceiving dysphagia at a single level, 84.1% accurately identified the level. The number and percentage of absolute match (single localisation level accurately identified by patient) among different diagnostic groups is shown in Table 2. χ^2 test showed this was statistically significant ($P < 0.001$), establishing a definite association between nature of the pathology and the patients' ability to localise the level of dysphagia.

Effect of level of obstructing lesion on localisation of dysphagia

When the actual level of the obstructing lesion was considered, it was noted that patients with pharyngeal obstruction were most likely to accurately localise the obstruction (Table 3).

Effect of patient gender on localisation of dysphagia

No association was found between gender and ability to detect the level of dysphagia. Similar figures were seen for males (183 of 386: 47.4% absolute match) and females (132 of 270: 48.9% absolute match), $P = 0.709$.

Effect of patient age on localisation of dysphagia

Three hundred and fifteen of 656 patients who correctly identified their level of obstruction had a mean age of 71.28 ± 13.02 years. The mean age for patients who were incorrect in identifying the level of their dysphagia was 70.58 ± 13.17 years. Independent *t* test found the mean difference not significant ($P = 0.497$) indicating that patient age does not influence ability to identify the level of dysphagia (95%CI: -2.706-1.314).

Effect of "associated symptoms" on patients' ability to detect level of dysphagia

Patients with dysphagia presented with many associated symptoms. Symptoms were described as progressive (43.4%), intermittent (56.4%), dysphagia to solids (99.5%) or to liquids (17.1%). It was associated with regurgitation, reflux, odynophagia, chest pain, diet change due to dysphagia and weight loss of 2 kg or more as shown in Table 4. Duration was less than 8 wk (27.6%), 8-25 wk (35.2%) or greater than 25 wk

(37.1%) Statistical analysis indicated no association of any of these with the ability to localise the level of dysphagia.

DISCUSSION

To our knowledge this is the largest study comparing patient localisation of dysphagia with true level of obstruction defined by endoscopy or barium swallow examination. In 48% of patients, the dysphagic symptoms accurately predicted the location of the underlying obstructive oesophageal pathology and this was most accurate (81.7%) in patients with lesions in the upper oesophagus despite only 17.6% of patients presenting with pharyngeal level lesions. No factors in patient demographics or history predicted which patients more accurately localised their pathology.

Previous studies have shown a very wide range in the ability of patients to accurately identify the level of obstructing oesophageal pathology ranging from 17% to 74%[3,8,10,11]. Possible reasons for the differences between each of these studies and our own include small patient numbers in the studies where patient localisation appeared more accurate[10,11] and differences in the populations studied. For instance, Roeder *et al*[8] found patients with distal pathology most accurate (80%) but these comprised only a small percentage of their study (which may indicate problems with selection bias). They comprise 62.6% of a larger patient cohort in the present study population.

When comparing specific pathologies with the ability to localise, those with pharyngeal pouches were most accurate. This differs from previous studies which failed to show any difference based on underlying oesophageal pathology[8,11]. It provides some reassurance that our policy of performing barium swallow initially rather than gastroscopy for patients presenting with pharyngeal level dysphagia is correct[6].

Again there have been differences in the pathology studied. We elected to study conditions where the pathology was well defined and could be accurately localised. Some studies have included patients with motility disorders[8] and gastro-oesophageal reflux disease[11] which are more diffuse. There are differences in investigations performed. Some studies performed barium swallow alone[10], others manometry and gastroscopy[8], and others barium swallow and gastroscopy[11]. Manometry was not easily accessible for our patient cohort (the nearest centre during most of the study period being 60 miles (96 km) from our centre so few patients were studied in this way). Differences in conditions studied, investigations performed, differences in the manner in which patient localisation was recorded and in the final diagnoses studied as well as the size of the patient cohort could all influence the final outcome.

The current study is retrospective and has used only 3 areas for patient localisation unlike that of Wilcox[11] who used exact patient identified levels and determined how accurate they were in centimetres from the pathology found. But others have used 3-6 levels also[3,8].

We also acknowledge that the pathology seen at endoscopy or on barium study is not always the underlying reason for a patient to experience dysphagia eg a patient with a Schatzki ring could have oesophageal dysmotility underlying their dysphagia, but it seems highly likely that the recorded pathology (pharyngeal pouches, achalasia, oesophageal cancer and peptic strictures) would have been responsible in the majority. Our study has included only patients with obstructing oesophageal pathology, which represents 18% of the 3668 consecutive cases presenting with dysphagia to our rapid-access dysphagia hotline clinic. Patients with dysphagia and non-obstructive oesophageal pathology were excluded from analysis. This is an important point to bear in mind, as although we have demonstrated that 48% of patients accurately predict their level of obstructing oesophageal pathology, in clinical practice when faced with a patient with dysphagic symptoms the predictive value of level of dysphagia will likely be an order of magnitude less than we observed in our selected population.

It is recognised that 11.3% of upper gastrointestinal cancers have been missed on an examination within 3 years of their diagnosis[12]. Whilst oesophageal lesions account for only 9% of this cohort it is possible that endoscopists or radiologists could be misled by patient symptoms and focus on the area of patient localisation. With this in mind, we disagree with the advice of Wright and Ellis[10] to tailor the examination to focus on the patient localised area of dysphagia.

In summary, we have shown that in 48% of patients the level of dysphagic symptoms accurately predicted the location of the underlying obstructive oesophageal pathology and this was most accurate in patients with upper oesophageal pathology. The clinical utility of the level of dysphagia in an unselected dysphagic population is likely to be low.

Patients are relatively inaccurate in localising obstructing oesophageal pathologies causing dysphagia. Those with pharyngeal pouches or pharyngeal level pathology in general are most accurate but a full examination of the oesophagus is essential in all patients presenting with dysphagia to prevent missing pathology.

ACKNOWLEDGMENTS

We are grateful to Dr Stig B. Laursen for his expert biostatistical advice.

COMMENTS

Background

Previous studies investigating patients with dysphagia and their ability to determine the level of obstructive lesions have produced discordant results. Some have shown good correlation between actual and perceived level,

others have not. Some have shown distal pathology is better correlated, others pharyngeal level.

Research frontiers

Dysphagia is a common symptom which can be secondary to malignancy. If patients are able to determine the level of their dysphagia, limited endoscopic or fluoroscopic examination may be feasible. It is also important to determine if patients with pharyngeal pouches in particular are able to determine the level of their dysphagia since some centres will investigate patients with pharyngeal level dysphagia initially by barium swallow to avoid endoscopy and risk of perforation.

Innovations and breakthroughs

This study is much larger than any previous studies investigating patient perception of dysphagia level and investigates only those conditions where there is a definite level of pathology. Detailed history obtained prospectively was available and used to determine if associated features help with patient perception. Patient perceived dysphagia level with actual level correlate in 48% of patients overall but in 84.1% of those with pharyngeal pouches and 81.7% of those with pharyngeal level pathology. Ability to perceive level of obstruction was unrelated to patient age or gender or any associated symptoms.

Applications

Patients with pharyngeal level dysphagia and pharyngeal pouches in particular are most able to accurately determine the level of their dysphagia. Patients with pharyngeal level dysphagia should therefore be considered for barium swallow as their initial investigation. Endoscopists and radiologists should be aware that they need to investigate the whole of the oesophagus for causes of dysphagia and should not be reliant on patient derived level of dysphagia.

Peer-review

This theme of this article is unique, though more information and discussion is required. The detailed complaints of dysphagia should be discussed and the factors for the decreasing accuracy should be analyzed carefully. More information of the obstructive lesion or considering the cause in combination with the background of each subject may provide some clue.

REFERENCES

1 **Castell DO**, Donner MW. Evaluation of dysphagia: a careful history is crucial. *Dysphagia* 1987; **2**: 65-71 [PMID: 3507297]

2 **Edwards DA**. Discriminatory value of symptoms in the differential diagnosis of dysphagia. *Clin Gastroenterol* 1976; **5**: 49-57

3 **Edwards DA.** History and symptoms of esophageal disease in the esophagus. In Vantrappen G, Hellemans J (eds): Disease of the Esophagus. New York: Springer-Verlag, 1974.

4 **Murray IA**, Palmer J, Waters C, Dalton HR. Predictive value of symptoms and demographics in diagnosing malignancy or peptic stricture. *World J Gastroenterol* 2012; **18**: 4357-4362 [PMID: 22969199 DOI: 10.3748/wjg.v18.i32.4357]

5 **Murray IA**, Joyce S, Palmer J, Lau M, Schultz M. Incidence and features of eosinophilic esophagitis in dysphagia: a prospective observational study. *Scand J Gastroenterol* 2016; **51**: 257-262 [PMID: 26446708 DOI: 10.3109/00365521.2015.1093166]

6 **Grimes DR**, Wilde A, Palmer J, Waters C, Dalton HR, Murray IA. Incidence and predictive features of pharyngeal pouch in a dysphagic population. *Dysphagia* 2014; **29**: 305-309 [PMID: 24385219 DOI:10.1007/s00455-013-9507-4]

7 **Rhatigan E**, Tyrmpas I, Murray G, Plevris JN. Scoring system to identify patients at high risk of oesophageal cancer. *Br J Surg* 2010; **97**: 1831-1837 [PMID: 20737538 DOI: 10.1002/bjs.7225]

8 **Roeder BE**, Murray JA, Dierkhising RA. Patient localization of esophageal dysphagia. *Dig Dis Sci* 2004; **49**: 697-701 [PMID: 15185881 DOI: 10.1023/B: DDAS.0000026321.02927.39]

9 **Jones B**, Ravich WJ, Donner MW, Kramer SS, Hendrix TR. Pharyngoesophageal interrelationships: observations and working concepts. *Gastrointest Radiol* 1985; **10**: 225-233 [PMID: 4029538 DOI: 10.1007/BF01893105]

10 **Wright RE**, Ellis PK. Patient perception and localization of dysphagia -- barium study correlation. *Dis Esophagus* 1997; **10**: 211-214; discussion 211-214; [PMID: 9280082]

11 **Wilcox CM**, Alexander LN, Clark WS. Localization of an obstructing esophageal lesion. Is the patient accurate? *Dig Dis Sci* 1995; **40**: 2192-2196 [PMID: 7587788 DOI: 10.1007/BF02209005]

12 **Menon S,** Trudgill N. How commonly is upper gastrointestinal cancer missed at endoscopy? A meta-analysis. *Endosc Int Open* 2014; **2**: E46-50 [PMID: 26135259 DOI: 10.1055/s-0034-1365524]

13 **Murray IA,** Water C, Maskell G, Despott EJ, Palmer J, Dalton HR. Improved clinical outcomes and efficacy with a nurse-led dysphagia hotline service. *Frontline Gastroenterol* 2013; **4**: 102-107 [DOI: 10.1136/flgastro-2012-100244]

14 **National Cancer Institute SEER Training Modules**. Anatomy of the Esophagus. Available from: URL: http: //training.seer.cancer.gov/ugi/anatomy/esophagus.html

15 **IBM Corp.** Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.

16 **Cohen J.** A coefficient of agreement for nominal scales. *Educ Psychol Measure* 1960; **20**: 37-46 [DOI: 10.1177/001316446002000104]

P- Reviewer: Konaka K, Pescatori M **S- Editor**: Qi Y
L- Editor: A **E- Editor**: Liu WX

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v23.i6.1044

World J Gastroenterol 2017 February 14; 23(6): 1044-1050
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Retrospective Study

Eosinophilic cholangitis is a potentially underdiagnosed etiology in indeterminate biliary stricture

Dirk Walter, Sylvia Hartmann, Eva Herrmann, Jan Peveling-Oberhag, Wolf O Bechstein, Stefan Zeuzem, Martin-Leo Hansmann, Mireen Friedrich-Rust, Jörg G Albert

Dirk Walter, Stefan Zeuzem, Mireen Friedrich-Rust Department of Internal Medicine I, Johann Wolfgang Goethe-University Hospital, 60590 Frankfurt, Germany

Sylvia Hartmann, Martin-Leo Hansmann, Dr. Senckenberg Institute for Pathology, Johann Wolfgang Goethe-University Hospital, 60590 Frankfurt, Germany

Eva Herrmann, Institute of Biostatistics and Mathematical Modeling, Johann Wolfgang Goethe-University Hospital, 60590 Frankfurt, Germany

Jan Peveling-Oberhag, Jörg G Albert, Department for Gastroenterology, Hepatology and Endocrinology, Robert-Bosch Hospital, 70376 Stuttgart, Germany

Wolf O Bechstein, Department of General and Visceral Surgery, Johann Wolfgang Goethe-University Hospital, 60590 Frankfurt, Germany

Author contributions: Walter D and Albert JG designed the concept of the study; Data were gathered by Walter D, Peveling-Oberhag J, Bechstein WO, Zeuzem S and Friedrich-Rust M; Hartmann S and Hansmann ML performed histopathological review; Statistical analysis was performed from Herrmann E; The manuscript was drafted by Walter D, Hartmann S, Peveling-Oberhag J and Albert JG; all authors critically revised the manuscript.

Institutional review board statement: The study protocol was approved by the institutional review board (No. 478/15) of the local ethics committee of the University Hospital Frankfurt.

Informed consent statement: Patient informed consent was waived due to the retrospective design of the study.

Conflict-of-interest statement: The authors have no conflicts of interest to declare or other disclosures of record.

Data sharing statement: No additional data are available.

Manuscript source: Invited manuscript

Correspondence to: Dr. Dirk Walter, Department of Internal Medicine I, Johann Wolfgang Goethe-University hospital, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany. dirk.walter@kgu.de
Telephone: +49-69-63015122
Fax: +49-69-63016448

Received: October 6, 2016
Peer-review started: October 10, 2016
First decision: October 20, 2016
Revised: November 17, 2016
Accepted: January 11, 2017
Article in press: January 11, 2017
Published online: February 14, 2017

Abstract

AIM

To investigate presence and extent of eosinophilic cholangitis (EC) as well as IgG4-related disease in patients with indeterminate biliary stricture (IBS).

METHODS

All patients with diagnosis of sclerosing cholangitis (SC) and histopathological samples such as biopsies or surgical specimens at University Hospital Frankfurt from 2005-2015 were included. Histopathological diagnoses as well as further clinical course were reviewed. Tissue samples of patients without definite diagnosis after complete diagnostic work-up were reviewed regarding

presence of eosinophilic infiltration and IgG4 positive plasma cells. Eosinophilic infiltration was as well assessed in a control group of liver transplant donors and patients with primary sclerosing cholangitis.

RESULTS

One hundred and thirty-five patients with SC were included. In 10/135 (13.5%) patients, no potential cause of IBS could be identified after complete diagnostic work-up and further clinical course. After histopathological review, a post-hoc diagnosis of EC was established in three patients resulting in a prevalence of 2.2% (3/135) of all patients with SC as well as 30% (3/10) of patients, where no cause of IBS was identified. 2/3 patients with post-hoc diagnosis of EC underwent surgical resection with suspicion for malignancy. Diagnosis of IgG4-related cholangitis was observed in 7/135 patients (5.1%), whereas 3 cases were discovered in post-hoc analysis. 6/7 cases with IgG4-related cholangitis (85.7%) presented with eosinophilic infiltration in addition to IgG4 positive plasma cells. There was no patient with eosinophilic infiltration in the control group of liver transplant donors (n = 27) and patients with primary sclerosing cholangitis (n = 14).

CONCLUSION

EC is an underdiagnosed benign etiology of SC and IBS, which has to be considered in differential diagnosis of IBS.

Key words: Indeterminate biliary stricture; Endoscopy; Endoscopic retrograde cholangiopancreatography; Eosinophilic cholangitis; Bile duct stenosis; IgG4-related disease; Primary sclerosing cholangitis

Core tip: To differentiate benign from malignant disease in indeterminate biliary strictures (IBS) is crucial for clinical management. To date, data on eosinophilic cholangitis (EC) as a potential cause of IBS are lacking. In this retrospective study, we demonstrate that EC occurs in up to 30% of patients presenting with IBS and unclear clinical and histopathological findings at the end of diagnostic work-up. We thereby demonstrate that EC is a potentially underdiagnosed benign disease, which has to be considered in differential diagnoses of IBS to prevent these patients from surgery.

Walter D, Hartmann S, Herrmann E, Peveling-Oberhag J, Bechstein WO, Zeuzem S, Hansmann ML, Friedrich-Rust M, Albert JG. Eosinophilic cholangitis is a potentially underdiagnosed etiology in indeterminate biliary stricture. *World J Gastroenterol* 2017; 23(6): 1044-1050 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i6/1044.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i6.1044

INTRODUCTION

Sclerosing cholangitis (SC) can arise from various underlying diseases, such as infectious, immunological, toxic or ischemic etiology as well as due to mechanical obstruction. Different types of SC may look alike at cross sectional imaging and radiography of the bile ducts, unified by a characteristic narrowing of intrahepatic and/or extrahepatic bile duct system. For clinical management, especially differentiation of benign from malignant strictures is fundamental. However, a definite diagnosis can solely be established by histopathological analysis of the bile duct alterations. Clinical data together with percutaneous ultrasound, computed tomography (CT), magnetic resonance imaging (MRI) and endoscopic retrograde cholangiopancreatography (ERCP) contribute to a probable diagnosis, not equaling the security of histopathology. Differentiating indeterminate biliary strictures (IBS) and establishing the diagnosis of SC *vs* cholangiocarcinoma (CCA) is challenging in many patients. This is represented by studies reporting a benign diagnosis in patients with IBS after surgery in up to 17%[1-3].

In recent years, an increasing number of cases were reported with SC caused by eosinophilic cholangitis (EC): a benign condition first described by Leegaard in 1980[4]. EC is characterized by (1) a wall thickening or stenosis of the biliary system; (2) histopathological findings of eosinophilic infiltration; and (3) reversibility of biliary abnormalities without treatment or following steroid treatment[5]. Peripheral eosinophilia was observed often but not necessarily in case reports (65%[6]). EC as cause of SC is of special interest, since it can appear as Klatskin-mimicking lesion and is often only diagnosed after bile duct resection, although conservative treatment leads to resolution of the stricture. However, an underlying cause of EC could not be identified to date and data on prevalence of EC are lacking.

In the present study, we performed a retrospective analysis to evaluate prevalence of EC and IgG4-RD in patients with IBS and inconclusive histopathological findings.

MATERIALS AND METHODS

Patients

Patients with diagnosis of SC (according to ICD-10 Code) between 2005 and 2015 at University Hospital Frankfurt were screened and all patients with histopathological specimen available from biopsies or surgical resections were included. Thereby, patients with IBS that were surgically treated for suspicion of malignancy were included as well as inconclusive findings at biopsies. To evaluate the subsequent clinical course of the patients with inconclusive histopathological findings, electronic medical records were investigated and

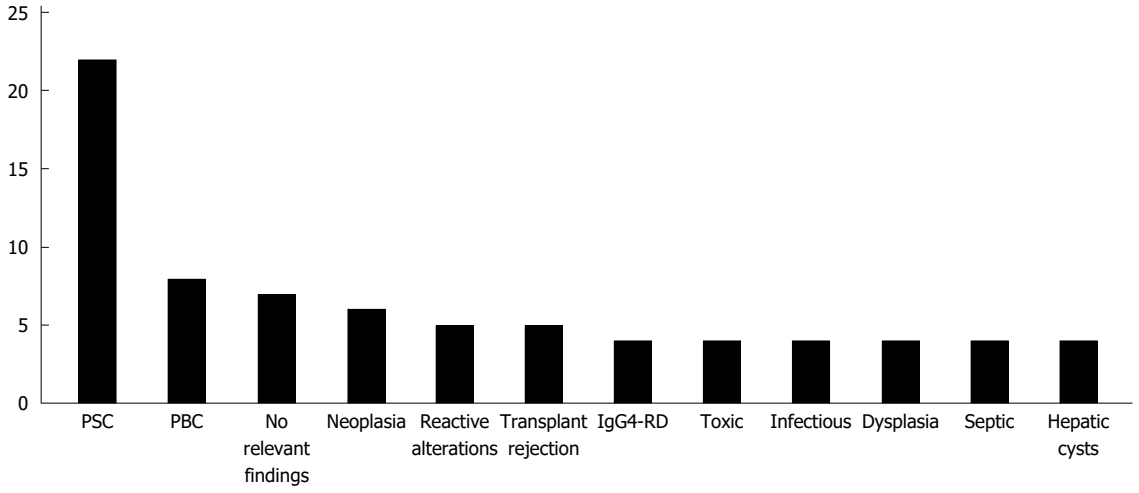

Figure 1 Underlying etiologies of patients with clear diagnoses in histopathological reports. IgG4-RD: IgG4-related disease; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cirrhosis.

standardized. Extracted data were: age, gender and final diagnosis from clinical documents.

Histopathological and clinical review

In all patients with inconclusive histological and clinical findings after full diagnostic work-up, hematoxylin-eosin stained slides of surgical or bioptical specimens were reevaluated by an expert pathologist. Eosinophilic granulocytes were counted per high power field (HPF) in areas of cholangitis with the highest density up to an eosinophilic count of 30/HPF. All cases with ⩾ 15 eosinophilic granulocytes/HPF were assessed as positive according to the threshold for eosinophilic esophagitis[7]. In addition, a representative block was chosen and staining with an IgG4-antibody (Mouse anti-IgG4, Zytomed Systems, Berlin, Germany) was performed. Cases were considered as IgG4-positive, when > 30% of plasma cells stained positive for IgG4. Furthermore, all patients with inconclusive findings after full diagnostic work-up were reviewed for presence and appearance of biliary stricture in cross sectional imaging (CT, MRI) and ERCP.

After the review of histopathological and clinical data, patients were classified into consistent with EC, consistent with IgG4-RD or not consistent with either EC or IgG4-RD.

To evaluate eosinophilic infiltration in primary sclerosing cholangitis (PSC) and non-inflammatory bile ducts, samples with histopathological reports of biopsies or surgical specimens including the diagnosis PSC and liver transplant-donors were investigated as well.

For ERCP, standard duodenoscopes (Olympus V-Scopes, TJF 160VF, TJF-Q180 V; Olympus Europe, Hamburg, Germany) were used and the short-wire technique with locking the wire at the distal end of the duodenoscope was applied. In patient 4, cholangioscopy was used as well (duodenoscope TJF - Q180V, Olympus Medical, Tokyo, Japan).

Descriptive statistics were calculated using BiAS (version 11.01, BiAS for Windows; Epsilon-Verlag, Frankfurt, Germany). The study protocol was approved by the institutional review board (No. 478/15) of the local ethics committee of the University Hospital Frankfurt.

RESULTS

Patients

In total, 135 histopathological specimens were identified of patients treated for SC between 2005 and 2015. Mean age of the patients at the date of initial histopathological investigation was 54.0 (SD 15.2, range 22-86) and 87/135 (64%) of the patients were men. Histopathological reports were based on liver biopsies (n = 64), intraductal bile duct biopsies (n = 33), biliary brush cytology (n = 16), surgical specimen from liver or bile duct resection (n = 15), liver transplant (n = 6) or autopsy (n = 1).

In 69 of 135 patients (51%), the histopathological investigation reported a strong suspicion of a specific disease or a definite diagnosis (all diagnoses are shown in Figure 1). In 7 patients, no relevant pathological findings were reported. 6/7 of these patients were clinically diagnosed with PSC and one patient with CCA in further clinical course.

In the group of patients with inconclusive histopathological diagnosis, investigation of clinical reports revealed a specific diagnosis in 56/66 patients (85%). In the remaining ten patients (15%), clinical diagnosis was either unclear cholangitis (n = 4) or patients had surgery with suspicion for malignancy but inconclusive findings in histopathological examination (n = 6). The study design, including final diagnoses, is documented in Figure 2.

Histopathological review

To further characterize these ten patients with unclear

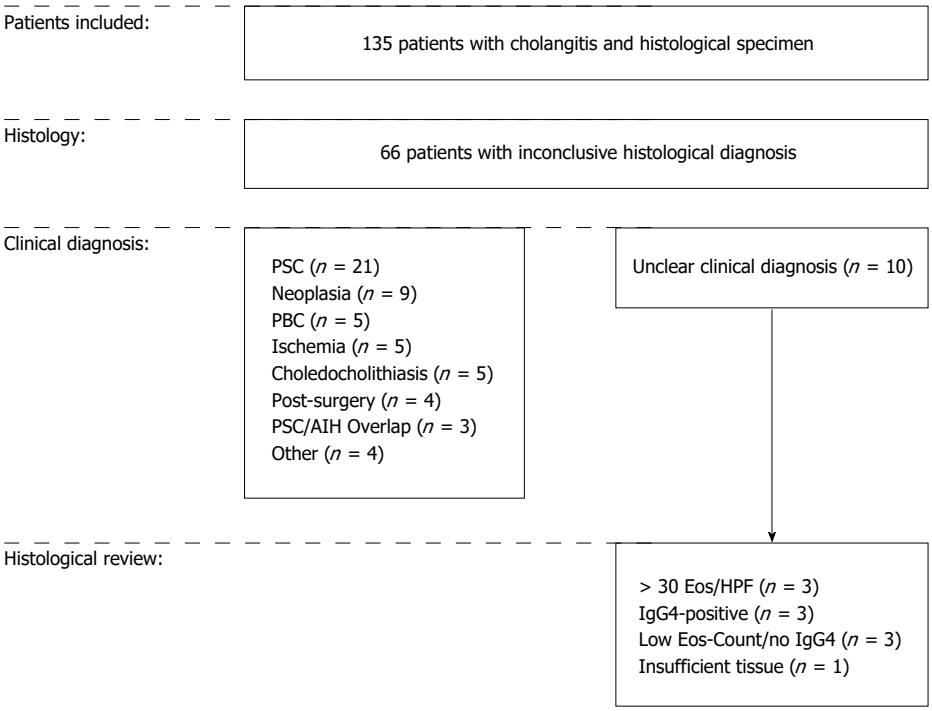

Figure 2 Study design. AIH: Autoimmune hepatitis; Eos: Eosinophilic granulocytes; HPF: High power field; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cirrhosis.

Table 1 Clinical data of all patients with suspicion for IgG4-related disease (Pat1-Pat3) or eosinophilic cholangitis (Pat4-Pat6)

Patient ID	Age	Sex	Year	Eos (histology)	Eos (serum)	IgG4-staining	Biliary stenosis	Clinical course
Pat1	40	M	2008	+	-	+	Perihilar	Hemihepatectomy
Pat2	52	M	2011	+	-	+	Perihilar	Hemihepatectomy
Pat3	73	M	2005	-	NA	+	Perihilar	CBD-resection
Pat4	40	F	2015	+	-	-	Distal	Steroids
Pat5	63	M	2008	+	-	-	Distal	PPPD
Pat6	65	F	2006	+	-	-	Perihilar	CBD-resection

Eosinophilic infiltrate was assessed as positive, if ⩾ 15 eosinophilic granulocytes/high power field were present. Cut off for peripheral eosinophilia was ⩾ 4% of leukocytes. Plasma cells were assessed as positive, if > 30% stained positive for IgG4. CBD: Common bile duct; PPPD: Pylorus preserving pancreaticoduodenectomy; Year: The year the histological specimen was obtained; Eos: Eosinophilic granulocytes; NA: Not available.

clinical as well as histopathological diagnosis and to exclude EC and IgG4-RD as possible underlying etiology, we performed a histological review quantifying eosinophilic infiltration and determining IgG4-status. Thereby, we identified 3/10 (30%) patients with dense eosinophilic infiltrate (⩾ 30 eosinophilic granulocytes/HPF) but without IgG4-positive plasma cells. We also found 3/10 patients (30%) to have a distinct infiltration of plasma cells with positive IgG4 staining. Notably, 2 of these 3 patients additionally showed as well a dense eosinophilic infiltrate (> 30/HPF; Figure 3). Of the remaining 4 patients, 3 did neither have an elevated eosinophilic count nor a positive staining for IgG4 and 1 patient had insufficient material for a reliable histopathological review.

Since 2/3 newly diagnosed IgG4-positive samples had a marked eosinophilic infiltrate, a review of the 4 routinely diagnosed IgG4-RD cases was performed. Notably, 3/4 of these patients had > 30 eosinophilic granulocytes/HPF, whereas one patient had 25 eosinophilic granulocytes/HPF. Thus, of all cases with IgG4-RD, 6/7 (85.7%) showed a marked infiltration with eosinophilic granulocytes.

Clinical review

All six patients with suspicion of EC or IgG4-RD were investigated for alterations in the biliary tract. Patients with suspicion of EC had strictures in both the extrahepatic and intrahepatic biliary tree, whereas all strictures of patients with suspicion of IgG4-RD were located in the perihilar region. Besides anatomical location of the stricture, further clinical course of these patients was investigated. 5/6 of these patients underwent surgical resection, whereas one patient with EC was treated conservatively with oral steroids. Clinical data of the 6 patients consistent with diagnoses of EC or IgG4-RD are shown in Table 1.

Thereby, after histological and clinical review,

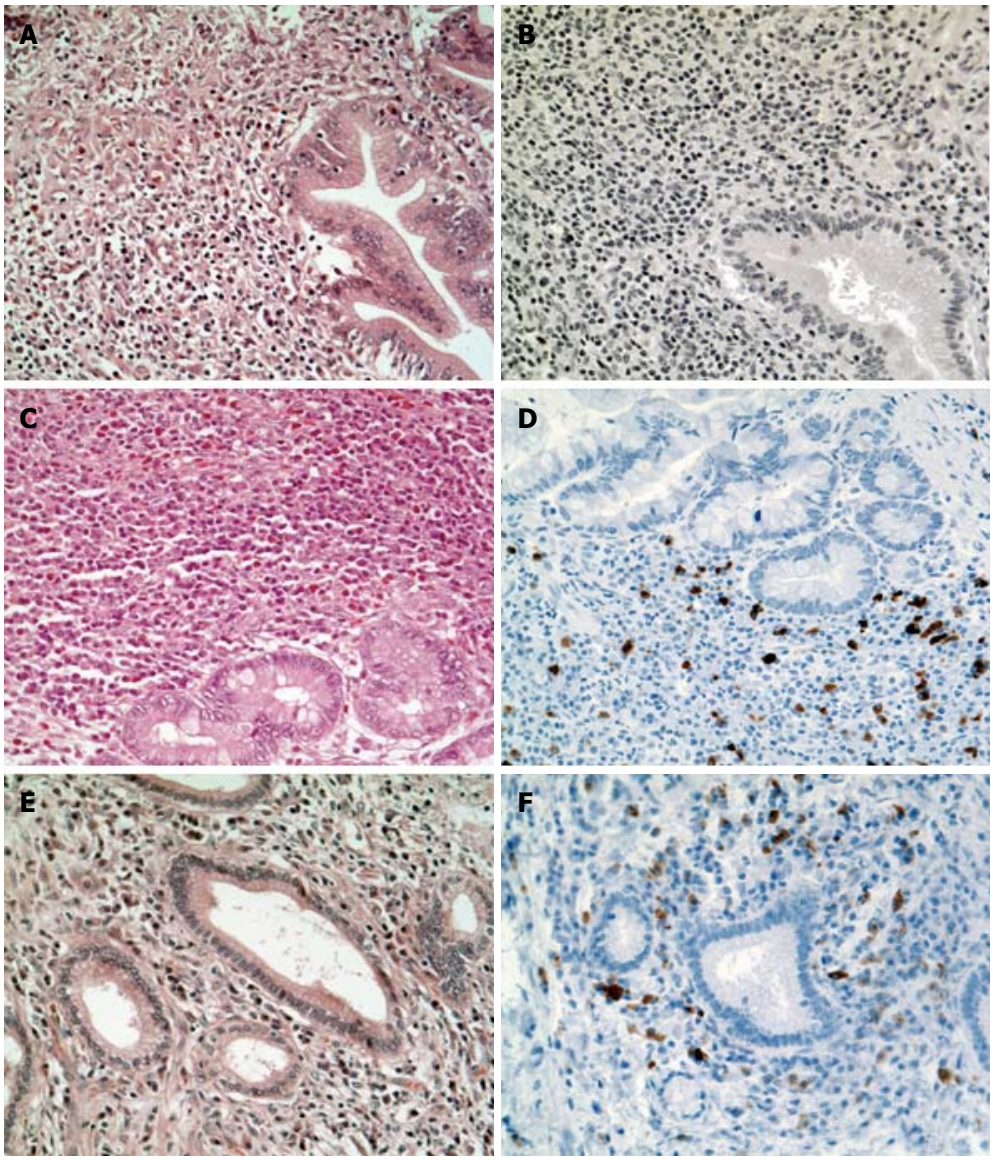

Figure 3 Infiltration of eosinophilic granulocytes and IgG4 positive plasma cells of patients with eosinophilic cholangitis without IgG4 positive plasma cells (A and B); as well as patients with IgG4-related cholangitis with eosinophilic infiltration (C and D); and without eosinophilic infiltration (E and F). A, C and E: × 200, hematoxylin-eosin; B, D and F: × 200, IgG4-Ab.

prevalence of cases consistent with EC was 3/135 (2%) in all patients with cholangitis and biopsy or surgical specimen, 3/66 (5%) in cases with inconclusive histological findings and 3/10 (30%) in patients with no clear clinical diagnosis in further course after full diagnostic work-up. Further clinical course of these patients was investigated as well: the two patients with EC treated with bile duct resection had several episodes of transient cholestasis or cholangitis after surgery, which were managed with temporary stent placement if necessary. The patient treated with oral steroids is currently off treatment since 12 mo and has not experienced a relapse so far.

Taking into account the growing knowledge about IgG4-RD during the period of this study, the date of histological examination of this entity was investigated as well: Samples with IgG4-RD being diagnosed only within the retrospective histopathological review in this study were taken from 2005-2011, whereas patients with diagnosis of IgG4-RD in initial histopathological examination were taken between 2012 and 2014.

Eosinophilic infiltration in primary sclerosing cholangitis and non-inflammatory bile duct tissue

To further characterize EC in comparison to other potential differential diagnoses, eosinophilic infiltration was analyzed in 14 patients with PSC (mean histological stage was 2.0, range 1-3). Mean eosinophilic count/HPF was 1.8 (range 0-6, SD 2.0). In addition, non-inflammatory biliary tissue from liver transplant donors was investigated for degree of eosinophilic infiltration: 27 samples were examined and mean eosinophilic count was 0.1/HPF (range 0-3, SD 0.6; Supplementary Figure 1).

DISCUSSION

Management of patients presenting with IBS is a frequent challenge for the clinician and the strategic choice of whether and when to perform surgery might have an important effect on these patients' quality of live. In the present study we report results from a retrospective prevalence study on EC, a benign and conservatively treatable differential diagnosis of IBS, which so far has only been described in case reports.

We evaluated all patients with diagnosis of sclerosing cholangitis and available histopathological specimen and found a prevalence of EC of 3/135 (2.2%) as well as 3/10 (30%) in the group of patients without clear clinical diagnosis in further course. 2/3 patients with EC were treated surgically. Of note, also in case reports of EC, 14/35 (40%) patients had cholecystectomy and 10/35 (28.6%) patients were treated with major bile duct resections due to suspicion for malignancy (Supplementary Table 1). The considerable part of patients being retrospectively diagnosed with EC in our study combined with the high rate of surgeries in literature indicates that EC is most likely an underdiagnosed disease, which is often only identified after possibly avoidable bile duct resection. These data stress the important role EC plays as potential underlying disease of SC and point out that surgeons, interventional endoscopists and gastrointestinal pathologists should be aware of this conservatively treatable condition in cases with IBS.

All cases consistent with EC in this study had a dense infiltrate of eosinophilic granulocytes with $\geqslant$ 30 per HPF which was considerably higher than the threshold of $\geqslant$ 15 eosinophilic granulocytes/HPF we defined according to diagnostic criteria for eosinophilic esophagitis[7]. In view of the frequent submucosal infiltration with eosinophilic granulocytes, we recommend taking preferably intraductal biopsies instead of biliary brushings in case of suspicion for EC. Moreover, as recommended in IBS and eosinophilic esophagitis, we suggest to take multiple biopsies to reduce risk of false negative results[8,9]. Since some cases with EC were reported to be associated with eosinophilic infiltration in other parts of the gastrointestinal tract, taking additional biopsies in the stomach or colon should be considered as well[10-12]. In addition, cholangioscopy can be another helpful tool to diagnose EC[13].

IgG4-RD was identified to be the underlying disease of SC in 7/135 (5.2%) cases. Three of these cases were diagnosed only after retrospective histopathological review within this study. Of note, these three cases were originally diagnosed 2011 and earlier whereas the four routinely diagnosed cases of IgG4-RD were between 2012 and 2014, pointing out the growing knowledge about this disease during the period of our study. Of note, since prevalence data about IgG4-related cholangitis in western countries is very limited, a comparison of our data with other studies is hindered[14,15].

Notably, 6/7 (85.7%) cases with IgG4-RD additionally showed a marked eosinophilic infiltration suggesting a potential overlap in pathogenesis of these two conditions. This finding correlates to a prospective study from 2014, where a cohort of patients with eosinophilic esophagitis was shown to have significantly elevated IgG4-positive plasma cells compared to a control group[16]. Furthermore, IgG4-positive plasma cells and serum levels reacted to specific foods suggesting an IgG4-associated pathogenesis of eosinophilic esophagitis. However, three patients with diagnosis of EC in our study did not show IgG4-positive plasma cells indicating that common features in pathogenesis of EC and IgG4-RD need further characterization.

Another entity important to differentiate from EC is PSC. For instance, in a Japanese study, 27% of PSC patients were reported to have peripheral eosinophilia and hepatic infiltration with eosinophilic granulocytes was described in a patient with PSC[17,18]. Even so, in the present study we performed a histological investigation of 27 cases of PSC and did not find evidence for eosinophilic infiltration in this cohort suggesting a rather easy differentiation of these entities in western patients by histopathological investigation.

To interpret the data of this study it has to be considered that our study was designed retrospectively and monocentric including mostly Caucasian patients. Moreover, Frankfurt University Hospital is a tertiary referral center, which might have raised the number of patients with inconclusive findings.

In conclusion, the findings of this study suggest that to date, EC is most likely an underdiagnosed etiology of SC, which is important to have in mind in patients presenting with IBS.

ACKNOWLEDGMENTS

The excellent technical assistance of Yvonne Michel, Katharina Sandkühler and Damaris Zaubzer is greatly acknowledged.

COMMENTS

Background

Differentiation of benign and malignant biliary stenosis in indeterminate biliary stricture (IBS) is a common interdisciplinary challenge. Data on eosinophilic cholangitis as a potential cause of IBS are lacking.

Research frontiers

This study represents the first retrospective analysis on prevalence of eosinophilic cholangitis.

Innovations and breakthroughs

This study revealed eosinophilic cholangitis as a most likely underdiagnosed differential diagnosis of sclerosing cholangitis.

Applications

This study suggests that eosinophilic cholangitis has to be kept in mind in patients presenting with IBS. In suspicious cases, multiple biopsies should be

obtained and cholangioscopy should be considered.

Terminology

Eosinophil granulocytes are a subgroup of leukocytes. IgG4 is a subgroup of immunoglobulin G which represents the majority of serum antibodies. Infiltration of the bile duct with eosinophilic granulocytes or IgG4-positive plasma cells can lead to sclerosing cholangitis and thereby mimic biliary cancer.

Peer-review

Excellent effort in contributing important information on the challenging problem of IBS. As the authors learn more about EC and similar entities from contributions such as this the authors will be able to offer more specific treatment and avoid surgery that is not needed.

REFERENCES

1 **Erdogan D**, Kloek JJ, ten Kate FJ, Rauws EA, Busch OR, Gouma DJ, van Gulik TM. Immunoglobulin G4-related sclerosing cholangitis in patients resected for presumed malignant bile duct strictures. *Br J Surg* 2008; **95**: 727-734 [PMID: 18418862 DOI: 10.1002/bjs.6057]

2 **Domagk D**, Poremba C, Dietl KH, Senninger N, Heinecke A, Domschke W, Menzel J. Endoscopic transpapillary biopsies and intraductal ultrasonography in the diagnostics of bile duct strictures: a prospective study. *Gut* 2002; **51**: 240-244 [PMID: 12117887 DOI: 10.1136/gut.51.2.240]

3 **Juntermanns B**, Kaiser GM, Reis H, Saner FH, Radunz S, Vernadakis S, Heuer M, Kuehl H, Paul A, Treckmann J. Klatskin-mimicking lesions: still a diagnostical and therapeutical dilemma? *Hepatogastroenterology* 2011; **58**: 265-269 [PMID: 21661379]

4 **Leegaard M**. Eosinophilic cholecystitis. *Acta Chir Scand* 1980; **146**: 295-296 [PMID: 7468056]

5 **Matsumoto N**, Yokoyama K, Nakai K, Yamamoto T, Otani T, Ogawa M, Tanaka N, Iwasaki A, Arakawa Y, Sugitani M. A case of eosinophilic cholangitis: imaging findings of contrast-enhanced ultrasonography, cholangioscopy, and intraductal ultrasonography. *World J Gastroenterol* 2007; **13**: 1995-1997 [PMID: 17461504 DOI: 10.3748/wjg.v13.i13.1995]

6 **Nashed C**, Sakpal SV, Shusharina V, Chamberlain RS. Eosinophilic cholangitis and cholangiopathy: a sheep in wolves clothing. *HPB Surg* 2010; **2010**: 906496 [PMID: 21076681 DOI: 10.1155/2010/906496]

7 **Kavitt RT**, Hirano I, Vaezi MF. Diagnosis and Treatment of Eosinophilic Esophagitis in Adults. *Am J Med* 2016; **129**: 924-934 [PMID: 27155108 DOI: 10.1016/j.amjmed.2016.04.024]

8 **Walter D**, Peveling-Oberhag J, Schulze F, Bon D, Zeuzem S, Friedrich-Rust M, Albert JG. Intraductal biopsies in indeterminate biliary stricture: Evaluation of histopathological criteria in fluoroscopy- vs. cholangioscopy guided technique. *Dig Liver Dis* 2016; **48**: 765-770 [PMID: 27067926 DOI: 10.1016/j.dld.2016.03.013]

9 **Nielsen JA**, Lager DJ, Lewin M, Rendon G, Roberts CA. The optimal number of biopsy fragments to establish a morphologic diagnosis of eosinophilic esophagitis. *Am J Gastroenterol* 2014; **109**: 515-520 [PMID: 24445569 DOI: 10.1038/ajg.2013.463]

10 **Schoonbroodt D**, Horsmans Y, Laka A, Geubel AP, Hoang P. Eosinophilic gastroenteritis presenting with colitis and cholangitis. *Dig Dis Sci* 1995; **40**: 308-314 [PMID: 7851195 DOI: 10.1007/BF02065415]

11 **Jimenez-Saenz M**, Villar-Rodriguez JL, Torres Y, Carmona I, Salas-Herrero E, Gonzalez-Vilches J, Herrerias-Gutierrez JM. Biliary tract disease: a rare manifestation of eosinophilic gastroenteritis. *Dig Dis Sci* 2003; **48**: 624-627 [PMID: 12757181 DOI: 10.1023/A:1022521707420]

12 **Sussman DA**, Bejarano PA, Regev A. Eosinophilic cholangiopathy with concurrent eosinophilic colitis in a patient with idiopathic hypereosinophilic syndrome. *Eur J Gastroenterol Hepatol* 2008; **20**: 574-577 [PMID: 18467919 DOI: 10.1097/MEG.0b013e3282f1cc11]

13 **Walter D**, Hartmann S, Albert JG. Indeterminate biliary stricture with suspicion for malignancy unmasked as eosinophilic cholangitis by cholangioscopy. *Gastrointest Endosc* 2017; **85**: 265-266 [PMID: 26902844 DOI: 10.1016/j.gie.2016.02.019]

14 **Hubers LM**, Maillette de Buy Wenniger LJ, Doorenspleet ME, Klarenbeek PL, Verheij J, Rauws EA, van Gulik TM, Oude Elferink RP, van de Graaf SF, de Vries N, Beuers U. IgG4-associated cholangitis: a comprehensive review. *Clin Rev Allergy Immunol* 2015; **48**: 198-206 [PMID: 24958363 DOI: 10.1007/ s12016-014-8430-2]

15 **Joshi D**, Webster GJ. Biliary and hepatic involvement in IgG4-related disease. *Aliment Pharmacol Ther* 2014; **40**: 1251-1261 [PMID: 25312536 DOI: 10.1111/apt.12988]

16 **Clayton F**, Fang JC, Gleich GJ, Lucendo AJ, Olalla JM, Vinson LA, Lowichik A, Chen X, Emerson L, Cox K, O'Gorman MA, Peterson KA. Eosinophilic esophagitis in adults is associated with IgG4 and not mediated by IgE. *Gastroenterology* 2014; **147**: 602-609 [PMID: 24907494 DOI: 10.1053/j.gastro.2014.05.036]

17 **Takikawa H**, Manabe T. Primary sclerosing cholangitis in Japan--analysis of 192 cases. *J Gastroenterol* 1997; **32**: 134-137 [PMID: 9058310 DOI: 10.1007/BF01213311]

18 **Watanabe H**, Ohira H, Kuroda M, Takagi T, Ishikawa H, Nishimaki T, Kasukawa R, Takahashi K. Primary sclerosing cholangitis with marked eosinophilic infiltration in the liver. *J Gastroenterol* 1995; **30**: 524-528 [PMID: 7550866 DOI: 10.1007/BF02347572]

P- Reviewer: Hokuto D, Jacob H, Peng SY **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Liu WX

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v23.i6.1051

World J Gastroenterol 2017 February 14; 23(6): 1051-1058
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Retrospective Study

Clinical impact of surveillance for head and neck cancer in patients with esophageal squamous cell carcinoma

Hiroyuki Morimoto, Tomonori Yano, Yusuke Yoda, Yasuhiro Oono, Hiroaki Ikematsu, Ryuichi Hayashi, Atsushi Ohtsu, Kazuhiro Kaneko

Hiroyuki Morimoto, Tomonori Yano, Yusuke Yoda, Yasuhiro Oono, Hiroaki Ikematsu, Kazuhiro Kaneko, Department of Gastroenterology and Endoscopy, National Cancer Center Hospital East, Kashiwa, Chiba 277-8577, Japan

Hiroyuki Morimoto, Atsushi Ohtsu, Course of Advanced Clinical Research of Cancer, Juntendo University Graduate School of Medicine, Kashiwa, Chiba 277-8577, Japan

Ryuichi Hayashi, Department of Head and Neck Surgery, National Cancer Center Hospital East, Kashiwa, Chiba 277-8577, Japan

Atsushi Ohtsu, National Cancer Center Hospital East, Kashiwa, Chiba 277-8577, Japan

Kazuhiro Kaneko, Division of Science and Technology for Endoscopy, National Cancer Center Hospital East, Kashiwa, Chiba 277-8577, Japan

Author contributions: Morimoto H and Yano T contributed equally to this work; Morimoto H and Yano T designed the research; Morimoto H, Yano T, Yoda Y, Oono Y, Ikematsu H and Kaneko K acquired data and performed the research; Morimoto H, Yano T and Kaneko K wrote the draft; Hayashi R and Ohtsu A supervised the report.

Institutional review board statement: This study was reviewed and approved by National Cancer Center Institutional Review Board (2012-331).

Informed consent statement: All study participated patients provided written informed consent before enrollment and any intervention and examination in this study. For full disclosure, the details of the study are published on the home page of National Cancer Center.

Conflict-of-interest statement: We have no financial relationships to disclose.

Data sharing statement: No additional data are available.

Manuscript source: Invited manuscript

Correspondence to: Tomonori Yano, MD, Chief, Department of Gastroenterology and Endoscopy, National Cancer Center Hospital East, Kashiwanoha 6-5-1, Kashiwa, Chiba 277-8577, Japan. toyano@east.ncc.go.jp
Telephone: +81-4-71331111
Fax: +81-4-71314724

Received: September 13, 2016
Peer-review started: September 16, 2016
First decision: October 28, 2016
Revised: November 24, 2016
Accepted: January 18, 2017
Article in press: January 18, 2017
Published online: February 14, 2017

Abstract

AIM

To evaluate the clinical impact of surveillance for head and neck (HN) region with narrow band imaging (NBI) in patients with esophageal squamous cell carcinoma (ESCC).

METHODS

Since 2006, we introduced the surveillance for HN region using NBI for all patients with ESCC before

treatment, and each follow-up. The patients with newly diagnosed stage Ⅰ to Ⅲ ESCC were enrolled and classified into two groups as follows: Group A (no surveillance for HN region); between 1992 and 2000), and Group B (surveillance for HN region with NBI; between 2006 and 2008). We comparatively evaluated the detection rate of superficial head and neck squamous cell carcinoma (HNSCC), and the serious events due to metachronous advanced HNSCC during the follow-up.

RESULTS

A total 561 patients (group A: 254, group B: 307) were enrolled. Synchronous superficial HNSCC was detected in 1 patient (0.3%) in group A, and in 12 (3.9%) in group B (P = 0.008). During the follow up period, metachronous HNSCC were detected in 10 patients (3.9%) in group A and in 30 patients (9.8%) in group B (P = 0.008). All metachronous lesions in group B were early stage, and 26 patients underwent local resection, however, 6 of 10 patients (60%) in group A lost their laryngeal function and died with metachronous HNSCC.

CONCLUSION

Surveillance for the HN region by using NBI endoscopy increase the detection rate of early HNSCC in patients with ESCC, and led to decrease serious events related to advanced metachronous HNSCC.

Key words: Esophageal squamous cell carcinoma; Head and neck squamous cell carcinoma; Narrow band imaging; Endoscopic resection; Surveillance; Metachronous cancer

Core tip: This is a retrospective study to evaluate the clinical impact of intensive surveillance for head and neck (HN) region by using narrow band imaging (NBI) endoscopy in patients with esophageal squamous cell carcinoma. The detection rate of superficial head and neck squamous cell carcinoma (HNSCC) which could be easily treated with endoscopic resection was dramatically increased after introduction of surveillance for HN region with NBI, and the serious events (loss of laryngeal function, death) due to metachronous advanced HNSCC were led to decrease when comparing with historical control. Surveillance for HN region with NBI might have a clinical impact at the point of reduction of head and neck cancer death in esophageal cancer survivor.

Morimoto H, Yano T, Yoda Y, Oono Y, Ikematsu H, Hayashi R, Ohtsu A, Kaneko K. Clinical impact of surveillance for head and neck cancer in patients with esophageal squamous cell carcinoma. *World J Gastroenterol* 2017; 23(6): 1051-1058 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i6/1051.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i6.1051

INTRODUCTION

Most of patients with esophageal squamous cell carcinoma (ESCC) have a high prevalence of second primary head and neck squamous cell carcinoma (HNSCC)[1]. Matsubara *et al*[2] reported an assessment of the risk of a second primary cancer in patients with ESCC undergoing esophagectomy. In that report, HNSCC was the highest risk after esophagectomy and the prognosis after the detection of HNSCC was significantly unfavorable compared to that of other malignancies because of the difficulty in the early detection of HNSCC. An image-enhanced endoscopic technology system, narrow-band imaging (NBI), was reported to be useful for the detection of superficial HNSCC[3-6]. These superficial lesions are depicted clearly as well-demarcated brownish areas without magnification, and increased intraepithelial papillary capillary loops (IPCL) with irregularity are visible as the endoscopic features of superficial HNSCC with magnification[3]. Muto *et al*[3] reported that both detection rate and diagnostic accuracy of HNSCC were higher in NBI than in white light imaging. Furthermore, several studies have also reported that minimally invasive treatment, such as peroral endoscopic resection (ER) of superficial pharyngeal cancer, was a feasible and effective treatment with curative intent[7-9].

At the beginning of 2006, we introduced intensive surveillance program for the head and neck (HN) region, including the oropharynx, hypopharynx, and larynx, using NBI for all ESCC patients before treatment and at every follow up visit. If early HNSCCs were detected, these lesions were mainly treated with ER after the confirmation of cured esophageal cancer. However, it has not been yet clarified whether prompt detection and intervention for early HNSCC in patients with ESCC would decrease the death rate or the loss of laryngeal function related to metachronous advanced HNSCC. In this study, we compared the detection rate of early HNSCC, and the number of serious adverse events related to metachronous advanced HNSCC, in periods before and after the commencement of NBI surveillance for the head and neck region.

MATERIALS AND METHODS

Patients

Patients were recruited from our database of patients who have received definitive treatments, such as ER, surgery and chemoradiotherapy (CRT), for ESCC in the National Cancer Center Hospital East. Selection criteria of this study were as follows: (1) initial treatment histologically confirmed ESCC; (2) clinical stage Ⅰ to Ⅲ; (3) no prior HNSCC; (4) absence of synchronous advanced cancer containing HNSCC; (5) no recurrence or metastasis of ESCC detected within 6 mo after initial ER or surgery for ESCC; (6) complete response (CR) was achieved and a recurrence after achieving

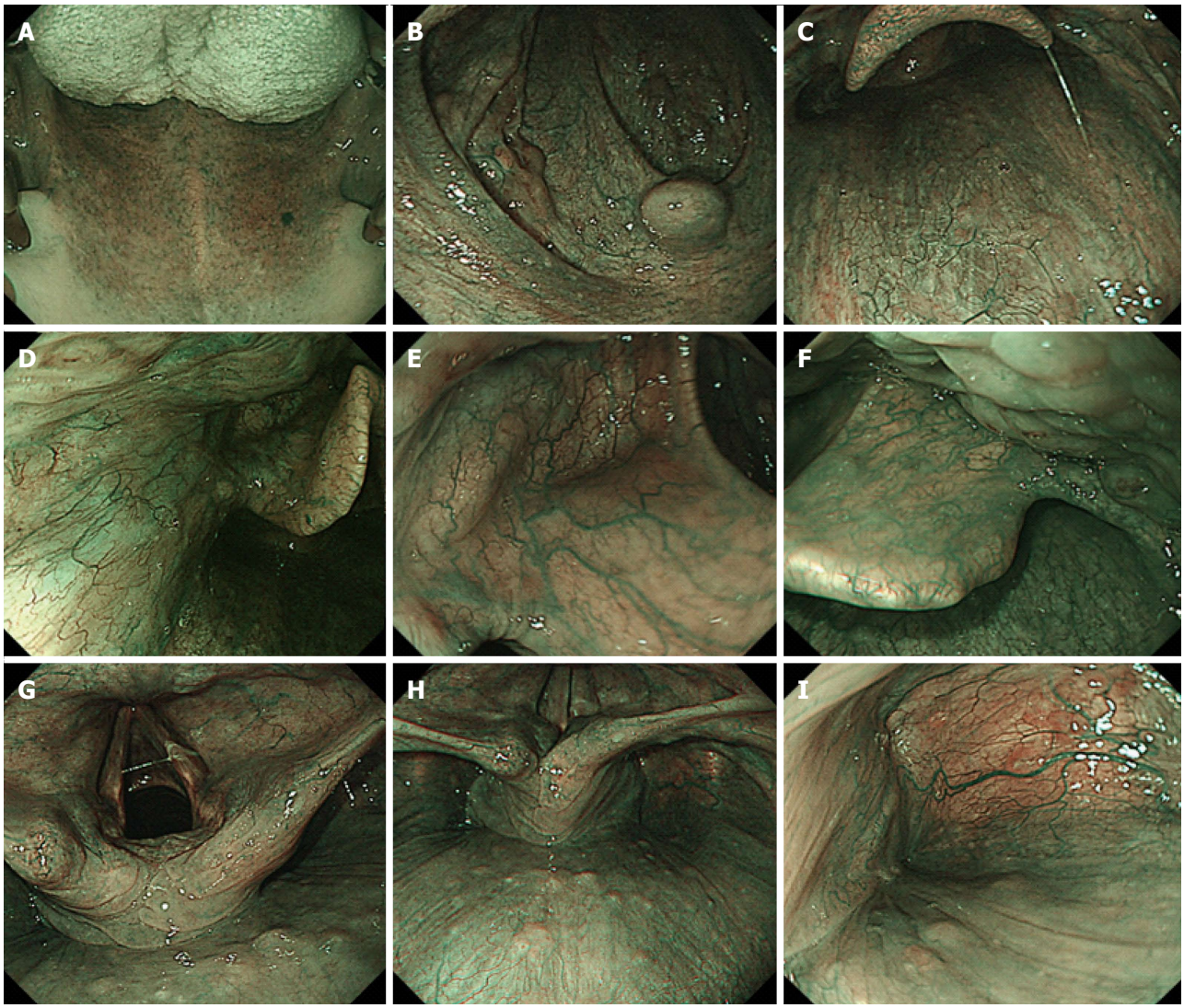

Figure 1 Narrow Band Imaging observations in individual regions from the oropharynx to the pharynx. A : The view seen from the entrance of the oral cavity: dorsal side of tongue, hard palate and soft palate; B: Uvula, palatoglossal arch and lateral walls of oropharynx; C: Posterior wall of oropharynx; D: The lateral wall of oropharynx; E: Vallecula of epiglottis, median glossoepiglottic fold; F: The right side of epiglottis, base of tongue; G: Vocal cord, arytenoids and aryepiglottic fold; H: Post-cricoid area and posterior wall of hypopharynx; I: The lateral wall and apex of right piriform sinus.

CR or metastasis was not detected within 6 mo of CRT; (7) an observation period longer than a year after treatment for ESCC; and (8) provided written informed consent before all endoscopic evaluation and treatment.

Two groups were classified between the periods before and after commencement of surveillance for the HN region. ESCC patients who were treated between October 1992 and December 2000, with a follow-up until December 2004 were defined as a control group (group A). During this period, the HN region was not intensively observed to detect superficial lesion with endoscopy before treatment or during follow up periods. Group B was defined patients who were treated between January 2006 and December 2008 and followed until December 2014. These patients in group B routinely received intensive surveillance using magnified NBI endoscopy in the HN region.

Endoscopic examination and follow-up schedule

Since the patients in group A underwent conventional endoscopy with white light illumination, intensive surveillance to detect superficial HNSCC was not performed actively. In contrast, the HN regions including the oropharynx, hypopharynx and larynx, were observed using endoscope equipped with a NBI system (Olympus Medical Science, Tokyo, Japan) in group B. The endoscopic observation order in the HN region using NBI endoscopy in our hospital is shown in Figure 1[10].

The follow-up schedule of endoscopic observation after the treatment for esophageal cancer was as follows. Patients who received CRT were evaluated every 6 mo after achieving CR. The initial examination was performed at 3 mo in patients after ER, and every 6 mo thereafter, and an annual examination was performed in patients after esophagectomy.

Table 1 Characteristics of patients with esophageal squamous cell carcinoma *n* (%)

	Group A (*n* = 254)	Group B (*n* = 307)	*P* value
Sex			
Male	214 (84)	260 (85)	0.907
Female	40 (16)	47 (15)	
Age (yr), median (range)	64 (39-81)	66 (41-86)	< 0.001
Baseline clinical TNM-stage			
Ⅰ	119 (47)	155 (50)	0.39
Ⅱ	66 (26)	88 (29)	0.50
Ⅲ	69 (27)	64 (21)	0.09
Treatment for primary ESCC			
Surgery	84 (33)	124 (40)	0.079
Endoscopic resection	77 (30)	113 (37)	0.010
Chemoradiotherapy	93 (37)	70 (23)	0.001
Follow-up period			
Median months (range)	60 (13-145)	67 (12-107)	0.150

ESCC: Esophageal squamous cell carcinoma.

Pathological evaluation of HNSCC

The histologic diagnosis was made according to criteria proposed by World Health Organization[11]. Clinical staging was determined according to the Japan Society for Head and Neck Cancer, same as the TNM classification 7th edition. Superficial cancers without lymph node or distant metastasis were defined as early cancer and cancers invading muscularis propria and deeper layers were defined as advanced cancer.

Treatment strategy and details of treatment for HNSCCs

Treatment for early HNSCC was provided after initial treatment for ESCC was completed. However, second primary HNSCC was not treated if the ESCC was not cured, because there was few possibility that the treatment for HNSCC affected the prognosis.

Endoscopic resection for HNSCC under general anesthesia was introduced in our hospital at the beginning of 2003. Subsequently, early HNSCC was mainly treated with ER after the confirmation of cured ESCC. When a lesion was small (approximately 10 mm in diameter or less), endoscopic mucosal resection with the cap technique was performed[8,12-14], and endoscopic submucosal dissection (ESD) was performed for larger lesions (over 10 mm in diameter)[7,9]. The procedure of ESD was as follows. A videoendoscope with a water jet system (JIFQ-260J, Olympus, Tokyo, Japan) was used for the entire procedure. And then 7 mL to 10 mL of 2.0% glycerin-free Lugol iodine solution, consisting of 2.0 g potassium iodine and 4.0 g iodine in 100 mL distilled water was sprayed to delineate the margins of the lesion. Markings were placed outside the margin of the lesion with a dual knife (Olympus KD-650L) and an electrosurgical current generator (ICC200, Erbe, Tübingen, Germany) set at 25 W for forced coagulation mode. A saline solution with epinephrine and indigo carmine dye was injected into the subepithelial layer. A circumferential incision around the lesion was performed and then the subepithelial tissue was dissected by the dual knife with 50 W current for forced coagulation mode. After the lesion was resected, a temporary tracheostomy was performed by a head and neck surgeon if laryngeal edema was severe.

Analysis and statistics

The detection rate of superficial HNSCC was evaluated for each group. The incidence rate of metachronous HNSCC and serious events related to metachronous HNSCC, such as death or loss of laryngeal function, were also evaluated.

All information was collected from the medical records or was provided by the patients' physicians. This retrospective study was approved by the institutional review board of the National Cancer Center in accordance with the Declaration of Helsinki.

SPSS Statistics 22 was used for statistical analysis. The results were expressed as medians. The Fisher's exact test was used to analyze categorical data to compare proportions. Risks of metachronous HNSCC were estimated by using the Kaplan-Meier method. *P* value of < 0.05 was considered statistically significant.

RESULTS

Patient characteristics

A total of 470 patients with stage Ⅰ to Ⅲ ESCC were initially treated with definitive treatments (ER: 125, surgery: 119, CRT: 173) between October 1992 and December 2000 as group A, whereas 443 patients with stage Ⅰ to Ⅲ ESCC were initially treated (ER: 159, surgery: 161, CRT: 123) between January 2006 and December 2008 as group B. Patients consisting of 254 in group A and 307 in group B were recruited in this study according to the eligibility criteria. The characteristics of these patients are shown in Table 1. The male-to-female ratio, clinical stage of ESCC, and the follow up period were not significantly different between group A and group B, however, median age was significantly higher in patients of group B than in patients of group A. There was a significant difference in a treatment for ESCC in both groups (P = 0.025): the frequency of CRT were higher in group A (group A: 37%, group B: 23%).

Synchronous HNSCC

Synchronous superficial HNSCC was detected in only 1 patient (0.3%) in group A. In contrast, the synchronous superficial HNSCC was found in 12 (3.9%) patients in group B (P = 0.008) (Table 2). Among these all 13 patients, 9 patients (69%) were cured of ESCC and 7 of the 9 patients with synchronous HNSCC were treated after the treatment for ESCC. In these 7 patients who were treated for HNSCC, 5 patients underwent organ preserved local resection (ER or surgery). One patient with hypopharyngeal cancer in

Table 2 Synchronous superficial head and neck squamous cell carcinoma lesions *n* (%)

	Group A (*n* = 254)	Group B (*n* = 307)	*P* value
Synchronous HNSCC			
No. of patients	1 (0.3)	12 (3.9)	0.008
No. of lesions	1	14	0.010
Location of cancer			
Oropharynx	0	5	
Hypopharynx	1	8	
Larynx	0	1	
Treatment for synchronous HNSCC			
ER or surgical local resection	0	7 (58)	
TPLE	0	1 (8)	
Radiation and/or chemotherapy	1 (100)	0	
No treatment	0	4 (33)	
Death due to synchronous HNSCC	0	0	

ER: Endoscopic resection; TPLE: Total pharyngo-laryngo-esophagectomy; HNSCC: Head and neck squamous cell carcinoma.

Table 3 Characteristics of metachronous head and neck squamous cell carcinoma *n* (%)

	Group A (*n* = 254)	Group B (*n* = 307)	*P* value
Metachronous HNSCC			
No. of patients	10 (3.9)	30 (9.8)	0.008
No. of lesions per patients			0.404
1	9	22	
⩾ 2	1	8	
Total number of cancers	11	53	0.007
Location of cancer			
Oropharynx	3	13	
Hypopharynx	7	34	
Larynx	1	6	
Clinical stage			< 0.001
Ⅰ / Ⅱ	4 (36)	53 (100)	
Ⅲ / Ⅳ	7 (64)	0	
Interval between ESCC and HNSCC			
Median months (range)	56 (7-80)	31 (7-107)	0.130

HNSCC: Head and neck squamous cell carcinoma.

group A underwent radiotherapy and 1 patient with hypopharyngeal cancer in group B underwent total pharyngo-laryngo-esophagectomy (TPLE) because the tumor was located in a position where treatment to preserve laryngeal function was impossible. The remaining 4 patients did not receive any treatment for synchronous HNSCC because their ESCC was not cured. Most of the patients who were cured of ESCC and received treatment for superficial HNSCC had preserved laryngeal function. No patient died due to synchronous HNSCC in both groups.

Metachronous HNSCC

Metachronous HNSCC lesions were detected in 10 patients (3.9%) in group A and in 30 patients (9.8%) in group B (P = 0.008; Table 3). The cumulative risk of metachronous HNSCC after treatment of ESCC is shown in Figure 2. The 5-year cumulative risk of developing metachronous HNSCC after treatment for ESCC was only 2.5% in group A, whereas it was 8.7% in group B (P < 0.001).

The characteristics of metachronous HNSCCs are shown in Table 3. Eleven metachronous HNSCC lesions were detected in 10 patients in group A, and 53 lesions in 30 patients in group B (P = 0.008). In the clinical stages of metachronous HNSCC, only 4 (36%) lesions were superficial type and stage Ⅰ / Ⅱ in group A, however, all 53 lesions were superficial lesions in group B (P < 0.001), and these lesions were stage Ⅰ / Ⅱ.

Table 4 Clinical course of patients with metachronous head and neck squamous cell carcinoma *n* (%)

	Group A, patients (*n* = 10)	Group B, patients (*n* = 30)	*P* value
Metachronous HNSCC	11 lesions	53 lesions	< 0.001
Ⅰ / Ⅱ	4 (36)	53 (100)	
Ⅲ / Ⅳ	7 (64)	0 (0)	
Treatment			
Local resection	2 (18)	49 (92)	< 0.001
Endoscopic resection	0	44	
Surgical local resection	2	5	
TPLE	3 (27)	0 (0)	0.001
Radiotherapy alone and/or chemotherapy	4 (36)	2 (4)	0.006
No treatment	2 (18)	2 (4)	0.133
Laryngeal function			< 0.001
Maintained	4 (40)	30 (100)	
Lost	6 (60)	0	
Outcome			0.001
Alive	3 (30)	26 (87)	
Death	7 (70)	4 (13)	
Death with metachronous HNSCC	6 (60)	0 (0)	< 0.001

HNSCC: Head and neck squamous cell carcinoma; TPLE: Total pharyngo-laryngo-esophagectomy.

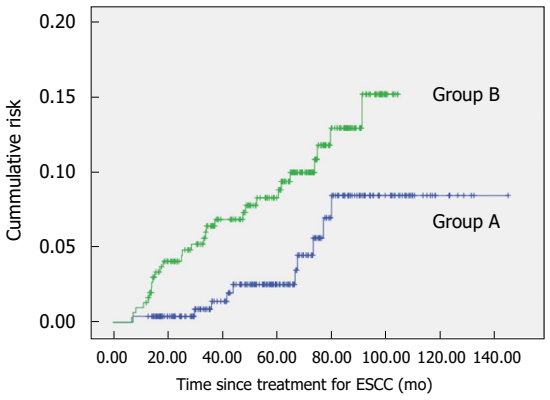

Figure 2 Cumulative risk of metachronous head and neck squamous cell carcinoma after treatment of esophageal squamous cell carcinoma. ESCC: Esophageal squamous cell carcinoma.

Clinical course of patients with metachronous HNSCC

The clinical course of patients with metachronous HNSCC is shown in Table 4. There were no patients in group A who underwent ER as an initial therapy. Of 10 patients in group A, 7 (70%) who were detected

Table 5 Clinical outcome of all patients with esophageal squamous cell carcinoma *n* (%)

	Group A (*n* = 254)	Group B (*n* = 307)	*P* value
Occurrence of advanced metachronous HNSCC	7 (2.8)	0	0.003
Loss of laryngeal function	6 (2.4)	0	0.008
Outcome			< 0.001
Alive	172 (68)	254 (83)	
Dead	82 (32)	53 (17)	
ESCC	43 (17)	41 (13)	0.284
HNSCC	6 (2.4)	0	0.018
Other cancer	6 (2.4)	4 (1.3)	0.360
Gastric cancer	3 (1.2)	0	0.092
Lung cancer	0	2 (0.7)	0.0503
Lymphoma	1 (0.4)	1 (0.3)	> 0.999
HCC	1 (0.4)	1 (0.3)	> 0.999
Prostate cancer	1 (0.4)	0	0.452
Other/unknown	27 (11)	8 (2.6)	< 0.0001
Radiation pneumonia	8 (8.1)	1 (0.3)	0.013
Heart failure	6 (2.4)	1 (0.3)	0.050

HNSCC: Head and neck squamous cell carcinoma; ESCC: Esophageal squamous cell carcinoma; HCC: Hepatocellular carcinoma.

metachronous HNSCC had stage Ⅲ/Ⅳ HNSCC at diagnosis. In these 7 patients, only one patient who received radiotherapy achieved a cure for HNSCC.

In contrast, metachronous HNSCC was found in 30 patients with 53 lesions in group B (Table 4). Furthermore, all the 53 lesions were superficial cancer alone. ER was performed in 44 of the 53 lesions and only 2 of 44 lesions had local recurrence. One of the 2 patients had re-ER and was cured, and another patient has not receive any active treatments for the superficial cancer. There were no patients who developed lymph node or distant metastasis within the observation period. In addition, 2 patients were received RT alone, and the remaining 2 were not treated for HNSCC because their ESCC recurred after the initial treatment for ESCC.

As serious events, the 7 of 10 patients in group A died due to cancer, and 6 of the 7 patients died due to metachronous HNSCC (group A: 60%, group B: 0%. $P < 0.001$; Table 4). Furthermore, 6 of the 10 patients (60%) in group A who were detected in metachronous HNSCC lost laryngeal function due to intensive treatment, otherwise none of the 30 patients with the 53 lesions in group B lost laryngeal function ($P < 0.001$).

Clinical outcome of all patients with ESCC

Clinical outcome is shown in Table 5. A total of 82 (32%) patients in group A and 53 (17%) patients in group B died during the follow up periods. While there was no significant difference in the frequency of deaths due to the progression of ESCC between both groups [group A *vs* B: 43 (17%) *vs* 41 (13%), $P = 0.28$], the deaths related to metachronous HNSCC were more frequent in group A (group A *vs* B: 6 (2.4%) *vs* 0 (0%), $P = 0.008$). In contrast, other noncancerous diseases or unknown sudden death which might be late toxicity of RT for ESCC were more frequent in group A [27 (11%) *vs* 8 (2.6%), $P < 0.001$].

DISCUSSION

This is the first study to investigate the clinical significance of early detection and intervention to second primary HNSCC in ESCC patients. The innovation of NBI has allowed for the early diagnosis of head and neck cancer. The NBI technique could significantly improve the efficacy of screening and surveillance of HN region, especially the lesions at oropharyngeal and hypopharyngeal mucosal sites. In previous reports, NBI screening was undertaken for the HN region (10%-13%) in ESCC patients[3,4]. In this study, we classified into two groups whether intervention of NBI surveillance was present or not, and detection rate of superficial HNSCC was clarified. Few superficial HNSCCs were detected using conventional endoscopy with white light illumination alone, however, many superficial HNSCCs were detected synchronously (3.9%) and metachronously (9.4%) after commencement of NBI surveillance. Furthermore, multiple metachronous HNSCCs were also detected. One of the main reason of lower HNSCC detection rate is considered that we did not perform NBI surveillance with magnifying endoscopy in all cases. One important point was that almost all of metachronous HNSCC could be detected as superficial cancer by NBI surveillance once from six months to one year.

Furthermore, early detection of second primary HNSCC in ESCC patients brought to minimally invasive treatment, such as peroral ER. In this study, ER was performed in 83% of second primary HNSCCs due to NBI surveillance, and these patients did not lose laryngeal function. In contrast, most of the second primary HNSCCs were detected as advanced cancers in no NBI surveillance from 1992 to 2000, 60% of patients lost laryngeal function due to invasive treatment. Several studies have reported that peroral ER of superficial HNSCC is a feasible and effective treatment with curative intent[9,15,16]. Muto *et al*[9] reported that local recurrence or distant metastasis after ER or superficial pharyngeal cancer were only 8% and patients who underwent ER had an excellent prognosis, with a 5-year cause-specific survival rate of 97% (95%CI: 93%-100%). We believe development of ER for cancer of oral cavity would progress along with early detection of superficial HNSCC. Loss of laryngeal function is a serious problem, and decreases quality of life in patients with second primary HNSCC. In contrast, we clarified that second primary advanced HNSCC could become the risk of death if superficial HNSCC was not detected. HNSCC was 2.4% of various death factors in no NBI surveillance,

however, there was no HNSCC related death after NBI surveillance. In this study, while two groups were different periods (1992-2000 *vs* 2006-2008), we compared the detection rate of early HNSCC, and the number of serious adverse events related to metachronous advanced HNSCC, in periods before and after the commencement of NBI surveillance for the head and neck region. We suggested that early detection of metachronous HNSCC in ESCC patients led to minimally invasive ER without loss of laryngeal function, and avoided HNSCC related death. Regarding follow-up periods, NBI surveillance was performed in 6 mo for ER and CRT and in 1 year for operation. In our present results, 5 years have passed through NBI surveillance, however, advanced HNSCC was not detected. We believe that 6 mo follow-up periods would be appropriate.

Limitations of this retrospective study are that the data are taken from only a single institution, and historical background, the medical backgrounds of the ESCC patients, are different in each group. Moreover, it is uncertain whether the approximately 5 years of follow up in the present study is long enough to verify the serious events due to metachronous HNSCC. However, it seems impossible to conduct a randomized control study since the usefulness of endoscopic surveillance with NBI has been demonstrated.

In conclusion, endoscopic surveillance using NBI for the HN region improved detection of both synchronous and metachronous superficial HNSCC in patients with ESCC. The early detection and intervention for HNSCC might lead to the reduction of serious adverse events and the risk of death related to HNSCC.

COMMENTS

Background

Most of patients with esophageal squamous cell carcinoma (ESCC) have a high prevalence of second primary head and neck squamous cell carcinoma (HNSCC). The innovation of narrow-band imaging (NBI) has allowed for the early diagnosis of head and neck cancer. However, it has not been yet clarified whether prompt detection and intervention for early HNSCC in patients with ESCC would decrease the death rate or the serious events related to metachronous advanced HNSCC. In this study, the authors compared the detection rate of early HNSCC, and the number of serious adverse events related to metachronous advanced HNSCC, in periods before and after the commencement of NBI surveillance for the head and neck region.

Research frontiers

NBI is useful to detect the early HNSCC and minimally invasive treatment, such as peroral endoscopic resection (ER) of early HNSCC, is a feasible and effective treatment with curative intent. This results of this study contribute to clarifying the clinical impact of early intervention to metachronous NHSCCs in patient with ESCC.

Innovations and breakthroughs

In this study, many HNSCCs were detected synchronously (3.9%) and metachronously (9.4%) after commencement of NBI surveillance, and all 53 lesions could be detected as early stage. These results are in agreement with previous reports. Minimally invasive treatment (ER) was performed in 83% of these second primary HNSCCs due to NBI surveillance and these patients did not lose laryngeal function or death related to HNSCC. In contrast, most of the second primary HNSCCs were detected as advanced cancers in no NBI surveillance from 1992 to 2000, 60% of patients lost laryngeal function and were died due to invasive treatment.

Applications

This study suggested that early intervention for metachronous HNSCC is useful to reduce the serious adverse events and the risk of death related to HNSCC in patient with ESCC.

Terminology

NBI: A video endoscopic imaging technique that enhances the display of the microstructures and capillaries in the superficial mucosal layer using narrow band filters that change the spectral features of the observation light.

Peer-review

This study investigated the clinical usefulness of surveillance of head and neck cancer in patients with esophageal squamous cell carcinoma. Although the study is retrospectively performed, the results are well analyzed and clearly presented.

REFERENCES

1 **Noguchi T**, Kato T, Takeno S, Wada S, Yanagisawa S, Suzuki M. Necessity of screening for multiple primary cancers in patients with esophageal cancer. *Ann Thorac Cardiovasc Surg* 2002; **8**: 336-342 [PMID: 12517292]

2 **Matsubara T**, Yamada K, Nakagawa A. Risk of second primary malignancy after esophagectomy for squamous cell carcinoma of the thoracic esophagus. *J Clin Oncol* 2003; **21**: 4336-4341 [PMID: 14645422 DOI: 10.1200/JCO.2003.12.074]

3 **Muto M**, Minashi K, Yano T, Saito Y, Oda I, Nonaka S, Omori T, Sugiura H, Goda K, Kaise M, Inoue H, Ishikawa H, Ochiai A, Shimoda T, Watanabe H, Tajiri H, Saito D. Early detection of superficial squamous cell carcinoma in the head and neck region and esophagus by narrow band imaging: a multicenter randomized controlled trial. *J Clin Oncol* 2010; **28**: 1566-1572 [PMID: 20177025 DOI: 10.1200/JCO.2009.25.4680]

4 **Katada C**, Tanabe S, Koizumi W, Higuchi K, Sasaki T, Azuma M, Katada N, Masaki T, Nakayama M, Okamoto M, Muto M. Narrow band imaging for detecting superficial squamous cell carcinoma of the head and neck in patients with esophageal squamous cell carcinoma. *Endoscopy* 2010; **42**: 185-190 [PMID: 20195988 DOI: 10.1055/s-0029-1243963]

5 **Nonaka S**, Saito Y, Oda I, Kozu T, Saito D. Narrow-band imaging endoscopy with magnification is useful for detecting metachronous superficial pharyngeal cancer in patients with esophageal squamous cell carcinoma. *J Gastroenterol Hepatol* 2010; **25**: 264-269 [PMID: 19874445 DOI: 10.1111/j.1440-1746.2009.05993.x]

6 **Piazza C**, Dessouky O, Peretti G, Cocco D, De Benedetto L, Nicolai P. Narrow-band imaging: a new tool for evaluation of head and neck squamous cell carcinomas. Review of the literature. *Acta Otorhinolaryngol Ital* 2008; **28**: 49-54 [PMID: 18669067]

7 **Iizuka T**, Kikuchi D, Hoteya S, Yahagi N, Takeda H. Endoscopic submucosal dissection for treatment of mesopharyngeal and hypopharyngeal carcinomas. *Endoscopy* 2009; **41**: 113-117 [PMID: 19214888 DOI: 10.1055/s-0028-1119453]

8 **Suzuki H**, Saito Y, Oda I, Nonaka S, Nakanishi Y. Feasibility of endoscopic mucosal resection for superficial pharyngeal cancer: a minimally invasive treatment. *Endoscopy* 2010; **42**: 1-7 [PMID: 20066588 DOI: 10.1055/s-0029-1243807]

9 **Muto M**, Satake H, Yano T, Minashi K, Hayashi R, Fujii S, Ochiai A, Ohtsu A, Morita S, Horimatsu T, Ezoe Y, Miyamoto S, Asato R, Tateya I, Yoshizawa A, Chiba T. Long-term outcome of transoral organ-preserving pharyngeal endoscopic resection for superficial pharyngeal cancer. *Gastrointest Endosc* 2011; **74**: 477-484 [PMID: 21704994 DOI: 10.1016/j.gie.2011.04.027]

10 **Kaneko K**, Yano T, Minashi K, Kojima T, Ito M, Satake H, Yajima Y, Yoda Y, Ikematsu H, Oono Y, Hayashi R, Onozawa M, Ohtsu A. Treatment strategy for superficial pharyngeal squamous cell carcinoma

synchronously combined with esophageal cancer. *Oncology* 2013; **84**: 57-64 [PMID: 23128894 DOI: 10.1159/0003 37981]
11 **Barnes D**, Eveson JW, Reichart P. World Health Organization Classification of Tumors. Pathology and genetics. Head and Neck tumors. Lyon: IARC Press, 2005
12 Japan Society for Head and Neck Cancer, General rules for clinical studies on head and neck cancer. Tokyo: Kanehara, 2005
13 **Inoue H**, Endo M, Takeshita K, Yoshino K, Muraoka Y, Yoneshima H. A new simplified technique of endoscopic esophageal mucosal resection using a cap-fitted panendoscope (EMRC). *Surg Endosc* 1992; **6**: 264-265 [PMID: 1465738]
14 **Inoue H**, Tani M, Nagai K, Kawano T, Takeshita K, Endo M, Iwai T. Treatment of esophageal and gastric tumors. *Endoscopy* 1999; **31**: 47-55 [PMID: 10082409 DOI: 10.1055/s-1999-13647]
15 **Muto M**, Nakane M, Katada C, Sano Y, Ohtsu A, Esumi H, Ebihara S, Yoshida S. Squamous cell carcinoma in situ at oropharyngeal and hypopharyngeal mucosal sites. *Cancer* 2004; **101**: 1375-1381 [PMID: 15368325 DOI: 10.1002/cncr.20482]
16 **Satake H**, Yano T, Muto M, Minashi K, Yoda Y, Kojima T, Oono Y, Ikematsu H, Aoyama I, Morita S, Miyamoto S, Fujii S, Yoshizawa A, Ochiai A, Hayashi R, Kaneko K. Clinical outcome after endoscopic resection for superficial pharyngeal squamous cell carcinoma invading the subepithelial layer. *Endoscopy* 2015; **47**: 11-18 [PMID: 25268310 DOI: 10.1055/s-0034-1378107]

P- Reviewer: Iijima K, Li CJ **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v23.i6.1059

World J Gastroenterol 2017 February 14; 23(6): 1059-1066
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Retrospective Study

Second-line bismuth-containing quadruple therapy for *Helicobacter pylori* eradication and impact of diabetes

Sung Eun Kim, Moo In Park, Seun Ja Park, Won Moon, Jae Hyun Kim, Kyoungwon Jung, Hae Koo Kim, Young Dal Lee

Sung Eun Kim, Moo In Park, Seun Ja Park, Won Moon, Jae Hyun Kim, Kyoungwon Jung, Hae Koo Kim, Young Dal Lee, Department of Internal Medicine, Kosin University College of Medicine, Busan 49267, South Korea

Author contributions: Kim SE interpreted and analyzed the data and wrote the manuscript; Park MI designed, organized, and supervised writing of the manuscript; Park SJ and Moon W helped with data interpretation that was used in the current study; Kim JH and Jung K provided input and organized the data for statistical analysis; Kim HK and Lee YD helped with data analysis; all authors approved the final version of the manuscript.

Institutional review board statement: The study was reviewed and approved by the Kosin University Gospel Hospital Institutional Review Board (IRB file No. 2015-03-018).

Conflict-of-interest statement: We have no conflicts of interest regarding the current paper.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Correspondence to: Moo In Park, MD, Professor, Department of Internal Medicine, Kosin University College of Medicine, 262 Gamcheon-ro, Seo-gu, Busan 49267, South Korea. mipark@kosinmed.or.kr
Telephone: +82-51-9906719
Fax: +82-51-9905055

Received: October 17, 2016
Peer-review started: October 18, 2016
First decision: October 28, 2016
Revised: November 21, 2016
Accepted: January 18, 2017
Article in press: January 18, 2017
Published online: February 14, 2017

Abstract

AIM

To investigate *Helicobacter pylori* (*H. pylori*) eradication rates using second-line bismuth-containing quadruple therapy and to identify predictors of eradication failure.

METHODS

This study included 636 patients who failed first-line triple therapy and received 7 d of bismuth-containing quadruple therapy between January 2005 and December 2015. We retrospectively demonstrated *H. pylori* eradication rates with respect to the year of therapy as well as demographic and clinical factors. *H. pylori* eradication was confirmed by a ^{13}C-urea breath test or a rapid urease test at least 4 wk after the completion of bismuth-based quadruple therapy: proton pump inhibitor, metronidazole, bismuth, and tetracycline.

RESULTS

The overall eradication rates by intention-to-treat analysis and per-protocol analysis were 73.9% (95%CI: 70.1%-77.4%) and 94.5% (95%CI: 92.4%-96.5%), respectively. Annual eradication rates from 2005 to 2015 were 100.0%, 92.9%, 100.0%, 100.0%, 100.0%, 97.4%, 100.0%, 93.8%, 84.4%, 98.9%, and 92.5%, respectively, by per-protocol analysis. A multivariate analysis showed that diabetes mellitus (OR = 3.99, 95%CI: 1.56-10.20, P = 0.004)

was associated with *H. pylori* eradication therapy failure.

CONCLUSION

The second-line bismuth-containing quadruple therapy for *H. pylori* infection is still effective in Korea, and diabetes mellitus is suggested to be a risk factor for eradication failure.

Key words: *Helicobacter pylori*; Disease eradication; Treatment failure; Bismuth; Diabetes mellitus

Core tip: This study investigated the efficacy of 7 d of second-line bismuth-containing quadruple therapy for *Helicobacter pylori* (*H. pylori*) infection and identified risk factors for eradication failure in South Korea. The overall eradication rate per-protocol analysis was 94.5% in the current study. Additionally, diabetes mellitus was related to *H. pylori* eradication therapy failure. Therefore, second-line bismuth-containing quadruple therapy for *H. pylori* infection is still worth considering in South Korea, and diabetes mellitus is suggested to be a risk factor for eradication failure.

Kim SE, Park MI, Park SJ, Moon W, Kim JH, Jung K, Kim HK, Lee YD. Second-line bismuth-containing quadruple therapy for *Helicobacter pylori* eradication and impact of diabetes. *World J Gastroenterol* 2017; 23(6): 1059-1066 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i6/1059.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i6.1059

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a global pathogen that causes gastritis, peptic ulcers, mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric cancer[1]. The International Agency for Research on Cancer, a branch of the World Health Organization, has declared that *H. pylori* is a definite gastric carcinogen (group I)[2,3]. Therefore, *H. pylori* eradication is crucial to maintain public health, especially in high *H. pylori* and gastric cancer prevalence areas.

Various combination therapies are recommended for *H. pylori* eradication due to a decrease in eradication rates. According to the Maastricht IV/Florence consensus report, clarithromycin-containing therapy [comprised of a proton pump inhibitor (PPI), amoxicillin, and clarithromycin] is recommended for first-line eradication treatment, and bismuth-containing quadruple therapy (comprised of a PPI, metronidazole, bismuth, and tetracycline) is recommended for second-line eradication treatment if first-line eradication therapy fails[4]. Guidelines for the treatment of *H. pylori* infection in South Korea are similar to recommendations in the Maastricht IV/Florence consensus report. Specifically, clarithromycin-containing triple therapy is the recommended first-line eradication therapy, and bismuth-containing quadruple therapy is recommended for the second-line eradication treatment if the clarithromycin-based triple therapy fails[5].

In general, clarithromycin-containing therapy is recommended for first-line eradication treatment in low (< 20%) clarithromycin resistance areas[4]. However, the eradication rates for clarithromycin-containing triple therapy have been decreasing significantly in Korea in recent years due to increased *H. pylori* antibiotic resistance[6,7]. In addition, there is controversy about the role of bismuth-containing quadruple therapy as a second-line therapy for *H. pylori* eradication due to a decrease in eradication rates for bismuth-containing quadruple therapy in Korea[8,9].

The aims of the present study were to identify the effects of second-line eradication therapy using bismuth-containing quadruple therapy at a single center over the past 11 years, and to evaluate risk factors associated with the failure of second-line eradication therapy.

MATERIALS AND METHODS

Study population

Patients who failed clarithromycin-containing triple therapy and received second-line bismuth-containing quadruple therapy at Kosin University Gospel Hospital from January 2005 to December 2015 were retrospectively enrolled in this study. *H. pylori* positivity was identified using a ^{13}C-urea breath test or a rapid urease test before and after eradication therapy. Patients lost to follow-up were defined as patients who received the second-line bismuth-containing quadruple therapy with unknown results regarding eradication success or failure. Compliance was classified as good or poor by pill count in the medical records. Patients who took 80% or more of the prescribed medicine were included in the good compliance group, and those who took less than 80% of the prescribed medicine were placed in the poor compliance group.

We investigated demographic features: area of residence, smoking and alcohol habits, diabetes mellitus, hypertension, endoscopic findings, and adverse effects of eradication therapy. Rural or urban residence was regarded as living or not living in the metropolitan cities of Korea, respectively. All patients underwent endoscopy, and endoscopic findings [such as gastric ulcers, duodenal ulcers, gastric and duodenal ulcers, a previous endoscopic submucosal dissection (ESD) state due to adenoma or early gastric cancer (EGC), MALT lymphoma, nodular gastritis, dyspepsia, gastric polyps, and intestinal metaplasia] were identified by endoscopy or by endoscopy with biopsy. Adverse effects after eradication therapy were identified by verification in the medical records. The Institutional Review Board (IRB) of Kosin University Gospel Hospital

Table 1 Baseline characteristics of the patients *n* (%)

Variable	Patients (n = 636[1])
Age (yr, mean ± SD)	54.6 ± 11.6
Gender	
Male	354 (55.7)
Female	282 (44.3)
Residence	
Rural	126 (19.8)
Urban	510 (80.2)
Cigarette smoking	174/605 (28.8)
Alcohol intake	279/605 (46.1)
Diabetes mellitus	61/605 (10.1)
Hypertension	121/605 (20.0)
Endoscopic findings	
Gastric ulcer	205 (32.2)
Duodenal ulcer	193 (30.3)
Gastric ulcer + Duodenal ulcer	40 (6.3)
Post ESD due to adenoma or EGC	91 (14.3)
Nodular gastritis	29 (4.6)
Others[2]	78 (12.2)

[1]Total number of enrolled patients; missing values are not included. The number behind the dash is the total number of subjects who answered each question. [2]Others include MALT lymphoma, dyspepsia, gastric polyp and intestinal metaplasia. ESD: Endoscopic submucosal dissection; EGC: Early gastric cancer; MALT lymphoma: Mucosa-associated lymphoid tissue lymphoma.

approved this study (IRB file No. 2015-03-018).

H. pylori eradication therapy and follow-up

Patients who failed the first-line clarithromycin-containing triple therapy (standard-dose PPI, 1.0 g amoxicillin, and 0.5 g clarithromycin twice daily for 7 d) were recommended for second-line eradication therapy. The latter was comprised of 20 mg rabeprazole twice daily, 500 mg metronidazole three times daily, 300 mg tripotassium dicitrato bismuthate, and 500 mg tetracycline four times daily for 7 d. Afterwards, a ^{13}C-urea breath test or a rapid urease test was conducted to assess *H. pylori* eradication at least 4 wk after the treatment completion, and at least 2 wk after cessation of PPIs or histamine (H_2) receptor antagonists.

^{13}C-urea breath test

Patients fasted for at least 4 h before the first breath sample was collected. Then, participants took tablets including 100 mg of ^{13}C-urea (UBiTkit™, Otsuka Pharmaceutical, Tokyo, Japan) with 100 mL of water orally, and the second breath sample was obtained 20 min after taking the tablets. *H. pylori* infection was analyzed using the ^{13}C-urea breath test (UBiT-IR300®; Otsuka Electronics, Osaka, Japan) on the collected breath samples. The cut-off value in the current procedure was set at 2.5‰.

Rapid urease test

To identify *H. pylori* infection with the rapid urease test (CLOtest®; Delta West, Bentley, WA, Australia), an endoscopic biopsy was conducted at the gastric mucosa. The site of gastric mucosal biopsy was antrum and/or corpus, and normal or near-normal gastric mucosa with little atrophy or intestinal metaplasia was removed. The tissue sample was immersed in rapid urea reagent. The result was positive when the reagent color changed from yellow to red at least 12 h later, and the result was negative when there was no change in reagent color.

Statistical analysis

All statistical analyses were conducted with the Statistical Package for the Social Sciences software version 20.0 (SPSS, Chicago, IL, United States). The *H. pylori* eradication rate was demonstrated by intention-to-treat (ITT) and per-protocol (PP) analyses. The trend in *H. pylori* eradication rates was analyzed with linear association. Patients lost to follow-up or those with poor compliance were excluded when we performed the PP analysis and univariate and multivariate logistic regression analyses. Categorical variables were analyzed using a χ^2-test, and continuous variables were analyzed using the Student's *t*-test. Univariate and multivariate logistic regression tests were used for the analysis of risk factors, which were expressed as an OR and 95%CI. A P value < 0.05 was considered statistically significant.

RESULTS

Patient characteristics

Between January 2005 and December 2015, 636 patients received 7 d of second-line bismuth-containing quadruple therapy after *H. pylori* eradication failure with clarithromycin-based triple therapy. Average age (mean ± SD) was 54.6 ± 11.6 years (range, 17-86 years), and 354 patients (55.7%) were male. Table 1 shows the clinical data and demographic information for enrolled patients. Among 636 patients receiving second-line bismuth-containing quadruple therapy, 138 patients were lost to follow-up, and three patients exhibited poor compliance. Finally, a total of 495 patients were included as subjects for PP analysis and multivariate logistic regression (Figure 1).

H. pylori eradication rates

In terms of eradication therapy success or failure, 468 patients achieved successful eradication. The eradication rates by ITT and PP analyses were 73.9% (95%CI: 70.1%-77.4%) and 94.5% (95%CI: 92.4%-96.5%) for second-line quadruple therapy, respectively. Annual eradication rates from 2005 to 2015 were 100.0%, 92.9%, 100.0%, 100.0%, 100.0%, 97.4%, 100.0%, 93.8%, 84.4%, 98.9% and 92.5%, consecutively by PP analysis. The eradication rate for first-line triple therapy decreased over the years (P = 0.01). Figure 2 presents the annual eradication rates for the last 11 years.

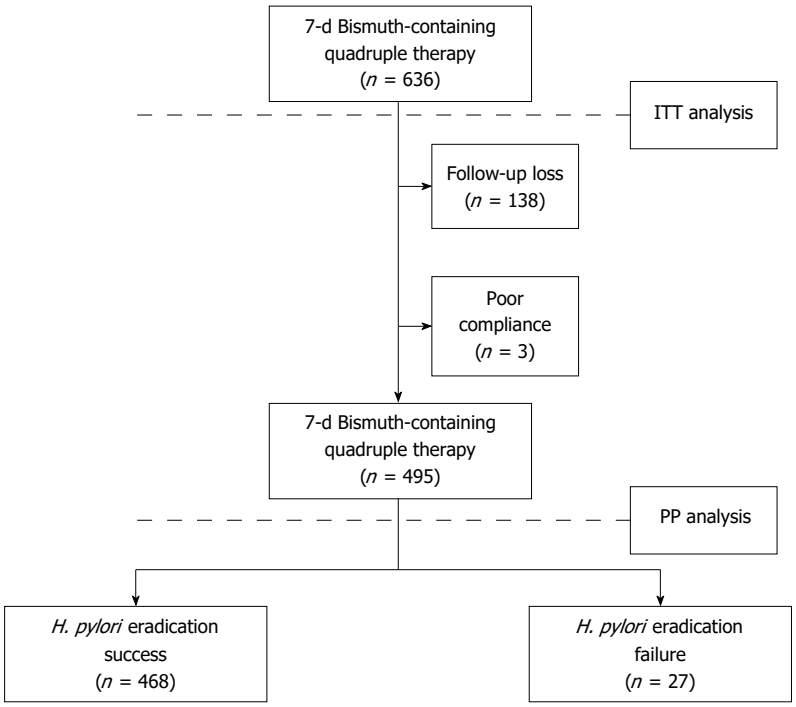

Figure 1 Flowchart of the study participants. ITT: Intention-to-treat; PP: Per-protocol; *H. pylori*: *Helicobacter pylori*.

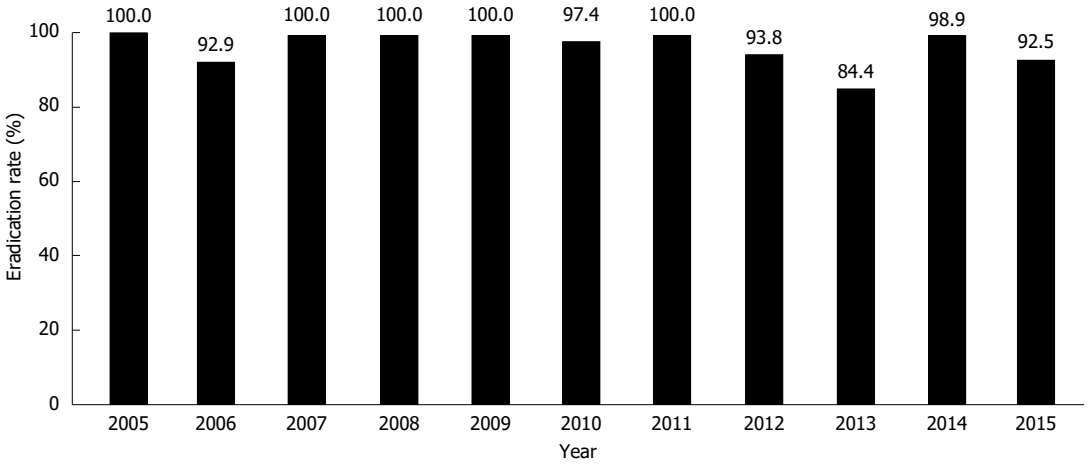

Figure 2 *Helicobacter pylori* eradication rates of second-line bismuth-based quadruple therapy according to years ($P = 0.01$).

Table 2 Adverse effects after bismuth-based quadruple therapy *n* (%)

	Patients (*n* = 495)
Side effect	74 (14.9)
Diarrhea	16 (3.2)
Bloating or abdominal pain	22 (4.4)
Nausea or vomiting	26 (5.3)
Others[1]	10 (2.0)

[1]Others include myalgia, headache and bitter sensation in the mouth.

Adverse effects of eradication therapy

Of the 495 patients, 74 patients (14.9%) complained of adverse events after bismuth-based quadruple therapy; fortunately, the adverse events were mild or moderate. Adverse events possibly related to treatment were diarrhea in 16 patients (3.2%), bloating or abdominal pain in 22 patients (4.4%), nausea or vomiting in 26 patients (5.3%), and others (such as myalgia, headache, and bitter sensation in the mouth) in 10 patients (2.0%; Table 2).

Associated factors for eradication failure

Associated factors for eradication failure are summarized in Table 3. Univariate and multivariate analyses demonstrated that only diabetes mellitus (OR = 3.99, 95%CI: 1.56-10.20, $P = 0.004$) was significantly related to eradication failure. There was no statistically significant relationship between eradication failure and

Table 3 Related factors about eradication failure of bismuth-based quadruple therapy *n* (%)

Variable	Eradication Success (*n* = 468[1])	Eradication Failure (*n* = 27[1])	*P* value[a]	*P* value[c]	Adjusted OR (95%CI)[c]
Age (yr)					
< 50	124 (95.4)	6 (4.6)			
⩾ 50	344 (94.2)	21 (5.8)	0.822	0.752	1.17 (0.44-3.09)
Gender					
Male	248 (93.2)	18 (6.8)			
Female	220 (96.1)	9 (3.9)	0.233	0.240	0.57 (0.22-1.46)
Residence					
Rural	90 (94.7)	5 (5.3)			
Urban	378 (94.5)	22 (5.5)	1.000	0.783	1.16 (0.41-3.24)
Cigarette smoking					
No	341 (95.0)	18 (5.0)			
Yes	111 (92.5)	9 (7.5)	0.359	0.435	1.48 (0.56-3.93)
Alcohol intake					
No	252 (94.4)	15 (5.6)			
Yes	200 (94.3)	12 (5.7)	1.000	0.522	0.74 (0.29-1.89)
Diabetes mellitus					
No	408 (95.6)	19 (4.4)			
Yes	44 (84.6)	8 (15.4)	0.005	0.004	3.99 (1.56-10.20)
Hypertension					
No	361 (94.5)	21 (5.5)			
Yes	91 (93.8)	6 (6.2)	0.806	0.638	0.78 (0.28-2.18)

[1]Total number of analyzed patients. Missing values are not included. [a]$P < 0.05$, univariate logistic regression test; [c]$P < 0.05$, multivariate logistic regression test. Logistic model including terms of age, gender, residence, cigarette smoking, alcohol intake, diabetes mellitus and hypertension.

other factors including age, gender, residence, smoking, alcohol, and hypertension.

DISCUSSION

In the current study, the *H. pylori* eradication rate for bismuth-containing quadruple therapy given for 7 d was < 80% by ITT analysis, but was > 90% by PP analysis in patients who failed clarithromycin-containing triple therapy. The frequency of adverse effects was less than 15%, which is consistent with the results of previous studies using bismuth-containing quadruple therapy[9,10].

As a second-line therapy, the effect of bismuth-containing quadruple therapy is controversial. Our PP eradication rate result was consistent with earlier studies, which reported that bismuth-containing quadruple therapy produced a high eradication rate in patients that failed *H. pylori* eradication therapy using clarithromycin-containing triple therapy. A recent multinational study in Europe reported the eradication rates for bismuth-containing quadruple therapy as rescue therapy for 10 d were 93.2%-93.8% by ITT analysis and 94.7%-95.0% by PP analysis[11]. Results with bismuth-containing quadruple therapy in China also demonstrated a 10-d bismuth-containing quadruple therapy eradication rate of 88.9% by ITT analysis and 90.9%-91.6% by PP analysis in patients that failed *H. pylori* eradication therapy[12,13]. However, eradication rates using second-line bismuth-containing quadruple therapy revealed diverse results in South Korea. Yoon *et al*[14] suggested that a 7-d bismuth-containing quadruple therapy might be as efficient as a 14-d bismuth-containing quadruple therapy for second-line eradication therapy, because a 7-d bismuth-containing quadruple therapy produced 83.5% and 87.7% eradication rates by ITT and PP analyses, respectively, and a 14-d bismuth-containing quadruple therapy produced 87.7% and 88.9% eradication rates by ITT and PP analyses, respectively. In contrast, another study reported that ITT eradication rates for a 7-d bismuth-containing quadruple therapy were 67.4%, and PP eradication rates were 78.2%, whereas ITT eradication rates for a 14-d bismuth-containing quadruple therapy were 72.8%, and PP eradication rates were 84.1%[9]. Usually, *H. pylori* eradication rates correlate with patient drug compliance and *H. pylori* antibiotic resistance. Unfortunately, studies to evaluate antibiotic resistance between different areas in South Korea are rare, and one small study determined there was no significant regional difference between *H. pylori* metronidazole and tetracycline resistance in South Korea[15]. Therefore, the reason for the high PP eradication rate in the current study is unclear. Although regional differences in antibiotic resistance may exist, bismuth-containing quadruple therapy achieved a more than 90% ITT eradication rate in patients who had *H. pylori* resistant to metronidazole (32.7%) and clarithromycin (63.3%)[11]. Thus, bismuth-containing quadruple therapy for second-line eradication therapy might even be effective in patients with antibiotic-resistant *H. pylori*.

In terms of adverse effects, most patients in the current study complained of gastrointestinal symptoms including nausea, vomiting, bloating, abdominal pain, or diarrhea. The symptoms were well tolerated, and no serious adverse events were observed. Only one patient wanted to be hospitalized for supportive care

due to nausea. With regard to neurologic symptoms, three patients complained of headache or dizziness, but the symptoms were mild. Severe neurological symptoms, such as bismuth-related encephalopathy, were not observed[11,16,17]. In accordance with previous studies, bismuth-containing therapy for the eradication of *H. pylori* is considered safe and well tolerated[9,18].

Several factors have been postulated as the cause of eradication failure, including age, gender, smoking, alcohol, and specific drug history (*e.g.*, aspirin)[6,19-22]. However, there was no significant relationship between these factors and eradication failure in the current study, except for diabetes mellitus. Diabetes mellitus has been presumed to be a risk factor for *H. pylori* eradication failure based on a recent meta-analysis (RR = 2.19, 95%CI: 1.65-2.90)[23]. It is hypothesized that microcirculatory complications related to diabetes mellitus could induce gastroparesis and reduce the absorption of antibiotics into the gastric mucosa, thereby influencing the effect of eradication therapy[24,25]. In addition, drug binding was revealed to be decreased by glycosylation, which was presumed to be associated with levels of blood glucose[26]. Concerning antibiotic resistance, the frequent use of antibiotics might increase antibiotic resistance[23,27]. A recent Danish nationwide cohort study found that the rates for community-based antibiotic prescriptions were higher in patients with diabetes mellitus compared to the general population[28]. Therefore, a more careful choice of *H. pylori* eradication therapy is needed for patients with diabetes mellitus.

Limitations of the present study are that it was performed at a single center and many patients were lost to follow-up, which might have influenced results of the ITT analysis. In addition, antibiotic susceptibility tests were not conducted in this study. Culturing *H. pylori* is difficult, and the response rates for antibiotic susceptibility tests are relatively low. Therefore, this was hard to inspect in all enrolled patients, and there were no standard criteria for identifying antibiotic resistance[9]. Furthermore, we did not diagnose *H. pylori* by histology before and after eradication therapy, as most patients underwent the ^{13}C-urea breath test or the rapid urease test for confirmation of *H. pylori* presence before and after therapy. These limitations could affect the eradication rate. According to the manufacturer, sensitivity and specificity of the rapid urease test were 90% to 95% and 95% to 100%, respectively. A recent meta-analysis reported that sensitivity and specificity of the ^{13}C-urea breath test were 95% to 97% and 91% to 94%, respectively, and that this test only rarely provided false-positive results[29-32]. We found that eradication rates based on the ^{13}C-urea breath test and the rapid urease test were 95.1% (327/344) and 93.4% (141/151), respectively (P = 0.519). Therefore, there was no significant difference between the two methods. The accuracy of both tests is high and very practical for clinical use[33], thus the absence of histology is unlikely to have had a significant effect on this study.

In conclusion, bismuth-containing quadruple therapy might be effective in patients that failed *H. pylori* eradication using clarithromycin-containing triple therapy, and might be worthy of consideration as a useful second-line therapy for *H. pylori* eradication in South Korea. Additionally, patients with diabetes mellitus are at higher risk for eradication failure with bismuth-containing quadruple therapy. Further studies on a larger scale evaluating the effects of second-line bismuth-containing quadruple therapy are needed in the near future in South Korea.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) has been classified as a definite gastric carcinogen (group I) by the International Agency for Research on Cancer. Therefore, *H. pylori* eradication is important to protect public health, especially in areas with high *H. pylori* prevalence. However, the eradication rate for proton pump inhibitor (PPI)-containing triple therapy has decreased worldwide, and an effective rescue treatment is needed.

Research frontiers

There is controversy about the role of bismuth-containing quadruple therapy as a second-line therapy for *H. pylori* eradication due to a decrease in eradication rates for bismuth-containing quadruple therapy in South Korea. In addition, risk factors related to the failure of second-line eradication therapy are obscure.

Innovations and breakthroughs

This retrospective study was performed to investigate the effects of second-line eradication therapy using bismuth-containing quadruple therapy at a single center over the past 11 years, and to evaluate the risk factors associated with the failure of second-line eradication therapy. According to the high eradication rate and low adverse effects of the therapy, bismuth-containing quadruple therapy is worthy of consideration as a useful second-line therapy for *H. pylori* eradication in South Korea. Additionally, diabetes mellitus is suggested to be a risk factor for eradication failure.

Applications

This retrospective study's design and findings may be helpful for planning further prospective studies on a larger scale which can evaluate the effects of second-line bismuth-containing quadruple therapy and clarify additional risk factors for eradication failure.

Terminology

H. pylori: A global pathogen that causes gastritis, peptic ulcers, mucosa-associated lymphoid tissue lymphoma, and gastric cancer. Eradication of *H. pylori* infection is crucial to maintaining public health, especially in high *H. pylori* and gastric cancer prevalence areas.

Peer-review

This is a well-designed, although retrospective study including a high number of patients. The methods used are appropriate, the statistics is sound. The difference between intention-to-treat and per-protocol eradication rates reflects the real life, while a proportion of patients lost to follow up is high.

REFERENCES

1 **Moss SF**, Malfertheiner P. Helicobacter and gastric malignancies. *Helicobacter* 2007; **12** Suppl 1: 23-30 [PMID: 17727457 DOI: 10.1111/j.1523-5378.2007.00539.x]

2 **IARC working group**. IARC working group on the evaluation of carcinogenic risks to humans: some industrial chemicals. Lyon,

15-22 February 1994. *IARC Monogr Eval Carcinog Risks Hum* 1994; **60**: 1-560 [PMID: 7869568]
3 **Kim MS**, Kim N, Kim SE, Jo HJ, Shin CM, Lee SH, Park YS, Hwang JH, Kim JW, Jeong SH, Lee DH, Kim JM, Jung HC. Long-term follow-up Helicobacter pylori reinfection rate and its associated factors in Korea. *Helicobacter* 2013; **18**: 135-142 [PMID: 23066652 DOI: 10.1111/hel.12018]
4 **Malfertheiner P**, Megraud F, O'Morain CA, Atherton J, Axon AT, Bazzoli F, Gensini GF, Gisbert JP, Graham DY, Rokkas T, El-Omar EM, Kuipers EJ. Management of Helicobacter pylori infection--the Maastricht IV/ Florence Consensus Report. *Gut* 2012; **61**: 646-664 [PMID: 22491499 DOI: 10.1136/gutjnl-2012-302084]
5 **Kim SG**, Jung HK, Lee HL, Jang JY, Lee H, Kim CG, Shin WG, Shin ES, Lee YC. Guidelines for the diagnosis and treatment of Helicobacter pylori infection in Korea, 2013 revised edition. *J Gastroenterol Hepatol* 2014; **29**: 1371-1386 [PMID: 24758240 DOI: 10.1111/jgh.12607]
6 **Kim SE**, Park MI, Park SJ, Moon W, Choi YJ, Cheon JH, Kwon HJ, Ku KH, Yoo CH, Kim JH, Lee GW, Song SE. Trends in Helicobacter pylori eradication rates by first-line triple therapy and related factors in eradication therapy. *Korean J Intern Med* 2015; **30**: 801-807 [PMID: 26552455 DOI: 10.3904/kjim.2015.30.6.801]
7 **Shin WG**, Lee SW, Baik GH, Huh KC, Lee SI, Chung JW, Jung WT, Park MI, Jung HK, Kim HU, Kim JH, Seol SY, Yoon SM, Jeon SW, Hong SJ, Kim GH, Lee DH, Kim HS, Choi SC, Kang HM, Lee J, Kim JG, Kim JJ. Eradication Rates of Helicobacter pylori in Korea Over the Past 10 years and Correlation of the Amount of Antibiotics Use: Nationwide Survey. *Helicobacter* 2016; **21**: 266-278 [PMID: 26470999 DOI: 10.1111/hel.12279]
8 **Lee BH**, Kim N, Hwang TJ, Lee SH, Park YS, Hwang JH, Kim JW, Jeong SH, Lee DH, Jung HC, Song IS. Bismuth-containing quadruple therapy as second-line treatment for Helicobacter pylori infection: effect of treatment duration and antibiotic resistance on the eradication rate in Korea. *Helicobacter* 2010; **15**: 38-45 [PMID: 20302588 DOI: 10.1111/j.1523-5378.2009.00735.x]
9 **Hwang JJ**, Lee DH, Lee AR, Yoon H, Shin CM, Park YS, Kim N. Fourteen- vs seven-day bismuth-based quadruple therapy for second-line Helicobacter pylori eradication. *World J Gastroenterol* 2015; **21**: 8132-8139 [PMID: 26185386 DOI: 10.3748/wjg.v21.i26.8132]
10 **Lee ST**, Lee DH, Lim JH, Kim N, Park YS, Shin CM, Jo HJ, Song IS. Efficacy of 7-Day and 14-Day Bismuth-Containing Quadruple Therapy and 7-Day and 14-Day Moxifloxacin-Based Triple Therapy as Second-Line Eradication for Helicobacter pylori Infection. *Gut Liver* 2015; **9**: 478-485 [PMID: 25071068 DOI: 10.5009/gnl14020]
11 **Delchier JC**, Malfertheiner P, Thieroff-Ekerdt R. Use of a combination formulation of bismuth, metronidazole and tetracycline with omeprazole as a rescue therapy for eradication of Helicobacter pylori. *Aliment Pharmacol Ther* 2014; **40**: 171-177 [PMID: 24863854 DOI: 10.1111/apt.12808]
12 **Zheng Q**, Dai J, Li X, Lu H, Xiao S. Comparison of the efficacy of pantoprazole-based triple therapy versus quadruple therapy in the treatment of Helicobacter pylori infections: a single-center, randomized, open and parallel-controlled study. *Weichangbingxue* 2009; **14**: 8-11
13 **Zheng Q**, Chen WJ, Lu H, Sun QJ, Xiao SD. Comparison of the efficacy of triple versus quadruple therapy on the eradication of Helicobacter pylori and antibiotic resistance. *J Dig Dis* 2010; **11**: 313-318 [PMID: 20883428 DOI: 10.1111/j.1751-2980.2010.00457.x]
14 **Yoon JH**, Baik GH, Kim YS, Suk KT, Shin WG, Kim KH, Kim KO, Park CH, Baik IH, Jang HJ, Kim JB, Kae SH, Kim DJ, Kim HY. Comparison of the Eradication Rate between 1- and 2-Week Bismuth-Containing Quadruple Rescue Therapies for Helicobacter pylori Eradication. *Gut Liver* 2012; **6**: 434-439 [PMID: 23170146 DOI: 10.5009/gnl.2012.6.4.434]
15 **Kim JY**, Kim NY, Kim SJ, Baik GH, Kim GH, Kim JM, Nam RH, Kim HB, Lee DH, Jung HC, Song IS. Regional difference of antibiotic resistance of helicobacter pylori strains in Korea. *Korean J Gastroenterol* 2011; **57**: 221-229 [PMID: 21519175 DOI: 10.4166/2011.57.4.221]
16 **Tillman LA**, Drake FM, Dixon JS, Wood JR. Review article: safety of bismuth in the treatment of gastrointestinal diseases. *Aliment Pharmacol Ther* 1996; **10**: 459-467 [PMID: 8853750 DOI: 10.1046/j.1365-2036.1996.22163000.x]
17 **Serfontein WJ**, Mekel R. Bismuth toxicity in man II. Review of bismuth blood and urine levels in patients after administration of therapeutic bismuth formulations in relation to the problem of bismuth toxicity in man. *Res Commun Chem Pathol Pharmacol* 1979; **26**: 391-411 [PMID: 392661]
18 **Ford AC**, Malfertheiner P, Giguere M, Santana J, Khan M, Moayyedi P. Adverse events with bismuth salts for Helicobacter pylori eradication: systematic review and meta-analysis. *World J Gastroenterol* 2008; **14**: 7361-7370 [PMID: 19109870 DOI: 10.3748/wjg.v14.i48.7361]
19 **Suzuki T**, Matsuo K, Ito H, Sawaki A, Hirose K, Wakai K, Sato S, Nakamura T, Yamao K, Ueda R, Tajima K. Smoking increases the treatment failure for Helicobacter pylori eradication. *Am J Med* 2006; **119**: 217-224 [PMID: 16490464 DOI: 10.1016/j.amjmed.2005.10.003]
20 **Moayyedi P**, Chalmers DM, Axon AT. Patient factors that predict failure of omeprazole, clarithromycin, and tinidazole to eradicate Helicobacter pylori. *J Gastroenterol* 1997; **32**: 24-27 [PMID: 9058291 DOI: 10.1007/BF01213292]
21 **Cho DK**, Park SY, Kee WJ, Lee JH, Ki HS, Yoon KW, Cho SB, Lee WS, Joo YE, Kim HS, Choi SK, Rew JS. The trend of eradication rate of Helicobacter pylori infection and clinical factors that affect the eradication of first-line therapy. *Korean J Gastroenterol* 2010; **55**: 368-375 [PMID: 20571304 DOI: 10.4166/kjg.2010.55.6.368]
22 **Graham DY**, Lew GM, Malaty HM, Evans DG, Evans DJ, Klein PD, Alpert LC, Genta RM. Factors influencing the eradication of Helicobacter pylori with triple therapy. *Gastroenterology* 1992; **102**: 493-496 [PMID: 1732120 DOI: 10.1016/0016-5085(92)90095-G]
23 **Horikawa C**, Kodama S, Fujihara K, Hirasawa R, Yachi Y, Suzuki A, Hanyu O, Shimano H, Sone H. High risk of failing eradication of Helicobacter pylori in patients with diabetes: a meta-analysis. *Diabetes Res Clin Pract* 2014; **106**: 81-87 [PMID: 25110103 DOI: 10.1016/j.diabres.2014.07.009]
24 **Groop LC**, Luzi L, DeFronzo RA, Melander A. Hyperglycaemia and absorption of sulphonylurea drugs. *Lancet* 1989; **2**: 129-130 [PMID: 2567896 DOI: 10.1016/S0140-6736(89)90184-0]
25 **Kong MF**, Macdonald IA, Tattersall RB. Gastric emptying in diabetes. *Diabet Med* 1996; **13**: 112-119 [PMID: 8641114 DOI: 10.1002/(SICI)1096-9136(199602)13:2<112::AID-DIA37>3.0.CO;2-H]
26 **Gwilt PR**, Nahhas RR, Tracewell WG. The effects of diabetes mellitus on pharmacokinetics and pharmacodynamics in humans. *Clin Pharmacokinet* 1991; **20**: 477-490 [PMID: 2044331 DOI: 10.2165/00003088-199120060-00004]
27 **Marhoffer W**, Stein M, Maeser E, Federlin K. Impairment of polymorphonuclear leukocyte function and metabolic control of diabetes. *Diabetes Care* 1992; **15**: 256-260 [PMID: 1547682 DOI: 10.2337/diacare.15.2.256]
28 **Mor A**, Berencsi K, Nielsen JS, Rungby J, Friborg S, Brandslund I, Christiansen JS, Vaag A, Beck-Nielsen H, Sørensen HT, Thomsen RW. Rates of Community-based Antibiotic Prescriptions and Hospital-treated Infections in Individuals With and Without Type 2 Diabetes: A Danish Nationwide Cohort Study, 2004-2012. *Clin Infect Dis* 2016; **63**: 501-511 [PMID: 27353662 DOI: 10.1093/cid/ciw345]
29 **Versalovic J**. Helicobacter pylori. Pathology and diagnostic strategies. *Am J Clin Pathol* 2003; **119**: 403-412 [PMID: 12645343 DOI: 10.1309/5DTF5HT7NPLNA6J5]
30 **Gatta L**, Vakil N, Ricci C, Osborn JF, Tampieri A, Perna F, Miglioli M, Vaira D. Effect of proton pump inhibitors and antacid therapy on 13C urea breath tests and stool test for Helicobacter pylori infection. *Am J Gastroenterol* 2004; **99**: 823-829 [PMID: 15128344 DOI: 10.1111/j.1572-0241.2004.30162.x]
31 **Mégraud F**, Lehours P. Helicobacter pylori detection and

antimicrobial susceptibility testing. *Clin Microbiol Rev* 2007; **20**: 280-322 [PMID: 17428887 DOI: 10.1128/CMR.00033-06]

32 **Ferwana M**, Abdulmajeed I, Alhajiahmed A, Madani W, Firwana B, Hasan R, Altayar O, Limburg PJ, Murad MH, Knawy B. Accuracy of urea breath test in Helicobacter pylori infection: meta-analysis. *World J Gastroenterol* 2015; **21**: 1305-1314 [PMID: 25632206 DOI: 10.3748/wjg.v21.i4.1305]

33 **Leodolter A**, Wolle K, Malfertheiner P. Current standards in the diagnosis of Helicobacter pylori infection. *Dig Dis* 2001; **19**: 116-122 [PMID: 11549820 DOI: 10.1159/000050665]

P- Reviewer: Buzas GM, Karatapanis S, Slomiany BL, Suzuki H
S- Editor: Gong ZM **L- Editor**: A **E- Editor**: Liu WX

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v23.i6.1067

World J Gastroenterol 2017 February 14; 23(6): 1067-1075
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Observational Study

Disease impact on the quality of life of children with inflammatory bowel disease

Giorgos Chouliaras, Daphne Margoni, Konstantina Dimakou, Smaragdi Fessatou, Ioanna Panayiotou, Eleftheria Roma-Giannikou

Giorgos Chouliaras, Daphne Margoni, Konstantina Dimakou, Smaragdi Fessatou, Ioanna Panayiotou, Eleftheria Roma-Giannikou, First Department of Pediatrics, National and Kapodistrian University of Athens, "Aghia Sophia" Children's Hospital, 11527 Athens, Greece

Author contributions: Chouliaras G and Margoni D contributed equally to this work; Roma-Giannikou E conceptualized and designed the study, supervised the analyses and wrote the manuscript; Chouliaras G is a medical biostatistician and performed the main statistical analysis; Chouliaras G and Margoni D designed the study, analysed the data and wrote the manuscript; Dimakou K, Fessatou S and Panayiotou I participated in study design, data collection and manuscript preparation; all authors approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the Ethics Committee of "Aghia Sophia" Children's Hospital, Athens, Greece

Informed consent statement: All study participants, or their legal guardian, provided informed consent prior to study enrolment.

Conflict-of-interest statement: There are no conflicts of interest to report.

Data sharing statement: No other data are available.

Manuscript source: Invited manuscript

Correspondence to: Giorgos Chouliaras, MD, PhD, First Department of Pediatrics, National and Kapodistrian University of Athens, "Aghia Sophia" Children's Hospital, Thivon and Papadiamantopoulou Street, 11527 Athens, Greece. georgehouliaras@msn.com
Telephone: +30-210-7467129
Fax: +30-21-32013237

Received: September 12, 2016
Peer-review started: September 12, 2016
First decision: October 20, 2016
Revised: November 16, 2016
Accepted: January 11, 2017
Article in press: January 11, 2017
Published online: February 14, 2017

Abstract

AIM

To assess the impact of disease characteristics on the quality of life (QOL) in children with inflammatory bowel diseases (IBD).

METHODS

This was a cross-sectional study conducted at the First Department of Pediatrics of the University of Athens at the "Aghia Sophia" Children's Hospital. Children diagnosed with Crohn's disease (CD) or ulcerative colitis (UC), who were followed as outpatients or during a hospitalization, participated, after informed consent was obtained from their legal representative. QOL was assessed by the IMPACT-III questionnaire. Demographic data and disease characteristics were also collected. Statistical analyses included parametric (Student's *t*-test and Pearson's *r*) and non-parametric (Mann-Whitney test, Fisher's test and Spearman's rho) procedures.

RESULTS

Ninety-nine patients (UC: 37, 73.0% females, CD: 62, 51.6% females), aged 12.8 ± 2.6 years were included.

Overall, as well as, sub-domain scores did not differ between UC and CD (overall score: 73.9 ± 13.3 *vs* 77.5 ± 11.2, respectively, $P = 0.16$). In the entire sample, total score was related to physician's global assessment (PGA, patients classified as "mild/moderate" active disease had, on average, 14.8 ± 2.7 points lower total scores compared to those "in remission", $P < 0.001$) and age at IMPACT completion (Pearson's $r = 0.29$, $P = 0.05$). Disease activity assessed by the indices Pediatric Ulcerative Colitis activity index, Pediatric Crohn's disease activity index or PGA was significantly associated with all subdomains scores. Presence of extraintestinal manifestations had a negative impact on emotional and social functioning domains.

CONCLUSION

Disease activity is the main correlate of QOL in children with IBD, underlining the importance of achieving and sustaining clinical remission

Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Quality of life; IMPACT-III; Children

Core tip: This study demonstrated that disease activity is the main correlate of quality of life (QOL) in children with inflammatory bowel diseases (IBD). Furthermore, several factors, that pose increased risks for impaired QOL for children with IBD, were identified. In brief, children of younger age, the early years after the diagnosis and the presence of extra-intestinal manifestations were inversely related to IMPACT-III scores. Therefore, in children with these specific features, physicians should be more vigilant in order to recognize and address issues related to their QOL promptly.

Chouliaras G, Margoni D, Dimakou K, Fessatou S, Panayiotou I, Roma-Giannikou E. Disease impact on the quality of life of children with inflammatory bowel disease. *World J Gastroenterol* 2017; 23(6): 1067-1075 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i6/1067.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i6.1067

INTRODUCTION

Inflammatory bowel diseases (IBD) are immune mediated disorders with a genetic component, characterized by chronic inflammation of the gastrointestinal tract. Although patients suffering from these conditions have a normal life expectancy, the need for long-term medication, frequent hospitalizations, surgeries and the relapsing nature of the disease significantly negatively affect their quality of life (QOL). The term QOL encompasses the patients' subjective perception of their health state, as well as the impact of their disease on their physical, social and emotional wellbeing[1].

There are several studies published on the subject, the majority of which support that patients with IBD, as with other chronic diseases, have an impaired QOL compared to the normal population[1-3]. Most researchers have concluded that in patients with IBD the disease activity is the main predictive factor of QOL; however it is not yet defined how other disease characteristics, such as disease duration, recurrent hospitalizations or different treatment modalities affect their QOL.

In this study we assessed the QOL in Greek pediatric patients with IBD and attempted to identify how it is affected by disease characteristics.

MATERIALS AND METHODS

The study was conducted at the First Department of Pediatrics of the University of Athens, Greece after approval by the Ethics Committee of the "Aghia Sophia" Children's Hospital in Athens, Greece. Informed consent was obtained from all legal representatives of the children who participated.

This was a cross-sectional study. Candidates for inclusion were children diagnosed with UC or CD according to the revised Porto criteria[4], who were hospitalized or followed in the outpatient IBD clinic. All patients had undergone at least one full ileocolonoscopy with biopsies, esophago-gastro-duodenoscopy with biopsies and magnetic resonance enterography for small bowel assessment.

At the time of evaluation, a number of parameters were recorded: Demographic data, disease activity, disease duration, current treatment and number of hospitalizations in the previous 3 mo. For the disease activity evaluation the Pediatric Crohn's disease activity index (PCDAI)[5] or the Pediatric Ulcerative Colitis activity index (PUCAI)[6] were used. For PCDAI the score ranges from 0 to 100, whereas for PUCAI the score ranges from 0 to 85 points. Based on the activity indices patients were classified as being in remission (PCDAI $\leqslant$ 10 or PUCAI $\leqslant$ 10), in relapse with mild activity (10 < PCDAI $\leqslant$ 30 or 10 < PUCAI $\leqslant$ 34) and in relapse with moderate/severe activity (PCDAI > 30 or PUCAI > 34). Physician's global assessment (PGA) was also recorded at the time of evaluation. PGA is a validated instrument through which the physician is able to evaluate disease activity clinically, on a 4 point scale (inactive, mild, moderate and severe disease).

QOL was assessed by the IMPACT-III questionnaire, which is a 35-item self-report tool that assesses QOL in children and adolescents with IBD. Children indicate on a 5-point Likert scale the extent to which they are bothered by specific aspects of their health condition. It consists of 6 subscales: bowel symptoms, systemic symptoms, emotional functioning, social functioning, body image and treatment/interventions. Scores range from 35 to 175, with higher scores

Table 1 Demographic data, disease characteristics and medications at the time of the evaluation *n* (%)

	Overall (*n* = 99)	CD (*n* = 62)	UC (*n* = 37)	*P* value
Gender, females	59 (59.6)	32 (51.6)	27 (73.0)	0.06[3]
Age (yr)	12.8 ± 2.6	13.4 ± 2.4	11.6 ± 2.5	0.001[1]
Age at diagnosis (yr)	9.9 ± 3.1	10.7 ± 2.9	8.6 ± 3.0	< 0.001[1]
Number of hospitalizations[4]	2 (1-4)	2 (1-4)	3 (1-6)	0.09[2]
Disease duration at IMPACT completion (yr)	2.8 ± 2.6	2.7 ± 2.5	3.0 ± 2.7	0.48[1]
Disease activity	NA	7.2 ± 10.3	14.0 ± 20.5	NA
Disease status, in remission	70 (70.7)	45 (72.6)	25 (67.6)	0.65[3]
Physician global assessment				0.9[3]
Clinical remission	80 (80.8)	50 (80.6)	30 (81.1)	
Mild	14 (14.1)	9 (14.5)	5 (13.5)	
Moderate	5 (5.1)	3 (4.8)	2 (5.4)	
Medications at IMPACT completion				
Antibiotics	2 (2.0)	2 (3.2)	0 (0.0)	0.52[3]
Steroids	39 (39.4)	24 (38.7)	15 (40.5)	0.99[3]
Immunomodulators	68 (68.7)	47 (75.8)	21 (56.8)	0.11[3]
Biologic agents	31 (31.3)	25 (40.3)	6 (16.2)	0.03[3]
Enteral nutrition	0 (0.0)	0 (0.0)	0 (0.0)	NA
Aminosalicylates	52 (52.5)	15 (24.2)	37 (100.0)	< 0.001[3]

[1]*t*-test; [2]Mann-Whitney test; [3]Fisher's exact test; [4]Three months prior to completing IMPACT, median (interquartile range). NA: Not applicable; CD: Crohn's disease; UC: Ulcerative colitis.

suggesting better QOL. We used the Greek version of the questionnaire, created by one of the authors (Roma-Giannikou E), which has been translated from the original one designed by Otley and the Pediatric Inflammatory Bowel Disease Working Group on Quality of Life in 2002[7]. The patients completed the IMPACT-III questionnaires, in the presence of their parent(s), in approximately 20 min.

Statistical analysis

Statistical analysis was performed by a medical biostatistician (GC). Summary statistics for continuous variables are presented as mean ± SD and compared by *t*-test, whereas in cases of small samples (< 30) or skewed distributions, median and interquartile range are presented and the Mann-Whitney test was used. Categorical data are presented as absolute (*n*) and relative (%) frequencies and compared by Fisher's exact test. Correlations were assessed by Pearson's correlation coefficient (*r*), or Spearman's rho. Due to the small number of observations in the PGA-category «moderate», categories «mild» and «moderate» were collapsed into one class and compared to patients in «clinical remission». Similarly, as the numbers of subjects in the moderate/severe categories, according to PCDAI/PUCAI classification, were very small, patients were grouped as "in remission" or "in relapse with mild/moderate activity" and a new joined variable was formed including all individuals. After univariate analyses were performed, a stepwise backward regression analysis was performed to assess significant parameters at the multivariate level, grouping all IBD patients. Level of statistical significance, for univariate analyses, was set to 0.05. For the stepwise approach, the level for entering a covariate into the model was 0.051 whereas for removing, it was 0.05. All analyses were performed with Stata 11.0 MP statistical software (Stata Corp, TX, United States).

RESULTS

Descriptive statistics

A total of 99 patients (62 CD, 37 UC) were included in the analysis. Demographic data and disease characteristics are shown in Table 1. Patients with CD were older at the time of diagnosis, as well as at the time of IMPACT completion and were more likely to be treated with an anti-TNFα agent. Table 2 illustrates total and sub-domain IMPACT scores, overall as well as according to diagnosis. In general, patients with CD scored higher in all scales compared to ulcerative colitis group; however the results did not reach statistical significance, not even after adjusting for PGA level (all *P* values > 0.05). The only exception was emotional functioning domain, where among patients in clinical remission, according to PGA, CD children scored significantly higher compared to UC children (79.3 ± 16.2 *vs* 70.0 ± 21.1, *P* = 0.05).

Total score of correlates of IMPACT scores

In the entire study sample of IBD patients, the total IMPACT score was positively related to disease duration (Pearson's *r* = 0.20, *P* = 0.04), age at IMPACT completion (Pearson's *r* = 0.19, *P* =0.05) and inversely related to disease activity assessed by PUCAI and PCDAI ("in remission" *vs* "mild/moderate": 79.6 ± 11.4 *vs* 68.0 ± 10.0, respectively, *P* < 0.001). Sub-analyses confirmed the relation between total IMPACT score and disease activity in both IBD groups (UC: Pearson's *r* = -0.45, *P* = 0.005, CD: Pearson's *r* = -0.42, *P* < 0.001) and between total IMPACT score and age at IMPACT completion only in the UC group (Pearson's *r* = 0.29,

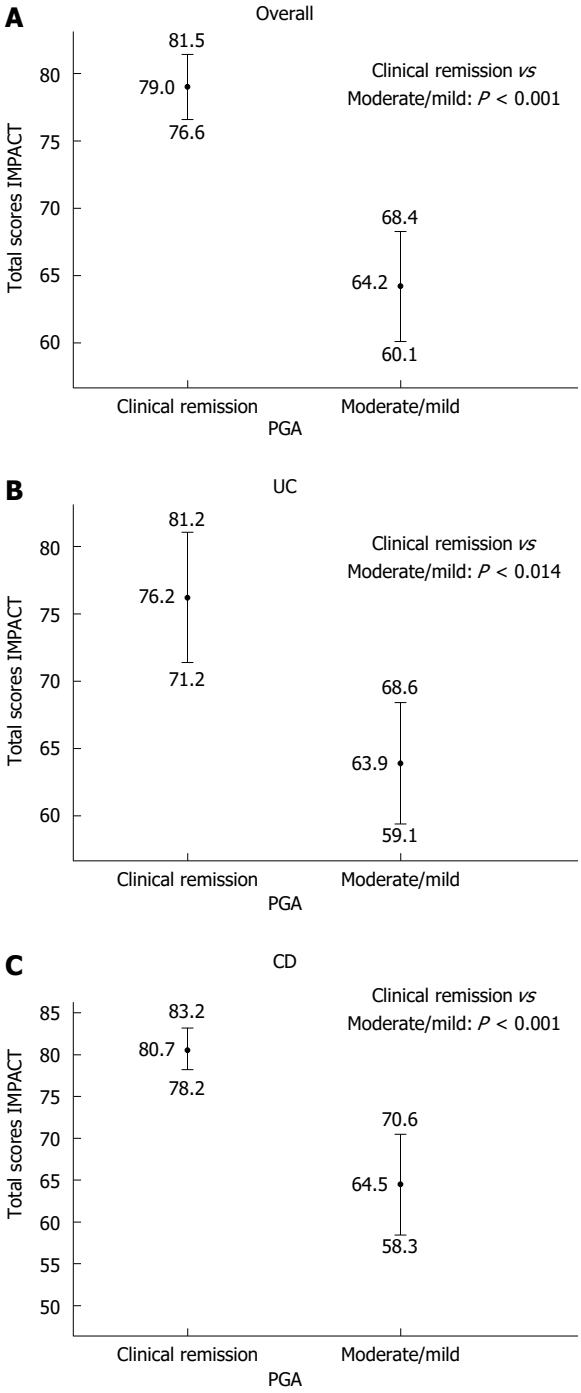

Figure 1 Total IMPACT scores (mean and 95%CI) according to physician's global assessment in the entire inflammatory bowel disease study sample (A), ulcerative colitis (B) and Crohn's disease (C). Statistical comparisons were performed by the Mann-Whitney test. PGA: Physician's global assessment; UC: Ulcerative colitis; CD: Crohn's disease.

P = 0.05). Disease duration was not a significant predictor in either group, in stratified analyses. Overall, total scores showed a significant trend across the PGA scale, with lower scores corresponding to worst clinical assessment (Mann-Whitney test, *P* < 0.001; Figure 1A). The same result occurred in the stratified analyses (Mann-Whitney test, UC: *P* = 0.014 and CD: *P* < 0.001; Figure 1B and C respectively).

Gender, number of hospitalizations in the previous 3 mo and type of medication were not correlated to total IMPACT scores neither overall or per group (all *P* values > 0.10).

Finally a multivariate stepwise regression analysis was performed in the entire study sample. In the final model, PGA and age at IMPACT-III completion remained significant. More specifically, patients classified as "mild/moderate" had, on average, 14.5 ± 2.7 points lower total scores compared to those "in remission" (*P* < 0.001). In addition, it was estimated that one year increase in age at IMPACT-III completion increases the total score by an average of 0.8 ± 0.4 points, regardless of PGA classification (*P* = 0.044).

Sub-domain scores of correlates of IMPACT scores

Bowel symptoms: Overall, the bowel symptoms domain score was significantly related to disease duration (Pearson's *r* = 0.27, *P* = 0.005), age at IMPACT completion (Pearson's *r* = 0.23, *P* = 0.018) and disease activity (in remission *vs* mild/moderate activity: 81.4 ± 12.7 *vs* 65.3 ± 10.0, respectively, *P* < 0.001). In the stratified analysis the direction of the correlations was retained; however in UC only disease activity remained a statistically significant predictor (Pearson's *r* = -0.56, *P* < 0.001), whereas in the CD group disease duration (Pearson's *r* = 0.31, *P* = 0.013) and disease activity (Pearson's *r* = -0.54, *P* < 0.001) reached statistical significance. Patients with CD who were being treated with steroids had significantly lower scores in the bowel symptoms domain compared to steroid-free CD patients (on-steroids *vs* no-steroids: 73.8 ± 13.8 *vs* 80.7 ± 13.9, *P* = 0.047). After stratification according to PGA, significance was marginally not achieved for patients in remission (on-steroids *vs* no-steroids: 77.8 ± 11.3 *vs* 83.7 ± 12.1, *P* = 0.09). No other significant associations between bowel symptoms domain scores and type of medications were observed. As shown in Tables 3, 4 and 5, across the study population, overall as well as in the sub-group analyses, there was a clear trend for bowel symptoms scores resulting in significantly lower scores for patients in the moderate/mild category compared to those classified in remission. The regression analysis showed that PGA, disease activity and disease duration were significant covariates in the final model. Based on PGA, children classified as "mild/moderate" had, on average, 9.6 ± 4.1 points lower total scores compared to those "in remission" (*P* = 0.022), whereas according to activity indices a similar difference was estimated between the two categories (9.8 ± 3.6, *P* = 0.008). An interesting point is that these results are adjusted for the level of the other covariate. For example, patients classified "in remission", according to PGA, have significantly different scores depending on their disease status based on activity indices. In the regression analysis it was, also, estimated that one year increase in disease duration increases the total score

Table 2 IMPACT scores (total and domain scores) in the study population

	Overall (n = 99)	CD (n = 62)	UC (n = 37)	P value[1]
Total score	76.2 ± 12.1	77.5 ± 11.2	73.9 ± 13.3	0.16
Individual domains				
Bowel symptoms	76.6 ± 14.8	78.0 ± 14.0	74.2 ± 15.5	0.22
Systemic symptoms	80.8 ± 17.4	82.0 ± 16.7	78.8 ± 18.5	0.36
Emotional functioning	72.4 ± 19.0	75.2 ± 18.3	67.9 ± 19.6	0.06
Social functioning	79.9 ± 12.4	80.6 ± 12.0	78.6 ± 13.0	0.42
Body image	71.5 ± 17.9	72.6 ± 19.3	69.8 ± 15.4	0.46
Treatment/interventions	69.0 ± 19.9	70.0 ± 18.4	67.3 ± 22.4	0.51

[1]*t*-test. CD: Crohn's disease; UC: Ulcerative colitis.

Table 3 IMPACT scores per domain according to physician's global assessment classification (total population)

Domain	PGA		P value[1]
	Clinical remission	Mild/moderate	
Bowel symptoms	80.0 ± 13.3	62.4 ± 11.8	< 0.001
Systemic symptoms	84.2 ± 16.0	66.6 ± 15.9	< 0.001
Emotional functioning	75.8 ± 18.6	58.3 ± 13.5	< 0.001
Social functioning	82.5 ± 11.0	69.0 ± 11.9	< 0.001
Body image	73.2 ± 17.9	64.5 ± 16.4	0.032
Treatment/interventions	70.9 ± 20.1	61.0 ± 17.1	0.007

[1]Mann-Whitney test. PGA: Physician's global assessment.

Table 4 IMPACT scores per domain according to physician's global assessment classification (ulcerative colitis patients)

Domain	PGA		P value[1]
	Clinical remission	Mild/moderate	
Bowel symptoms	77.4 ± 15.1	60.7 ± 9.2	0.007
Systemic symptoms	82.8 ± 17.6	61.9 ± 11.6	0.002
Emotional functioning	70.0 ± 21.1	58.7 ± 4.5	0.050
Social functioning	80.8 ± 12.7	69.0 ± 10.0	0.032
Body image	70.3 ± 16.6	67.9 ± 8.9	0.600
Treatment/interventions	68.9 ± 24.2	60.7 ± 11.5	0.300

[1]Mann-Whitney test. PGA: Physician's global assessment.

by an average of 1.0 ± 0.5 points, irrespective of PGA classification or disease activity (P = 0.037).

Systemic symptoms: With respect to the systemic symptoms domain score, disease activity was a significant correlate overall (in remission *vs* mild/moderate activity: 84.3 ± 16.0 *vs* 72.4 ± 18.0, respectively, P = 0.001), as well as in the UC and CD groups (Pearson's r = -0.45, P = 0.005 and Pearson's r = -0.30, P = 0.015, respectively). Similarly to the bowel symptoms domain, PGA was significantly and negatively related to systemic symptoms domain (Tables 3, 4 and 5). With the exception of steroids, treatment was not related to systemic symptoms. Patients receiving steroids scored significantly lower in the systemic symptoms domain (on-steroids *vs* no-steroids: 76.5 ± 18.8 *vs* 83.6 ± 18.9, P = 0.045). When the analysis was repeated, after stratifying according to PGA class, the effect was retained for those patients in remission

Table 5 IMPACT scores per domain according to physician's global assessment classification (Crohn's disease patients)

Domain	PGA		P value[1]
	Clinical remission	Mild/moderate	
Bowel symptoms	81.6 ± 12.1	63.4 ± 13.4	< 0.001
Systemic symptoms	85.0 ± 15.1	69.4 ± 17.9	0.005
Emotional functioning	79.3 ± 16.2	58.0 ± 16.9	< 0.001
Social functioning	83.4 ± 9.9	68.9 ± 13.2	0.001
Body image	75.0 ± 18.6	62.5 ± 19.6	0.036
Treatment/interventions	72.2 ± 17.5	61.1 ± 20.2	0.070

[1]Mann-Whitney test.

(on-steroids *vs* no-steroids: 79.2 ± 19.8 *vs* 86.6 ± 18.9, P = 0.039) but not for those with active disease (P = 0.34). In the final model only PGA remained statistically significant ("mild/moderate" had, on average, 17.5 ± 4.1 points lower total scores compared to those "in remission" (P < 0.001)

Emotional functioning: The same pattern was observed for the emotional functioning domain score and disease activity, as well: (overall: in remission *vs* mild/moderate activity: 75.9 ± 19.4 *vs* 64.0 ± 15.2, respectively, P = 0.004, UC: Spearman's rho = -0.33, P = 0.04, CD: Spearman's rho = -0.32, P = 0.011). In addition, disease duration appeared to be positively and significantly related to the emotional functioning domain score overall, but not in the subgroup analyses (overall: Pearson's r = 0.20, P = 0.046). An interesting finding was the negative effect of extra-intestinal manifestations on emotional functioning (yes *vs* no: 55.0 ± 19.8 *vs* 73.4 ± 18.6, P = 0.046). In relation to PGA, in accordance to previous results, patients classified as "mild/moderate" had significantly worse scores compared to those "in remission" (Tables 3, 4 and 5). Therapeutic modalities did not affect emotional functioning. The multivariate analyses, where all candidate parameters were included and a stepwise process rejected the statistically insignificant, revealed a quite different final model. PGA and disease duration were, again, significant correlates [PGA: "mild/moderate" had, on average, 15.7 ± 4.4 points lower scores compared to those "in remission" (P = 0.001); one year increase in disease duration increases the score

by an average of 1.6 ± 0.7 points (0.019)]. Moreover, it was shown that patients with extra-intestinal manifestations had lower scores (average difference: -19.7 ± 0.7, P = 0.017) and the same was demonstrated for gender, with boys having significantly higher scores compared to girls (average difference: 7.4 ± 3.6, P = 0.045).

Social functioning: A positive trend was recorded between age and social functioning scores, overall (Pearson's r = 0.21, P = 0.030), but not in stratified analyses. The previously described inverse relation between domain scores and disease activity was also observed in the social functioning domain score not only in the entire IBD population (overall: in remission *vs* mild/moderate activity: 83.7 ± 10.7 *vs* 70.8 ± 11.5, respectively, P < 0.001), but also per IBD group (UC: Pearson's r = -0.52, P < 0.001 and CD: Pearson's r = -0.43, P < 0.001). Disease duration appeared to be positively and significantly related to social functioning domain score in the entire study population (Pearson's r = 0.21, P = 0.034). Patients in the "mild/moderate" group according to PGA classification had impaired social functioning compared to those "in remission" (Tables 3, 4 and 5). Medications were not associated with the social functioning score, overall nor in the stratified analyses. Nevertheless, in the final modeling, PGA was not a significant parameter. Higher disease activity assessed by PCDAI and PUCAI resulted in worse social functioning ("mild/moderate" *vs* "in remission", average difference -13.2 ± 2.3, P < 0.001) and the presence of extra-intestinal manifestations, also, was related to lower scores compared to no extra-intestinal manifestations (average difference: -12.1 ± 4.9, P = 0.015).

Body image and treatment/interventions: For both, body image and treatment/interventions domains, no statistically significant relationship to the assessed disease characteristics or prescribed medications was found. Regarding PGA, in general, the previously described trends were also observed, although correlations for body image and treatment/intervention domains in UC patients (P = 0.6 and P = 0.3, respectively), and treatment/intervention in CD patients (P = 0.07) did not reach statistical significance (Tables 3, 4 and 5).

DISCUSSION

The results of the present study indicate that disease activity is the major factor associated with low QOL in children with IBD. The analysis demonstrated a clear inverse relationship between disease activity and IMPACT-III, total and subdomain, scores. The same trend was observed for CD and UC patients, separately. Additionally, it was shown that physician's assessment (through the PGA scale) was a strong correlate of QOL. Interestingly, in the multivariate analysis, PGA absorbed the statistical significance of disease activity, as a correlate of IMPACT-III total score. This could reflect the greater ability of physician's perspective to detect subtle variations in the patient's physical and psychological status. These findings are in accordance with the majority of previous studies, which also found disease activity as the major negative predictor of QOL in IBD patients[8-17].

Published data have been inconsistent regarding comparisons of QOL between patients with CD and UC. Some reports suggest that no such differences exist[18,19], whereas, others describe poorer QOL in patients with CD, due to its worse clinical course, constant need of treatment and higher likelihood for surgery[20]. In our analysis, CD patients reported better QOL scores compared to UC patients, although the difference was not statistically significant. In the subdomain analyses statistical significance was reached for the emotional functioning domain. Notably, CD patients in our study were older and were more likely to be receiving an anti-TNF agent. Both these parameters have been shown to improve QOL, although for the latter no such association was recorded in our sample[21,22].

The assessment of the association between age and QOL in pediatric IBD populations has generated controversial results. We recorded a positive, statistically significant association between age and total IMPACT-III scores, independently of PGA classification. In 2002, Loonen *et al*[23] concluded that adolescents have impaired QOL scores compared to younger children, whereas Gallo *et al*[18] found no association. An interesting study published by Deepal *et al*[24] in 2012, supported that post-colectomy QOL in UC patients was better when the diagnosis was made under the age of twelve. A possible interpretation of our result is that, as children grow into adolescence, they may be able to develop more efficient coping mechanisms and therefore be less vulnerable to the psychological effect of a chronic disease.

An area which has been sparsely investigated is the impact of disease duration and received mediations on QOL. Although some studies failed to find any correlation[19,25], others have indicated improved QOL scores with longer disease duration[9,26,27]. Similarly to the latter, we also showed that disease duration is positively correlated to IMPACT-III total scores. Moreover, in subdomain-analyses, the same positive trend was observed for bowel symptoms, emotional functioning and social functioning. This finding could reflect an adaption process to a new life-style, which, particularly for growing, peri-pubertal children, may be cumbersome and prolonged. In pediatric IBD, bowel symptoms, emotional and social issues are of major concern and their course contribute significantly to QOL[28].

Our findings, also, suggest that, with the exception of steroids, type of medication had no effect on QOL. Nevertheless, patients on steroid therapy

scored significantly lower in the systemic symptoms domain and CD patients on steroids recorded impaired bowel symptoms domain subscores. Surprisingly, for systemic symptoms, the effect was retained for the group of patients in remission, after stratifying according to PGA class, whereas for bowel symptoms in CD it was marginally lost. This is probably due to the fact that patients usually receive steroids during and shortly after a flare, when systemic symptoms are present or recent, consequently affecting their QOL. Another interpretation could be that steroid therapy can cause mood impairments, although this should affect the emotional functioning of the patients as well. Furthermore, steroid therapy imposes dietary restrictions and requires supplemental medication (vitamin D). All the above might contribute to the observed effect. Previous studies have, also, demonstrated the negative impact of steroid therapy in the QOL of patients[29,30]. In contrast to recent reports, we did not find any association between the use of biological agents and IMPACT-III, total and subdomain, scores[21,22,31].

An interesting observation, not frequently reported in literature, was the negative association between extra-intestinal manifestations and emotional functioning. This seems to be in accordance with one previous study which concluded that musculoskeletal manifestations had a detrimental effect on QOL[32]. In our population, three patients had sclerosing cholangitis and two patients suffered from type Ⅱ peripheral arthritis. The latter has a clinical course independent of IBD activity[33]; therefore it could contribute to an impaired QOL even if a patient is in remission. The former is known to have an irreversible, progressive course leading ultimately to liver failure. It would be reasonable to assume that the knowledge of having such a destructive, chronic, untreatable disease, severely affects emotional functioning in these patients.

The main drawback of our study is the cross-sectional nature of the analysis that does not permit detection of causal relations. Furthermore small subsample sizes may have prevented some comparisons from reaching statistical significance due to reduced power.

In conclusion, The QOL of patients with IBD is directly and mainly dependent on the activity of their disease and this relationship is optimally assessed by PGA rather than activity indices. Extra-intestinal manifestations and use of steroids should always raise the concern of impaired QOL, even if clinical remission of intestinal disease has been achieved. Disease duration and age have a positive impact on QOL; therefore the first years after the diagnosis, particularly in younger children, is the most sensitive period requiring intensive, supportive interventions.

COMMENTS

Background

The inflammatory bowel diseases (IBD) are chronic conditions of the intestines requiring frequent hospitalizations and long-term medications. The risk for surgeries, complications and extra-intestinal manifestations is increased in children suffering from these disorders. All the above result in a significant psychosocial burden which negatively affects their quality of life (QOL).

Research frontiers

The spectrum of the clinical evolution of a child suffering from IBD presents many fluctuations and is affected by the age of onset, disease duration, medications and medication-related side effects, and disease-related complications, such as surgeries and extra-intestinal manifestations. The main research question, which is of direct clinical importance, is the identification of factors that predispose in impaired QOL. This would allow treating physicians to, timely, intervene in a timely manner and try to minimize the negative consequences, which is a top priority in growing adolescents.

Innovations and breakthroughs

Several, different, disease-related parameters were found to influence the QOL in pediatric IBD patients, such as disease activity, use of steroids and extra-intestinal manifestations. The significant relation of the physician's global assessment to the QOL underlines the importance of the clinician's subjective impression, apart from the standardized activity indices. The time frame of maximum vulnerability appears to be during the early years after the diagnosis, especially in younger children

Applications

The observations derived from the present study could serve as a guide for identifying IBD sub-groups at high risk for impaired QOL. Based on these results, physicians treating children with IBD, could implement early strategies in order to manage, or optimally prevent, significant deteriorations. On the other hand, focused research on these high-risk patients could help to clarify the biologic and psychological mechanisms underlying the above described processes.

Terminology

IBD is a group of chronic intestinal disorders, which may present in early childhood. QOL encompasses information from different aspects of a child's perception on everyday life. It can be assessed by the IMPACT-III questionnaire which is a self-reporting, validated, structured scale offering quantitative assessment on the QOL, overall, as well as on distinct sub-domains.

Peer-review

This study was well conducted and nicely written, it can be of assistance to the scientific community.

REFERENCES

1 **Kunz JH**, Hommel KA, Greenley RN. Health-related quality of life of youth with inflammatory bowel disease: a comparison with published data using the PedsQL 4.0 generic core scales. *Inflamm Bowel Dis* 2010; **16**: 939-946 [PMID: 19998462 DOI: 10.1002/ibd.21128]

2 **Timmer A**, Preiss JC, Motschall E, Rücker G, Jantschek G, Moser G. Psychological interventions for treatment of inflammatory bowel disease. *Cochrane Database Syst Rev* 2011; (**2**): CD006913 [PMID: 21328288 DOI: 10.1002/14651858.CD006913.pub2]

3 **Kilroy S**, Nolan E, Sarma KM. Quality of life and level of anxiety in youths with inflammatory bowel disease in Ireland. *J Pediatr Gastroenterol Nutr* 2011; **53**: 275-279 [PMID: 21865974 DOI: 10.1097/MPG.0b013e318214c131]

4 **Levine A**, Koletzko S, Turner D, Escher JC, Cucchiara S, de Ridder L, Kolho KL, Veres G, Russell RK, Paerregaard A, Buderus S, Greer ML, Dias JA, Veereman-Wauters G, Lionetti P, Sladek M, Martin de Carpi J, Staiano A, Ruemmele FM, Wilson DC. ESPGHAN revised porto criteria for the diagnosis of inflammatory bowel disease in children and adolescents. *J Pediatr Gastroenterol Nutr* 2014; **58**: 795-806 [PMID: 24231644 DOI: 10.1097/MPG.0000000000000239]

5 **Hyams JS**, Ferry GD, Mandel FS, Gryboski JD, Kibort PM, Kirschner BS, Griffiths AM, Katz AJ, Grand RJ, Boyle JT. Development and validation of a pediatric Crohn's disease activity index. *J Pediatr Gastroenterol Nutr* 1991; **12**: 439-447 [PMID: 1678008 DOI: 10.1097/00005176-199105000-00005]

6 **Turner D**, Otley AR, Mack D, Hyams J, de Bruijne J, Uusoue K, Walters TD, Zachos M, Mamula P, Beaton DE, Steinhart AH, Griffiths AM. Development, validation, and evaluation of a pediatric ulcerative colitis activity index: a prospective multicenter study. *Gastroenterology* 2007; **133**: 423-432 [PMID: 17681163 DOI: 10.1053/j.gastro.2007.05.029]

7 **Otley A**, Smith C, Nicholas D, Munk M, Avolio J, Sherman PM, Griffiths AM. The IMPACT questionnaire: a valid measure of health-related quality of life in pediatric inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 2002; **35**: 557-563 [PMID: 12394384 DOI: 10.1097/00005176-200210000-00018]

8 **Bernklev T**, Jahnsen J, Aadland E, Sauar J, Schulz T, Lygren I, Henriksen M, Stray N, Kjellevold O, Vatn M, Moum B. Health-related quality of life in patients with inflammatory bowel disease five years after the initial diagnosis. *Scand J Gastroenterol* 2004; **39**: 365-373 [PMID: 15125469 DOI: 10.1080/00365520310008386]

9 **Otley AR**, Griffiths AM, Hale S, Kugathasan S, Pfefferkorn M, Mezoff A, Rosh J, Tolia V, Markowitz J, Mack D, Oliva-Hemker M, Wyllie R, Rothbaum R, Bousvaros A, Del Rosario JF, Evans J, Blanchard W, Hyams J. Health-related quality of life in the first year after a diagnosis of pediatric inflammatory bowel disease. *Inflamm Bowel Dis* 2006; **12**: 684-691 [PMID: 16917222 DOI: 10.1097/00054725-200608000-00003]

10 **Graff LA**, Walker JR, Lix L, Clara I, Rawsthorne P, Rogala L, Miller N, Jakul L, McPhail C, Ediger J, Bernstein CN. The relationship of inflammatory bowel disease type and activity to psychological functioning and quality of life. *Clin Gastroenterol Hepatol* 2006; **4**: 1491-1501 [PMID: 17162241 DOI: 10.1016/j.cgh.2006.09.027]

11 **Hill R**, Lewindon P, Muir R, Grangé I, Connor F, Ee L, Withers G, Cleghorn G, Davies P. Quality of life in children with Crohn disease. *J Pediatr Gastroenterol Nutr* 2010; **51**: 35-40 [PMID: 20410845 DOI: 10.1097/MPG.0b013e3181c2c0ef]

12 **Hjortswang H**, Ström M, Almer S. Health-related quality of life in Swedish patients with ulcerative colitis. *Am J Gastroenterol* 1998; **93**: 2203-2211 [PMID: 9820397 DOI: 10.1111/j.1572-0241.1998.00537.x]

13 **Perrin JM**, Kuhlthau K, Chughtai A, Romm D, Kirschner BS, Ferry GD, Cohen SA, Gold BD, Heyman MB, Baldassano RN, Winter HS. Measuring quality of life in pediatric patients with inflammatory bowel disease: psychometric and clinical characteristics. *J Pediatr Gastroenterol Nutr* 2008; **46**: 164-171 [PMID: 18223375 DOI: 10.1097/MPG.0b013e31812f7f4e]

14 **Engelmann G**, Erhard D, Petersen M, Parzer P, Schlarb AA, Resch F, Brunner R, Hoffmann GF, Lenhartz H, Richterich A. Health-related quality of life in adolescents with inflammatory bowel disease depends on disease activity and psychiatric comorbidity. *Child Psychiatry Hum Dev* 2015; **46**: 300-307 [PMID: 24838299 DOI: 10.1007/s10578-014-0471-5]

15 **Gray WN**, Denson LA, Baldassano RN, Hommel KA. Disease activity, behavioral dysfunction, and health-related quality of life in adolescents with inflammatory bowel disease. *Inflamm Bowel Dis* 2011; **17**: 1581-1586 [PMID: 21674715 DOI: 10.1002/ibd.21520]

16 **Abdovic S**, Mocic Pavic A, Milosevic M, Persic M, Senecic-Cala I, Kolacek S. The IMPACT-III (HR) questionnaire: a valid measure of health-related quality of life in Croatian children with inflammatory bowel disease. *J Crohns Colitis* 2013; **7**: 908-915 [PMID: 23333037 DOI: 10.1016/j.crohns.2012.12.010]

17 **Szigethy E**, McLafferty L, Goyal A. Inflammatory bowel disease. *Child Adolesc Psychiatr Clin N Am* 2010; **19**: 301-318, ix [PMID: 20478501 DOI: 10.1016/j.chc.2010.01.007]

18 **Gallo J**, Grant A, Otley AR, Orsi M, MacIntyre B, Gauvry S, Lifschitz C. Do parents and children agree? Quality-of-life assessment of children with inflammatory bowel disease and their parents. *J Pediatr Gastroenterol Nutr* 2014; **58**: 481-485 [PMID: 24663034 DOI: 10.1097/MPG.0000000000000236]

19 **Kalafateli M**, Triantos C, Theocharis G, Giannakopoulou D, Koutroumpakis E, Chronis A, Sapountzis A, Margaritis V, Thomopoulos K, Nikolopoulou V. Health-related quality of life in patients with inflammatory bowel disease: a single-center experience. *Ann Gastroenterol* 2013; **26**: 243-248 [PMID: 24714279]

20 **Cohen RD**. The quality of life in patients with Crohn's disease. *Aliment Pharmacol Ther* 2002; **16**: 1603-1609 [PMID: 12197839 DOI: 10.1046/j.1365-2036.2002.01323.x]

21 **Szabó D**, Kökönyei G, Arató A, Dezsőfi A, Molnár K, Müller KE, Lakatos PL, Papp M, Lovász BD, Golovics PA, Cseh A, Veres G. Autoregressive cross-lagged models of IMPACT-III and Pediatric Crohn's Disease Activity Indexes during one year infliximab therapy in pediatric patients with Crohn's disease. *J Crohns Colitis* 2014; **8**: 747-755 [PMID: 24434181 DOI: 10.1016/j.crohns.2013.12.020]

22 **Vogelaar L**, Spijker AV, van der Woude CJ. The impact of biologics on health-related quality of life in patients with inflammatory bowel disease. *Clin Exp Gastroenterol* 2009; **2**: 101-109 [PMID: 21694833 DOI: 10.2147/ceg.s4512]

23 **Loonen HJ**, Grootenhuis MA, Last BF, Koopman HM, Derkx HH. Quality of life in paediatric inflammatory bowel disease measured by a generic and a disease-specific questionnaire. *Acta Paediatr* 2002; **91**: 348-354 [PMID: 12022311 DOI: 10.1080/08035250252834049]

24 **Dalal DH**, Patton D, Wojcicki JM, Clark AL, Garnett EA, Heyman MB. Quality of life in patients postcolectomy for pediatric-onset ulcerative colitis. *J Pediatr Gastroenterol Nutr* 2012; **55**: 425-428 [PMID: 22437468 DOI: 10.1097/MPG.0b013e318253f2f0]

25 **Pallis AG**, Vlachonikolis IG, Mouzas IA. Assessing health-related quality of life in patients with inflammatory bowel disease, in Crete, Greece. *BMC Gastroenterol* 2002; **2**: 1 [PMID: 11866863 DOI: 10.1186/1471-230x-2-1]

26 **Jäghult S**, Saboonchi F, Johansson UB, Wredling R, Kapraali M. Identifying predictors of low health-related quality of life among patients with inflammatory bowel disease: comparison between Crohn's disease and ulcerative colitis with disease duration. *J Clin Nurs* 2011; **20**: 1578-1587 [PMID: 21418363 DOI: 10.1111/j.1365-2702.2010.03614.x]

27 **Haapamäki J**, Turunen U, Roine RP, Färkkilä MA, Arkkila PE. Impact of demographic factors, medication and symptoms on disease-specific quality of life in inflammatory bowel disease. *Qual Life Res* 2009; **18**: 961-969 [PMID: 19629750 DOI: 10.1007/s11136-009-9514-y]

28 **Griffiths AM**, Nicholas D, Smith C, Munk M, Stephens D, Durno C, Sherman PM. Development of a quality-of-life index for pediatric inflammatory bowel disease: dealing with differences related to age and IBD type. *J Pediatr Gastroenterol Nutr* 1999; **28**: S46-S52 [PMID: 10204526 DOI: 10.1097/00005176-199904001-00009]

29 **Bernklev T**, Jahnsen J, Schulz T, Sauar J, Lygren I, Henriksen M, Stray N, Kjellevold Ø, Aadland E, Vatn M, Moum B. Course of disease, drug treatment and health-related quality of life in patients with inflammatory bowel disease 5 years after initial diagnosis. *Eur J Gastroenterol Hepatol* 2005; **17**: 1037-1045 [PMID: 16148548 DOI: 10.1097/00042737-200510000-00006]

30 **Romberg-Camps MJ**, Bol Y, Dagnelie PC, Hesselink-van de Kruijs MA, Kester AD, Engels LG, van Deursen C, Hameeteman WH, Pierik M, Wolters F, Russel MG, Stockbrügger RW. Fatigue and health-related quality of life in inflammatory bowel disease: results from a population-based study in the Netherlands: the IBD-South Limburg cohort. *Inflamm Bowel Dis* 2010; **16**: 2137-2147 [PMID: 20848468 DOI: 10.1002/ibd.21285]

31 **DeBoer MD**, Barnes BH, Stygles NA, Sutphen JL, Borowitz SM. Changes in inflammation and QoL after a single dose of infliximab during ongoing IBD treatment. *J Pediatr Gastroenterol Nutr* 2012; **54**: 486-490 [PMID: 21946833 DOI: 10.1097/MPG.0b013e3182382ee3]

32 **van der Have M**, Brakenhoff LK, van Erp SJ, Kaptein AA, Leenders M, Scharloo M, Veenendaal RA, van der Heijde DM, van der Meulen-de Jong AE, Hommes DW, Fidder HH. Back/joint pain, illness perceptions and coping are important predictors of quality of life and work productivity in patients with inflammatory

bowel disease: a 12-month longitudinal study. *J Crohns Colitis* 2015; **9**: 276-283 [PMID: 25547976 DOI: 10.1093/ecco-jcc/jju025]
33 **Peluso R**, Manguso F, Vitiello M, Iervolino S, Di Minno MN. Management of arthropathy in inflammatory bowel diseases. *Ther Adv Chronic Dis* 2015; **6**: 65-77 [PMID: 25729557 DOI: 10.1177/2040622314563929]

P- Reviewer: Capasso R, Gazouli M, Hokama A, Lakatos PL
S- Editor: Gong ZM **L- Editor**: A **E- Editor**: Liu WX

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v23.i6.1076

World J Gastroenterol 2017 February 14; 23(6): 1076-1089
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Observational Study

Simple pain measures reveal psycho-social pathology in patients with Crohn's disease

Shmuel Odes, Michael Friger, Ruslan Sergienko, Doron Schwartz, Orly Sarid, Vered Slonim-Nevo, Terri Singer, Elena Chernin, Hillel Vardi, Dan Greenberg; Israel IBD Research Nucleus

Shmuel Odes, Terri Singer, Department of Gastroenterology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer Sheba 84105, Israel

Michael Friger, Ruslan Sergienko, Elena Chernin, Hillel Vardi, Department of Public Health, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer Sheba 84105, Israel

Doron Schwartz, Department of Gastroenterology and Hepatology, Soroka Medical Center, Soroka Medical Center, Beersheba 84101, Israel

Orly Sarid, Vered Slonim-Nevo, Spitzer Department of Social Work, Ben-Gurion University of the Negev, Beer Sheba 84105, Israel

Dan Greenberg, Department of Health Systems Management, Faculty of Health Sciences Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

Author contributions: Odes S, Sergienko R, Schwartz D, Sarid O and Slonim-Nevo V contributed to study conception and design; Odes S, Sergienko R, Singer T, Schwartz D, Chernin E and Vardi H made contributions to acquisition of data; Odes S, Friger M and Sergienko R contributed to analysis and interpretation of data; Odes S and Sergienko R contributed to drafting of manuscript; all authors revised and made final approval of manuscript.

Supported by a generous grant from The Leona M. and Harry B. Helmsley Charitable Trust.

Conflict-of-interest statement: None to declare.

Data sharing statement: The principal author may be contacted at odes@bgu.ac.il for enquiries pertaining to the data-set. All data are anonymous.

Manuscript source: Unsolicited manuscript

Correspondence to: Shmuel Odes, MD, Department of Gastroenterology, Faculty of Health Sciences, Ben-Gurion University of the Negev, PO Box 653, Beer Sheba 84105, Israel. odes@bgu.ac.il
Telephone: +972-522700752
Fax: +972-86233083

Received: September 23, 2016
Peer-review started: September 26, 2016
First decision: November 9, 2016
Revised: November 20, 2016
Accepted: December 8, 2016
Article in press: December 8, 2016
Published online: February 14, 2017

Abstract

AIM

To determine whether pain has psycho-social associations in adult Crohn's disease (CD) patients.

METHODS

Patients completed demographics, disease status, Patient Harvey-Bradshaw Index (P-HBI), Short Form Health Survey (SF-36), Short Inflammatory Bowel Disease Questionnaire (SIBDQ), and five socio-psychological questionnaires: Brief Symptom Inventory, Brief COPE Inventory, Family Assessment Device, Satisfaction with Life Scale, and Work Productivity and Activity Impairment Questionnaire. Pain sub-scales in P-HBI, SF-36 and SIBDQ measures were recoded into 4 identical scores for univariate and multinomial logistic regression analysis of associations with psycho-social variables.

RESULTS

The cohort comprised 594 patients, mean age 38.6 ± 14.8 years, women 52.5%, P-HBI 5.76 ± 5.15. P-HBI, SF-36 and SIBDQ broadly agreed in their assessment of pain intensity. More severe pain was significantly associated with female gender, low socio-economic status, unemployment, Israeli birth and smoking. Higher pain scores correlated positively with psychological stress, dysfunctional coping strategies, poor family relationships, absenteeism, presenteeism, productivity loss and activity impairment and all WPAI sub-scores. Patients exhibiting greater satisfaction with life had less pain. The regression showed increasing odds ratios for psychological stress (lowest 2.26, highest 12.17) and female gender (highest 3.19) with increasing pain. Internet-recruited patients were sicker and differed from hardcopy questionnaire patients in their associations with pain.

CONCLUSION

Pain measures in P-HBI, SF-36 and SIBDQ correlate with psycho-social pathology in CD. Physicians should be aware also of these relationships in approaching CD patients with pain.

Key words: Crohn's disease; Psycho-social pathology; Pain

Core tip: Pain is a very important symptom in patients with Crohn's disease. Pain level and frequency are measurable with a series of simple questionnaires. We show that pain has demographic associations concerning gender, economic status, birthplace and smoking, as well as psycho-social associations such as disease coping strategies, family support, satisfaction with life, absenteeism and presenteeism related to the workplace, and leisure activity. Understanding these relationships will assist physicians in their approach to patients with pain.

Odes S, Friger M, Sergienko R, Schwartz D, Sarid O, Slonim-Nevo V, Singer T, Chernin E, Vardi H, Greenberg D; Israel IBD Research Nucleus. Simple pain measures reveal psycho-social pathology in patients with Crohn's disease. *World J Gastroenterol* 2017; 23(6): 1076-1089 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i6/1076.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i6.1076

INTRODUCTION

Crohn's disease (CD) is an idiopathic inflammatory condition of the gastrointestinal tract, most commonly affecting the small and large intestines, and causing diarrhea, pain, malaise, weight loss and anemia. Abdominal pain is the commonest form of pain in patients with CD. It constitutes a major diagnostic criterion of CD in epidemiological studies and the first therapeutic target in CD patient management[1-5]. Over 50% of adult patients with active CD reported having abdominal pain[6,7]. Interestingly, pain is also present when CD is not active. Pain was present in 20% to 50% of patients in clinical remission[1,8,9]. It has been suggested that in these cases the pain results from persistent peripheral sensitization after the acute CD episode has passed, and that this hypersensitivity is augmented by psychological stressors[10]. Concern about pain was reported to be higher in some countries than others; it is reportedly higher in patients in Israel compared to some other countries[11]. Up to a third of patients need to take analgesics for abdominal pain[9]. Medical cannabis is increasingly used to relieve abdominal pain in CD[12]. It was shown that dependence on medication for pain was associated with poorer health status[13]. Pain results in impaired socio-psychological functioning and a reduced quality of life[9,14]. Abdominal pain in CD is associated with depression and increased anxiety[15,16].

The above-quoted studies indicate that while the intensity of pain in CD is a consequence of the pathology of the disease, it is related also to the psychological functioning of these individuals in response to illness-induced stress, and may be moderated by the coping mechanisms used by patients to deal with their illness, and perhaps by demographic variables. These important relationships are as yet poorly understood, and further knowledge in this area may contribute to improving the treatment of these patients. We aimed to investigate the relationship of pain to psychological functioning and disease-coping in the broad spectrum of CD patients of different demographic status. We report here the results of our study performed in a country-wide large non-selected community cohort of CD patients.

MATERIALS AND METHODS

Patients

Consecutive adult (age 18 years and over) patients consenting to take part in an ongoing socio-economic study of CD in the Israeli adult patient population were studied using self-report questionnaires. Patients were eligible to participate whatever the duration or severity of their illness, and irrespective of their past and present treatments and surgery (if any). There were two methods of patient recruitment. Most patients (70%) were recruited on a consecutive basis when presenting for follow-up or acute non-hospitalized care at the out-patient Gastroenterology Departments of five participating university-affiliated tertiary care hospitals in Israel. These patients met the standard criteria for diagnosis as CD (ECCO), and were given the option of completing the questionnaires on paper or on the internet in their own time at home. The other patients were canvassed on the website of "The Israel Foundation for

Crohn's Disease and Ulcerative Colitis" and completed the questionnaires on-line. It was assumed that these patients would have established CD. Physicians and nurses did not assist in completing the questionnaires. All questionnaires were in the public domain and were made available in their validated Hebrew translations. Knowledge of Hebrew was a condition for inclusion in the study. Charts of hospital-recruited patients were checked to uncover any psychological or psychiatric disease, but this information could not be ascertained for patients recruited by the internet.

Study design

This was a cross-sectional study with data collection from July 2013 to June 2016. Patients reported socio-demographic and medical characteristics including gender, year and place of birth, education, economic status, marital status and number of children, religion and religiosity, current and past smoking habits, disease duration, current medications, anytime surgery for CD, and hospitalizations for CD in the past year. Data concerning co-morbidities were collectible from most patients attending at the hospitals. Patients completed the Patient Harvey-Bradshaw Index (P-HBI), Short Form Health Survey (SF-36) and Short Inflammatory Bowel Disease Questionnaire (SIBDQ), all of which include questions about pain. In addition, patients completed five socio-psychological questionnaires: Brief Symptom Inventory (BSI), Brief COPE Inventory (COPE), Family Assessment Device (FAD), Satisfaction with Life Scale (SWLS) and Work Productivity and Activity Impairment Questionnaire (WPAI).

P-HBI[17]: This clinical measure of the severity of disease was specifically designed for patients with CD. It consists of 4 items reflecting the previous day's symptoms and signs of CD; the question regarding the physician's assessment of the possible presence of an abdominal mass in the original HBI is removed in the P-HBI, making the questionnaire suitable for completion by the patients themselves. A total score < 5 indicates disease remission, 5-7 mild disease, 8-16 moderate disease, and > 16 severe disease.

SF-36[18]: This generic health-related quality of life measure is comprised of 36 items divided into eight domains, which in turn are grouped as Physical Health Summary Score (physical functioning, role-physical, bodily pain, general health) and Mental Health Summary Score (vitality, role-emotional, social functioning, mental health). Responses refer to the past four weeks. The range of the Physical or Mental Health Summary Score is 0-100. A higher score represents a better quality of life. The Hebrew version has been validated[19].

SIBDQ[20]: Is an inflammatory bowel disease-specific health-related quality of life tool measuring physical, social, and emotional status. It consists of 10 items: each item refers to the last two weeks, and is rated on a 7 degree scale (1 = all the time, 7 = never). The total score is in the range from 10-70. A higher value indicates a better quality of life. A validated Hebrew version was used[21].

BSI[22]: This instrument is a measure of psychological stress in the past month. It consists of 53-items that assess nine symptomatic dimensions (depression, somatization, obsession-compulsive, interpersonal sensitivity, anxiety, hostility, phobic anxiety, paranoid ideation, and psychoticism) on a 0-4 scale; a higher score implies more psychological distress. The General Severity Index (GSI) yields a useful global summary score called the GSI with range 0-4. In non-patient normal individuals the GSI was reported as 0.30 ± 0.31. The Hebrew version was validated[23].

Brief COPE Inventory[24]: This measure comprises 28 items; each item is rated on a 4 degree scale (1 = I do not do it at all, 4 = I do it very much). Items are grouped to yield 14 coping subscales that are grouped into 3 strategies: emotion-focused (emotional support use, positive reframing, humor, acceptance, religion), problem-focused (active coping, instrumental support use, planning), and dysfunctional coping (self-distraction, denial, substance use, behavioral disengagement, venting, self-blame). A greater score indicates more use of that coping strategy. The Brief COPE presents the present condition of the subject. We used the validated version in Hebrew[25].

FAD[26]: This is a scale that measures the level of perceptions of family functioning and communication. It consists of 12 items; each item can be rated on a 4 degree scale (1 = strongly agree, 4 = not agree at all). A higher value indicates a worse family functioning. This measure has a Cronbach's Alpha = 0.89. It has been validated in Hebrew[27].

SWLS[28]: This instrument measures the individual's level of satisfaction with life at that moment in time. It includes five questions (q): "q1, my life is close to ideal; q2, conditions of my life are excellent; q3, I am satisfied with my life; q4, I have gotten the important things I want in life; q5, if I could live my life over, I would change almost nothing." Each question is rated on a 7-point scale (1 = not agree at all with the item, 7 = strongly agree). The possible range of this scale is from 1-7 per question. The summary score has a range of 5-35, with a higher value indicating a higher level of satisfaction with life. Cronbach's alpha was 0.89. This measure has been validated in Hebrew[29].

WPAI[30]: This measure evaluates the effect of disease on the patient's ability to work and to perform regular

Table 1 Details of pain questions and scoring[1]

Questionnaire	Question about pain	Score in questionnaire	Recoded score
Patient Harvey-Bradshaw Index	Did you have abdominal pains yesterday?	0 None	0
		1 Mild	1
		2 Moderate	2
		3 Severe	3
MOS Short-Form Survey Instrument	How much bodily pain have you had during the past 4 wk?	1 None	0
		2 Very Mild	0
		3 Mild	1
		4 Moderate	1
		5 Severe	2
		6 Very Severe	3
Short Inflammatory Bowel Disease Questionnaire	How often during the past 2 wk have you been troubled by pain in the abdomen?	1 All of the time	3
		2 Most of the time	2
		3 A good bit of the time	2
		4 Some of the time	1
		5 A little of the time	1
		6 Hardly any of the time	0
		7 None of the time	0

[1]Pain questions in the three questionnaires with original scoring, and the recoded scores used in the analysis.

activities in the past 7 d (not including the present day). This instrument yields 4 scores: absenteeism (work time missed due to disease), presenteeism (impairment while working, *i.e.*, reduced on-the-job effectiveness due to disease) work productivity loss (overall work impairment, *i.e.*, the sum of absenteeism plus presenteeism) and activity impairment (degree that disease impairs regular activities). Scores are expressed as percentages. Higher scores indicate greater impairment at work or when performing activities. The Hebrew version of this measure was accessed from the internet[31].

Statistical analysis

All data from the questionnaires were pooled in a single database. The questions relating to pain were question 2 in P-HBI, question 4 in SIBDQ and question 21 in SF-36. These questions emphasized different aspects of pain and differed by the time period under review and the possible responses. Patients whose data were deemed eligible for analysis were required to have filled in all 3 questions patients; with any missing values were excluded. Based on the frequency of patients' responses to these questions, 4 sub-scores (no pain, mild pain, moderate pain, severe pain) were formulated for each pain scale and used in the analysis (Table 1). Results are expressed as means (± SD), and medians (IQR) where the data distribution was skewed. Univariate analysis was used to show the significance of associations of pain with demographic and socio-psychological variables. We used the Mann-Whitney test, Kruskal-Wallis test, *t*-test, and Spearman correlations to test the significance of associations depending on the type of distribution of the data. A multinomial logistic regression was used to examine the associations between the level of pain (in the three scales) and those demographic and socio-psychological variables that were significant on univariate analysis. Each pain questionnaire was examined separately, and the "no pain" state was the reference category. The model controlled for age, education, economic status and family status. Statistical significance was set at $P < 0.05$. Since the analysis revealed large differences between patients filling in the questionnaires by internet or hardcopy, these results are shown separately.

Ethical considerations

The study was approved by the Ethics Committees of all participating hospitals and the patients recruited at these hospitals signed an approved informed consent form. Patients recruited *via* the website were deemed to have consented to participate in the study when they completed the questionnaires electronically. The consent form contained a description of the study, its aims and scope. A similar explanation was posted on the website. All data were treated anonymously.

RESULTS

Patients

The total cohort comprised 594 patients with mean age (± SD) 38.6 ± 14.8 years, and 57.6% were women. Duration of disease was 11.05 ± 8.73 years in the entire cohort; 10.8% of patients reported a disease duration of 2 years or less. The P-HBI was 5.76 ± 5.15; 44.6% of the patients were in remission and 55.4% had various grades of active disease. Further demographic data of the cohort are given in Table 2. Very few patients (< 5%) were found to have mild psychological comorbidities and they were included in the analysis since this did not impact on the outcome of the study. In the entire cohort 45.1% of patients were on biologic medication. These patients reported more pain by the P-HBI ($P = 0.03$) compared with those not on biologic medication. However, there were no differences in respect of the level or frequency of pain by SF-36 or SIBDQ.

We compared the patients who completed the questionnaires by internet or as hardcopy (Table 2). Internet patients had a lower economic status, higher disease activity level by P-HBI score and worse quality of life compared to the hardcopy patients.

Questionnaires

Results of the socio-psychological questionnaires appear in Table 3. In the total cohort the SF-36 summary scores were: physical 42.09 ± 10.76, and mental 41.99 ± 11.33. The SIBDQ total score was 46.33 ± 13.83. Half the patients reported their economic status as moderate. The mean score for satisfaction with life was moderate at 22.06 ± 7.64. The GSI mean score of 0.98 ± 0.75 indicated a mild psychological distress level in the cohort, but the FAD mean score of 1.81 ± 0.55 revealed moderate disturbance of family

Table 2 Demographic parameters and disease characteristics of the Crohn's disease cohort

Patient characteristic	Total cohort *n* = 594	Internet questionnaire *n* = 370	Hardcopy questionnaire *n* = 224	*P* value[1]
Age (yr)				0.151
mean ± SD	38.56 ± 14.06	36.99 ± 12.65	39.48 ± 14.77	
Median (min-max) (IQR)	35 (18-79) (28-47)	35 (18-72) (26 -44)	35 (19-79) (28-49)	
Education (yr)				0.043
mean ± SD	14.81 ± 2.93	15.05 ± 2.65	14.66 ± 3.08	
Disease duration (yr)				0.234
mean ± SD	11.05 ± 8.73	10.39 ± 8.23	11.45 ± 9.00	
Median (min-max) (IQR)	10 (0-47) (4-15.5)	10 (0-41) (3-16)	10 (0-47) (5-15)	
Female gender	57.6%	59.78%	56.90%	0.521
Married/living together	58.6%	57.01%	60.16%	0.452
Economic status				
Good	29.8%	25.45%	33.15%	0.040
Moderate	49.8%	57.27%	46.58%	
Poor	18.9%	17.27%	20.27%	
Current cigarette smoking	18.9%	16.97%	21.55%	0.183
Biologic medication	45.1%	44.64%	45.41%	0.856
Surgery, ever	33.3%	32.59%	33.78%	0.765
Hospitalization in past year	25.3%	26.79%	24.32%	0.503
Patient Harvey-Bradshaw Index (P-HBI)	5.76 ± 5.15	6.70 ± 5.69	5.32 ± 4.83	0.002
P-HBI sub-groups				
Disease remission (score < 5)	44.60%	66 (40.00%)	199 (55.74%)	0.003
Mild disease (score 5-7)	20.00%	47 (28.48%)	72 (20.17%)	
Moderate disease (score 8-16)	19.40%	40 (24.24%)	75 (21.01%)	
Severe disease (score > 16)	3.90%	12 (7.27%)	11 (3.08%)	

[1]Statistical differences between internet and hardcopy source of questionnaires.

Table 3 Scores of the social questionnaires of the Crohn's disease cohort

Variables	Total cohort mean ± SD Median (min-max) (IQR)	Internet Questionnaire mean ± SD Median (min-max) (IQR)	Hardcopy Questionnaire mean ± SD Median (min-max) (IQR)	*P* value[1]
MOS Short-Form Survey Instrument				
Physical health	42.09 ± 10.76	40.88 ± 10.41	42.72 ± 10.90	0.041
Mental health	41.99 ± 11.33	39.23 ± 11.36	43.42 ± 11.05	< 0.001
Short Inflammatory Bowel Disease Questionnaire, total score	46.33 ± 13.83	42.02 ± 13.38	48.84 ± 13.48	< 0.001
SWLS	22.06 ± 7.64	20.81 ± 7.92	22.82 ± 7.37	0.004
	23 (5-35) (16-28)	21.0 (5-35) (15-27)	24.0 (5-35) (17-29)	
GSI	0.98 ± 0.75	1.11 ± 0.80	0.90 ± 0.70	0.002
	0.79 (0-3.92) (0.38-1.47)	0.9 (0.0-3.9) (0.4-1.6)	0.7 (0.0-3.2) (0.4-1.3)	
FAD	1.81 ± 0.55	1.90 ± 0.56	1.75 ± 0.53	0.001
	1.75 (1.0-4.0) (1.33-2.17)	1.9 (1.0-4.0) (1.4-2.3)	1.7 (1.0-4.0) (1.3-2.1)	
COPE: Emotion-focused strategies	24.23 ± 5.88	24.50 ± 5.77	24.07 ± 5.94	0.340
	24.5 (3-40) (20-29)	25 (6-39) (20-29)	24 (3-40) (20-28)	
COPE: Problem-focused strategies	16.10 ± 4.74	16.82 ± 4.51	15.67 ± 4.83	0.004
	16 (3-24) (13-20)	17 (4-24) (14-20)	16 (3-24) (12-19)	
COPE: Dysfunctional Strategies	22.28 ± 5.93	23.41 ± 5.86	21.60 ± 5.87	0.000
	22 (6-42) (18-26)	23 (8-41) (20-27)	21 (6-42) (17-25)	
WPAI: Absenteeism (%)	8.81 ± 19.26	11.12 ± 20.77	7.36 ± 18.16	0.021
	0 (0-100) (0-8.35)	0 (0-100.0) (0-15.2)	0 (0-100) (0-1.6)	
WPAI: Presenteeism (%)	29.16 ± 30.20	31.52 ± 30.44	27.54 ± 30.00	0.119
	20 (0-100) (0-50)	20 (0-100.0) (10.0-55.0)	20.0 (0-100) (0-50)	
WPAI: Work productivity loss (%)	29.19 ± 30.38	33.60 ± 31.57	26.50 ± 29.38	0.025
	20 (0-100) (0-50)	21.2 (0-100) (10-60)	20.0 (0-100) (0-40.7)	
WPAI: Activity impairment (%)	33.92 ± 30.61	37.60 ± 30.86	31.74 ± 30.30	0.021
	30 (0-100) (10-60)	30 (0-100) (10-60)	20.0 (0-100) (0-50)	

[1]Statistical differences between internet and hardcopy source of questionnaires. SWLS: Satisfaction with Life Scale; GSI: Global Severity Index; FAD: McMaster Family Assessment Device; COPE: Brief Cope Inventory; WPAI: Work Productivity and Activity Impairment Questionnaire.

functioning. Patients made greater use of emotion-focused and dysfunctional coping strategies compared with problem-focused strategies. Concerning the work productivity of the patients, 8.81% reported absenteeism from work and 29.19% reported loss of productivity while at work. Nearly 30% of patients reported

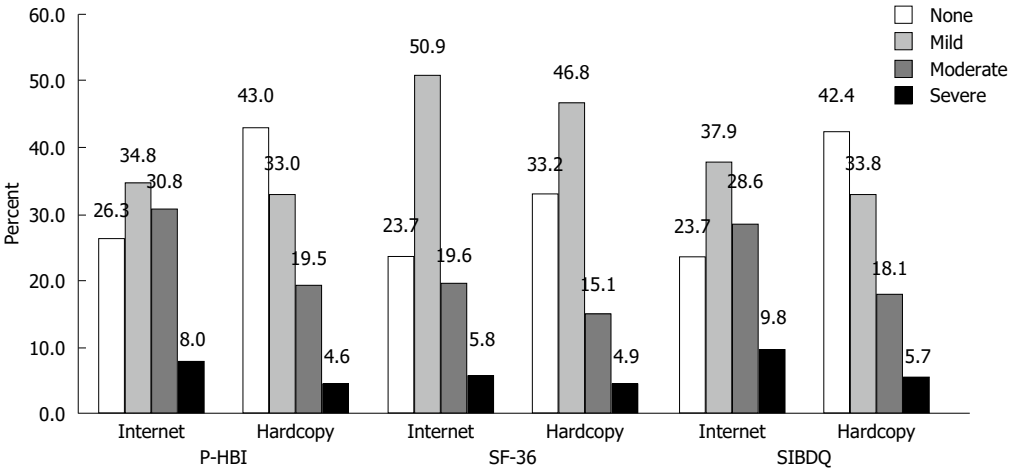

Figure 1 Responses to the pain questions by the Patient Harvey-Bradshaw Index, Short Form Health Survey and Short Inflammatory Bowel Disease Questionnaire, for patients completing the questionnaires by internet or hardcopy. Patient Harvey-Bradshaw Index (P-HBI) and Short Form Health Survey (SF-36) measure pain intensity whereas Short Inflammatory Bowel Disease Questionnaire (SIBDQ) measures pain frequency. *P* values for differences in responses to the pain questions are: by P-HBI $P < 0.001$, by SF-36 $P = 0.081$, by SIBDQ $P < 0.001$.

overall work impairment, and one third of the patients responded that their disease impaired regular daily activities. The data shown separately for internet and hardcopy patients also appear in Table 3. Significant differences between these groups are noted for quality of life measures, SWLS, GSI, FAD, problem-focused coping, dysfunctional coping, and three of the WPAI measures. Internet patients had lower quality and satisfaction of life scores and more psychological stress compared with hardcopy patients. Internet patients also reported having more problems with family support. Furthermore, internet patients made greater use of problem-focused and dysfunctional coping than hardcopy patients. The internet patients had more absenteeism from work, were less productive and had more activity impairment compared with hardcopy patients.

The mean scores (± SD) of the pain questions in the three questionnaires were the following: P-HBI 0.99 ± 0.92, SF-36 52.79 ± 28.44, and SIBDQ 4.46 ± 1.82. The median scores (IQR) of the pain questions were: P-HBI 1 (0-2), SF-36 40 (40-80), and SIBDQ 5 (3-6), respectively. The distribution of the patients' responses to the pain questions (Figure 1) indicated that the responses to P-HBI and SIBDQ were in close agreement, whereas the responses to SF-36 revealed relatively more patients reporting mild pain. Internet patients reported more pain intensity or frequency compared with hardcopy patients with respect to the pain scores by P-HBI and SIBDQ; the differences were statistically significant (both $P < 0.001$). Demographic variables associated significantly with the degree of reported pain by all three pain measures are shown in Table 4. By the P-HBI measure, females had more frequent moderate and severe pain than males (33.7% *vs* 24.2%, $P = 0.005$). Likewise, the P-HBI showed significantly more frequent moderate and severe pain in patient with poorer economic status, birthplace in Israel and not working. By the SF-36 pain measure, a similar result was noted for female gender, poorer economic status, Asia-Africa birthplace and not working. Again, by the SIBDQ pain measure, more frequent moderate and severe pain was noted for poor economic status, birthplace in Israel, current smoker and not working. In Tables 5 and 6 these data are shown separately for the internet and hardcopy patients. It will be noted that the statistically significant differences occur more in the hardcopy part of the cohort.

Pain measures

The results of the five socio-psychological measures were examined in relation to the results of the pain measures (Table 7). More intense pain (moderate and severe pain rather than no pain or mild pain) by P-HBI was noted for GSI, emotion-focused coping strategies, dysfunctional coping strategies, FAD, and all four WPAI analyses. For SF-36 the variables significantly associated with more intense pain were GSI, problem-focused strategies, dysfunctional coping strategies, FAD, and all four WPAI analyses. For the SIBDQ pain measure the significant associations with more intense pain were noted for GSI, dysfunctional coping strategies, FAD, and again all four WPAI analyses. On the other hand, a greater satisfaction with life score was significantly associated with less pain by P-HBI, SF-36 and SIBDQ pain measures (all $P < 0.0001$). The differences described here in the total cohort occurred in both the hardcopy and internet patients (Tables 8 and 9).

Regression analysis

A multinomial logistic regression analysis of demographic and social variables and intensity of pain was carried out. The results of the internet and hardcopy patients are shown separately in Table 10, which is designed in particular to show the differences between

Table 4 Comparison of demographic variables with the pain measures in the whole cohort *n* (%)

Variables	P-HBI					SF-36					SIBDQ				
	No pain	Mild pain	Moderate pain	Severe pain	*P* value	No pain	Mild pain	Moderate pain	Severe pain	*P* value	No pain	Mild pain	Moderate pain	Severe pain	*P* value
Education (yr)	15.1 ± 2.9	14.7 ± 2.8	14.8 ± 3.1	13.5 ± 3.1	0.017	14.8 ± 2.7	15.0 ± 2.7	14.6 ± 3.8	14.3 ± 3.1	0.463	15.1 ± 3.0	14.9 ± 2.8	14.7 ± 3.0	13.3 ± 2.5	0.001
Gender					0.005					< 0.001					
Female	100 (32.1)	107 (34.3)	87 (27.9)	18 (5.8)		74 (23.7)	156 (50.0)	62 (19.9)	20 (6.4)		103 (33.0)	105 (33.7)	81 (26.0)	23 (7.4)	0.054
Male	103 (45.4)	69 (30.4)	40 (17.6)	15 (6.6)		88 (38.8)	106 (46.7)	25 (11.0)	8 (3.5)		96 (42.3)	76 (33.5)	39 (17.2)	16 (7.0)	
Economic status					< 0.001					< 0.001					
Poor	22 (19.6)	36 (32.1)	40 (35.7)	14 (12.5)		11 (9.8)	57 (50.9)	33 (29.5)	11 (9.8)		17 (15.2)	36 (32.1)	38 (33.9)	21 (18.8)	< 0.001
Moderate	105 (35.5)	110 (37.2)	65 (22.0)	16 (5.4)		96 (32.4)	150 (50.7)	39 (13.2)	11 (3.7)		112 (37.8)	104 (35.1)	64 (21.6)	16 (5.4)	
Good	88 (49.7)	49 (27.7)	35 (19.8)	5 (2.8)		66 (37.3)	78 (44.1)	25 (14.1)	8 (4.5)		79 (44.6)	66 (37.3)	26 (14.7)	6 (3.4)	
Birthplace					0.048					0.015					
Western	19 (76.0)	3 (12.0)	2 (8.0)	1 (4.0)		12 (48.0)	9 (36.0)	1 (4.0)	3 (12.0)		19 (76.0)	4 (16.0)	1 (4.0)	1 (4.0)	0.013
Asia-Africa	14 (43.8)	12 (37.5)	5 (15.6)	1 (3.1)		7 (21.9)	16 (50.0)	9 (28.1)	0 (0)		13 (40.6)	13 (40.6)	6 (18.8)	0 (0.0)	
FSU	15 (44.1)	15 (44.1)	3 (8.8)	1 (2.9)		14 (41.2)	19 (55.9)	0 (0.0)	1 (2.9)		20 (58.8)	9 (26.5)	4 (11.8)	1 (2.9)	
Israel	111 (39.9)	92 (33.1)	61 (21.9)	14 (5.0)		90 (32.4)	128 (46.0)	46 (16.5)	14 (5.0)		105 (37.8)	98 (35.3)	56 (20.1)	19 (6.8)	
Current smoker					0.052					0.170					
No	175 (38.5)	152 (33.5)	104 (22.9)	23 (5.1)		140 (30.8)	220 (48.5)	75 (16.5)	19 (4.2)		172 (37.9)	156 (34.4)	102 (22.5)	24 (5.3)	< 0.001
Yes	29 (25.9)	41 (36.6)	32 (28.6)	10 (8.9)		29 (25.9)	52 (46.4)	21 (18.8)	10 (8.9)		26 (23.2)	43 (38.4)	25 (22.3)	18 (16.1)	
Working					< 0.001					< 0.001					
No	61 (32.8)	51 (27.4)	51 (27.4)	23 (12.4)		40 (21.5)	86 (46.2)	45 (24.2)	15 (8.1)		60 (32.3)	53 (28.5)	50 (26.9)	23 (12.4)	< 0.001
Yes	152 (39.9)	140 (36.7)	77 (20.2)	12 (3.1)		130 (34.1)	183 (48.0)	52 (13.6)	16 (4.2)		147 (38.6)	143 (37.5)	74 (19.4)	17 (4.5)	

P-HBI and SF-36 measure pain intensity whereas SIBDQ measures pain frequency. Data are mean ± SD or *n* (%). P-HBI: Patient Harvey-Bradshaw Index; SF-36: Short Form Health Survey; SIBDQ: Short Inflammatory Bowel Disease Questionnaire.

these patient groups with regard to the magnitude of the associations. The association was significant for GSI with all three pain measures and for all three results of pain in hardcopy patients, while for internet patients the associations were significant with all measures except mild pain by P-HBI and SF-36. Furthermore, there was a progressive increase in the odds ratio with increasing intensity of pain in all three measures for the internet and hardcopy patients. Female gender too demonstrated a significant association with SF-36 for moderate and severe intensities of pain, likewise with an increasing odds ratio, only for the hardcopy patients. For the P-HBI and SIBDQ measures, however, the regression with female gender was significant only for moderate pain, again limited to hardcopy patients. On regression analysis, the association of dysfunctional coping strategies was significant for mild pain by both P-HBI (OR = 1.07) and SF-36 (OR = 1.07), and for moderate pain by SIBDQ (OR = 1.10), for hardcopy patients. Problem-focused coping in hardcopy patients was associated with all levels of pain by P-HBI. Internet patients did not demonstrate any association of pain and coping strategies at the 5% statistical level, and few associations at the 10% level.

DISCUSSION

We have shown in the present study, carried out in a large cohort of CD patients with disease duration of 11 years, that the severity of pain as measured by the questions from the P-HBI, SF-36 and SIBDQ has significant associations with demographic and psycho-social measures. Patients with more intense pain tended to be females, poorer, unemployed, more stressed, less satisfied with life, and Israeli-born rather than immigrants. The pain questions from P-HBI, SF-36 and SIBDQ showed good general agreement in many of these associations. Internet patients had more active disease and lower scores for lower quality of life, and differed in their correlations with pain compared with the hardcopy patients.

Table 5 Comparison of demographic variables with the pain measures - Internet *n* (%)

Variables	P-HBI					SF-36					SIBDQ				
	No pain	Mild pain	Moderate pain	Severe pain	*P* value	No pain	Mild pain	Moderate pain	Severe pain	*P* value	No pain	Mild pain	Moderate pain	Severe pain	*P* value
Education (yr)	15.2 ± 2.5	15.1 ± 2.5	15.0 ± 2.7	14.4 ± 3.4	0.758	14.9 ± 2.7	15.2 ± 2.3	15.1 ± 2.9	13.8 ± 4.2	0.397	15.1 ± 2.9	15.5 ± 2.4	14.9 ± 2.6	13.6 ± 2.8	0.033
Gender															
Female	28 (54.9)	37 (62.7)	37 (63.8)	8 (50.0)	0.628	18 (41.9)	62 (65.3)	23 (63.9)	7 (70.0)	0.055	26 (55.3)	37 (60.7)	38 (65.5)	9 (50.0)	0.589
Male	23 (45.1)	22 (37.3)	21 (36.2)	8 (50.0)		25 (58.1)	33 (34.7)	13 (36.1)	3 (30.0)		21 (44.7)	24 (39.3)	20 (34.5)	9 (50.0)	
Economic status															
Bad	4 (6.9)	12 (16.0)	16 (23.2)	6 (33.3)	0.003	2 (3.9)	18 (15.9)	12 (27.9)	6 (46.2)	0.001	2 (3.8)	13 (15.5)	12 (19.4)	11 (50.0)	< 0.001
Medium	25 (43.1)	13 (17.3)	15 (21.7)	3 (16.7)		21 (41.2)	24 (21.2)	8 (18.6)	3 (23.1)		19 (36.5)	23 (27.4)	11 (17.7)	3 (13.6)	
Good	29 (50.0)	50 (66.7)	38 (55.1)	9 (50.0)		28 (54.9)	71 (62.8)	23 (53.5)	4 (30.8)		31 (59.6)	48 (57.1)	39 (62.9)	8 (36.4)	
Current Smoker															
Not Smoking	52 (91.2)	62 (81.6)	53 (76.8)	14 (87.5)	0.175	44 (84.6)	95 (84.1)	33 (80.5)	9 (75.0)	0.821	46 (90.2)	70 (83.3)	49 (79.0)	16 (76.2)	0.353
Smoking	5 (8.8)	14 (18.4)	16 (23.2)	2 (12.5)		8 (15.4)	18 (15.9)	8 (19.5)	3 (25.0)		5 (9.8)	14 (16.7)	13 (21.0)	5 (23.8)	
Working															
Not Working	13 (24.1)	21 (29.6)	17 (29.8)	10 (55.6)	0.093	8 (17.0)	31 (31.3)	15 (36.6)	7 (53.8)	0.044	13 (26.0)	18 (24.3)	19 (33.3)	11 (57.9)	0.033
Working	41 (75.9)	50 (70.4)	40 (70.2)	8 (44.4)		39 (83.0)	68 (68.7)	26 (63.4)	6 (46.2)		37 (74.0)	56 (75.7)	38 (66.7)	8 (42.1)	

P-HBI and SF-36 measure pain intensity whereas SIBDQ measures pain frequency. Data are mean ± SD or *n* (%). P-HBI: Patient Harvey-Bradshaw Index; SF-36: Short Form Health Survey; SIBDQ: Short Inflammatory Bowel Disease Questionnaire.

Table 6 Comparison of demographic variables with the pain measures - Hardcopy *n* (%)

Variables	P-HBI					SF-36					SIBDQ				
	No pain	Mild pain	Moderate pain	Severe pain	*P* value	No pain	Mild pain	Moderate pain	Severe pain	*P* value	No pain	Mild pain	Moderate pain	Severe pain	*P* value
Education (yr)	15.1 ± 3.0	14.5 ± 2.9	14.5 ± 3.3	12.5 ± 2.4	0.002	14.7 ± 2.7	14.8 ± 3.0	14.3 ± 4.4	14.6 ± 2.0	0.411	15.1 ± 3.1	14.5 ± 3.0	14.5 ± 3.3	12.9 ± 2.2	0.004
Gender															
Female	72 (47.4)	70 (59.8)	50 (72.5)	10 (58.8)	0.005	56 (47.1)	94 (56.3)	39 (76.5)	13 (72.2)	0.002	77 (50.7)	68 (56.7)	43 (69.4)	14 (66.7)	0.067
Male	80 (52.6)	47 (40.2)	19 (27.5)	7 (41.2)		63 (52.9)	73 (43.7)	12 (23.5)	5 (27.8)		75 (49.3)	52 (43.3)	19 (30.6)	7 (33.3)	
Economic status															
Bad	18 (11.5)	24 (20.0)	24 (33.8)	8 (47.1)	< 0.001	9 (7.4)	39 (22.7)	21 (38.9)	5 (29.4)	< 0.001	15 (9.6)	23 (18.9)	26 (39.4)	10 (47.6)	< 0.001
Medium	76 (48.4)	60 (50.0)	27 (38.0)	7 (41.2)		68 (55.7)	79 (45.9)	16 (29.6)	7 (41.2)		81 (51.9)	56 (45.9)	25 (37.9)	8 (38.1)	
Good	63 (40.1)	36 (30.0)	20 (28.2)	2 (11.8)		45 (36.9)	54 (31.4)	17 (31.5)	5 (29.4)		60 (38.5)	43 (35.2)	15 (22.7)	3 (14.3)	
Current Smoker															
Not Smoking	123 (83.7)	90 (76.9)	51 (76.1)	9 (52.9)	0.026	96 (82.1)	125 (78.6)	42 (76.4)	10 (58.8)	0.178	126 (85.7)	86 (74.8)	53 (81.5)	8 (38.1)	< 0.001
Smoking	24 (16.3)	27 (23.1)	16 (23.9)	8 (47.1)		21 (17.9)	34 (21.4)	13 (23.6)	7 (41.2)		21 (14.3)	29 (25.2)	12 (18.5)	13 (61.9)	
Working															
Not Working	48 (30.2)	30 (25.0)	34 (47.9)	13 (76.5)	< 0.001	32 (26.0)	55 (32.4)	30 (53.6)	8 (44.4)	0.003	47 (29.9)	35 (28.7)	31 (46.3)	12 (57.1)	0.007
Working	111 (69.8)	90 (75.0)	37 (52.1)	4 (23.5)		91 (74.0)	115 (67.6)	26 (46.4)	10 (55.6)		110 (70.1)	87 (71.3)	36 (53.7)	9 (42.9)	

P-HBI and SF-36 measure pain intensity whereas SIBDQ measures pain frequency. Data are mean ± SD or *n* (%). P-HBI: Patient Harvey-Bradshaw Index; SF-36: Short Form Health Survey; SIBDQ: Short Inflammatory Bowel Disease Questionnaire.

Table 7 Comparison of social questionnaires with the pain measures in the whole cohort

Variables	P-HBI					SF-36					SIBDQ				
	No pain	Mild pain	Moderate pain	Severe pain	*P* value	No pain	Mild pain	Moderate pain	Severe pain	*P* value	No pain	Mild pain	Moderate pain	Severe pain	*P* value
GSI	0.6 ± 0.5	1.0 ± 0.6	1.3 ± 0.8	1.8 ± 1.0	< 0.001	0.6 ± 0.5	1.0 ± 0.7	1.4 ± 0.8	1.6 ± 0.9	< 0.001	0.6 ± 0.5	1.0 ± 0.7	1.3 ± 0.7	1.8 ± 0.9	< 0.001
COPE: Emotion-focused Strategies	23.4 ± 5.9	24.7 ± 5.7	25.0 ± 5.8	24.2 ± 7.1	0.045	23.4 ± 5.9	24.6 ± 5.9	24.2 ± 5.7	26.0 ± 5.8	0.154	23.6 ± 5.9	24.8 ± 5.7	24.8 ± 5.5	23.0 ± 7.3	0.059
COPE: Problem-focused Strategies	15.6 ± 4.9	16.1 ± 4.7	16.8 ± 4.4	16.6 ± 4.9	0.249	15.4 ± 5.1	16.1 ± 4.5	16.5 ± 4.5	18.2 ± 4.3	0.042	15.6 ± 5.1	16.2 ± 4.7	17.0 ± 4.1	15.4 ± 4.6	0.067
COPE: Dysfunctional Strategies	20.0 ± 5.5	22.9 ± 5.5	24.0 ± 5.8	25.6 ± 6.6	< 0.001	20.0 ± 5.7	22.7 ± 5.7	24.0 ± 5.9	25.9 ± 5.5	< 0.001	19.9 ± 5.4	22.8 ± 5.4	24.5 ± 6.0	24.6 ± 6.9	< 0.001
FAD	1.7 ± 0.5	1.8 ± 0.6	1.9 ± 0.5	2.1 ± 0.7	0.003	1.7 ± 0.6	1.8 ± 0.5	1.9 ± 0.5	1.8 ± 0.7	0.081	1.7 ± 0.5	1.9 ± 0.5	1.8 ± 0.6	2.0 ± 0.7	< 0.001
SWLS	24.3 ± 6.6	22.0 ± 7.7	19.9 ± 7.5	17.7 ± 9.8	< 0.001	24.5 ± 6.7	22.3 ± 7.1	18.4 ± 8.4	17.9 ± 9.1	< 0.001	24.8 ± 6.4	22.2 ± 7.3	19.2 ± 7.4	16.9 ± 9.8	< 0.001
WPAI: Absenteeism	3.9 ± 11.9	5.9 ± 12.4	18.2 ± 27.2	43.4 ± 37.6	< 0.001	2.8 ± 10.6	8.3 ± 18.6	21.3 ± 25.2	29.4 ± 35.0	< 0.001	1.8 ± 5.5	7.8 ± 18.3	19.1 ± 22.7	38.5 ± 42.2	< 0.001
WPAI: Presenteesism	14.6 ± 22.8	29.9 ± 28.0	44.4 ± 29.7	77.1 ± 26.4	< 0.001	13.0 ± 20.7	29.2 ± 26.1	55.0 ± 32.9	70.7 ± 34.1	< 0.001	10.6 ± 18.2	31.6 ± 27.5	48.2 ± 29.4	69.0 ± 31.1	< 0.001
WPAI: Work productivity loss	15.7 ± 23.3	29.5 ± 26.9	46.4 ± 32.4	80.6 ± 27.1	< 0.001	13.7 ± 20.9	30.0 ± 27.0	58.6 ± 33.2	65.3 ± 35.5	< 0.001	10.2 ± 15.9	32.7 ± 27.9	52.2 ± 30.9	66.6 ± 34.7	< 0.001
WPAI: Activity Impairment	17.1 ± 23.6	32.9 ± 26.4	51.1 ± 27.9	78.3 ± 23.5	< 0.001	14.3 ± 22.0	33.4 ± 25.4	59.8 ± 27.8	76.1 ± 28.8	< 0.001	12.3 ± 19.6	34.9 ± 25.4	52.7 ± 26.2	78.9 ± 24.1	< 0.001

P-HBI and SF-36 measure pain intensity whereas SIBDQ measures pain frequency. Data are mean ± SD. P-HBI: Patient Harvey-Bradshaw Index; SF-36: Short Form Health Survey; SIBDQ: Short Inflammatory Bowel Disease Questionnaire; GSI: Global Severity Index; COPE: Brief Cope Inventory; FAD: McMaster Family Assessment Device; SWLS: Satisfaction with Life Scale; WPAI: Work Productivity and Activity Impairment Questionnaire.

Table 8 Comparison of social questionnaires with the pain measures - Internet

Variables	P-HBI					SF-36					SIBDQ				
	No pain	Mild pain	Moderate pain	Severe pain	*P* value	No pain	Mild pain	Moderate pain	Severe pain	*P* value	No pain	Mild pain	Moderate pain	Severe pain	*P* value
GSI	0.7 ± 0.7	1.0 ± 0.7	1.3 ± 0.8	2.1 ± 0.9	< 0.001	0.7 ± 0.7	1.0 ± 0.7	1.5 ± 0.7	1.9 ± 1.1	< 0.001	0.7 ± 0.6	1.0 ± 0.7	1.4 ± 0.7	1.9 ± 1.0	< 0.001
COPE: Emotion-focused Strategies	23.4 ± 6.1	24.8 ± 5.5	25.5 ± 5.6	23.2 ± 6.3	0.158	23.5 ± 6.1	24.8 ± 6.0	24.7 ± 4.6	25.5 ± 5.7	0.655	24.4 ± 5.6	24.3 ± 5.8	25.2 ± 5.7	23.4 ± 6.5	0.525
COPE: Problem-focused Strategies	15.7 ± 4.8	16.8 ± 4.5	17.5 ± 4.0	18.3 ± 4.8	0.096	15.8 ± 5.1	16.7 ± 4.4	17.5 ± 4.0	19.7 ± 4.1	0.051	16.1 ± 4.5	16.7 ± 4.8	17.7 ± 3.8	16.6 ± 5.0	0.384
COPE: Dysfunctional Strategies	20.8 ± 5.8	23.4 ± 5.8	24.4 ± 5.2	27.7 ± 5.4	< 0.001	20.9 ± 5.7	23.2 ± 5.8	25.4 ± 4.9	27.8 ± 5.5	< 0.001	20.9 ± 5.4	22.8 ± 5.5	24.8 ± 5.8	27.4 ± 5.6	< 0.001
FAD	1.8 ± 0.6	1.9 ± 0.5	1.9 ± 0.5	2.2 ± 0.7	0.116	1.8 ± 0.6	1.9 ± 0.5	2.0 ± 0.5	2.1 ± 0.8	0.264	1.8 ± 0.6	1.9 ± 0.5	2.0 ± 0.6	2.1 ± 0.7	0.074
SWLS	24.1 ± 7.7	20.8 ± 7.6	19.4 ± 7.3	15.5 ± 8.5	< 0.001	24.8 ± 7.7	21.2 ± 6.7	16.1 ± 7.6	16.8 ± 10.4	< 0.001	24.1 ± 7.7	21.3 ± 7.3	18.6 ± 7.3	17.2 ± 9.5	< 0.001
WPAI: Absenteeism	5.2 ± 12.1	6.9 ± 12.8	17.3 ± 25.2	38.1 ± 40.7	0.002	3.4 ± 9.7	11.3 ± 21.7	17.1 ± 21.5	46.3 ± 35.8	< 0.001	1.6 ± 4.9	9.1 ± 18.5	18.9 ± 21.1	35.4 ± 50.3	< 0.001
WPAI: Presenteesism	15.2 ± 25.8	27.1 ± 26.2	43.2 ± 26.7	76.7 ± 29.6	< 0.001	12.7 ± 17.9	31.5 ± 27.8	51.4 ± 32.1	80.0 ± 24.5	< 0.001	9.1 ± 13.9	31.4 ± 29.9	44.6 ± 26.4	68.0 ± 35.8	< 0.001
WPAI: Work productivity loss	18.0 ± 26.1	29.2 ± 26.7	47.3 ± 30.3	78.2 ± 32.2	< 0.001	15.5 ± 20.6	32.6 ± 29.3	59.3 ± 32.0	78.5 ± 17.5	< 0.001	9.5 ± 14.4	32.6 ± 29.4	52.2 ± 28.9	62.1 ± 42.4	< 0.001
WPAI: Activity Impairment	16.5 ± 23.1	34.2 ± 26.2	48.6 ± 27.0	80.6 ± 25.7	< 0.001	16.3 ± 21.9	34.2 ± 26.0	62.8 ± 25.4	88.0 ± 18.7	< 0.001	11.5 ± 16.4	34.2 ± 25.6	48.6 ± 25.9	82.5 ± 24.5	< 0.001

P-HBI and SF-36 measure pain intensity whereas SIBDQ measures pain frequency. Data are mean ± SD. P-HBI: Patient Harvey-Bradshaw Index; SF-36: Short Form Health Survey; SIBDQ: Short Inflammatory Bowel Disease Questionnaire; GSI: Global Severity Index; COPE: Brief Cope Inventory; FAD: McMaster Family Assessment Device; SWLS: Satisfaction with Life Scale; WPAI: Work Productivity and Activity Impairment Questionnaire.

Pain

Pain is an important symptom in CD patients and features prominently in Patient Reported Outcome scales like the Crohn's Disease Activity Index (CDAI) and the P-HBI, as well as the health-related quality of life measures SIBDQ and SF-36. While measurement of pain by the patient's subjective response to 4 questions as in the CDAI and the P-HBI has been disputed as to its reliability, it nevertheless remains a widely accepted practice and its brevity makes it quite acceptable to patients. P-HBI and SIBDQ both ask about abdominal pain, which is the commonest form of pain in CD patients, present in about 70% of women and 65% of men[8,32]. SF-36 however enquires about bodily pain, which would include in particular rheumatological pain that is present in 30%-40% of CD cases, particularly in women[8,32]. The recall period of the P-HBI is just one day, adding to its reliability. For the SIBDQ and SF-36 the recall period is longer, 2 and 4 wk respectively. This longer recall period may explain

Table 9 Comparison of social questionnaires with the pain measures - Hardcopy

Variables	P-HBI					SF-36					SIBDQ				
	No pain	Mild pain	Moderate pain	Severe pain	*P* value	No pain	Mild pain	Moderate pain	Severe pain	*P* value	No pain	Mild pain	Moderate pain	Severe pain	*P* value
GSI	0.6 ± 0.5	1.0 ± 0.6	1.4 ± 0.7	1.6 ± 1.0	< 0.001	0.5 ± 0.5	1.0 ± 0.7	1.3 ± 0.8	1.4 ± 0.7	< 0.001	0.5 ± 0.5	1.0 ± 0.6	1.3 ± 0.8	1.7 ± 0.8	< 0.001
COPE: Emotion-focused Strategies	23.4 ± 5.8	24.6 ± 5.8	24.5 ± 6.0	25.3 ± 7.9	0.234	23.4 ± 5.9	24.4 ± 5.8	23.8 ± 6.4	26.3 ± 6.0	0.302	23.3 ± 6.0	25.1 ± 5.6	24.4 ± 5.3	22.5 ± 8.3	0.076
COPE: Problem-focused Strategies	15.6 ± 5.0	15.6 ± 4.8	16.1 ± 4.7	14.6 ± 4.4	0.725	15.2 ± 5.2	15.8 ± 4.6	15.8 ± 4.8	17.1 ± 4.3	0.642	15.4 ± 5.3	15.8 ± 4.6	16.4 ± 4.3	14.0 ± 3.8	0.192
COPE: Dysfunctional Strategies	19.8 ± 5.4	22.6 ± 5.3	23.5 ± 6.4	23.1 ± 7.2	< 0.001	19.7 ± 5.7	22.3 ± 5.5	22.9 ± 6.3	24.5 ± 5.1	< 0.001	19.6 ± 5.4	22.8 ± 5.3	24.1 ± 6.3	21.5 ± 7.0	< 0.001
FAD	1.7 ± 0.5	1.8 ± 0.6	1.8 ± 0.5	1.9 ± 0.7	0.088	1.7 ± 0.5	1.8 ± 0.5	1.8 ± 0.6	1.6 ± 0.5	0.224	1.7 ± 0.5	1.8 ± 0.5	1.7 ± 0.5	1.9 ± 0.6	0.016
SWLS	24.4 ± 6.1	22.7 ± 7.6	20.3 ± 7.7	20.2 ± 10.8	0.003	24.4 ± 6.3	23.0 ± 7.2	20.1 ± 8.6	18.6 ± 8.2	0.002	25.1 ± 5.8	22.7 ± 7.4	19.8 ± 7.5	16.5 ± 10.3	< 0.001
WPAI: Absenteeism	3.4 ± 11.9	5.3 ± 12.2	19.2 ± 29.5	57.8 ± 29.1	< 0.001	2.5 ± 11.0	6.4 ± 16.0	25.6 ± 28.4	19.7 ± 33.1	< 0.001	1.8 ± 5.8	6.8 ± 18.3	19.4 ± 24.8	41.1 ± 37.8	< 0.001
WPAI: Presenteesism	14.4 ± 21.4	31.5 ± 29.1	46.0 ± 33.5	78.0 ± 22.8	< 0.001	13.2 ± 22.2	27.5 ± 24.6	58.3 ± 33.8	64.4 ± 39.4	< 0.001	11.2 ± 19.7	31.7 ± 25.8	53.0 ± 32.6	70.0 ± 27.5	< 0.001
WPAI: Work productivity loss	14.8 ± 22.2	29.7 ± 27.2	45.5 ± 34.9	86.1 ± 11.3	< 0.001	12.9 ± 21.2	28.3 ± 25.4	58.0 ± 34.9	57.7 ± 41.9	< 0.001	10.4 ± 16.4	32.7 ± 27.0	52.3 ± 33.7	70.5 ± 29.6	< 0.001
WPAI: Activity Impairment	17.3 ± 23.9	32.1 ± 26.6	53.5 ± 28.6	75.7 ± 21.4	< 0.001	13.4 ± 22.1	32.8 ± 25.1	57.6 ± 29.4	69.4 ± 31.7	< 0.001	12.6 ± 20.6	35.5 ± 25.4	56.3 ± 26.2	75.0 ± 23.8	< 0.001

P-HBI and SF-36 measure pain intensity whereas SIBDQ measures pain frequency. Data are mean ± SD. GSI: Global Severity Index; COPE: Brief Cope Inventory; FAD: McMaster Family Assessment Device; SWLS: Satisfaction with Life Scale; WPAI: Work Productivity and Activity Impairment Questionnaire.

Table 10 Multinomial logistic regression analysis, stratified by source of questionnaire, of General Severity Index, gender, the three coping strategies and the intensity of pain1

Characteristic	No pain	Mild pain		Moderate pain		Severe pain	
		Internet OR (*P* value)	Hardcopy OR (*P* value)	Internet OR (*P* value)	Hardcopy OR (*P* value)	Internet OR (*P* value)	Hardcopy OR (*P* value)
Pain by HBI							
GSI	Ref.	1.74 (0.14)	2.67 (< 0.001)	2.89 (0.01)	6.13 (< 0.001)	8.51 (< 0.001)	11.66 (< 0.001)
Gender (female)	Ref.	1.11 (0.80)	1.63 (0.07)	1.03 (0.95)	3.43 (0.00)	0.76 (0.69)	2.43 (0.14)
Emotion-focused	Ref.	1.01 (0.81)	1.04 (0.21)	1.04 (0.35)	1.06 (0.15)	0.96 (0.56)	1.15 (0.03)
Problem-focused	Ref.	1.04 (0.53)	0.91 (0.02)	1.05 (0.44)	0.91 (0.04)	1.14 (0.19)	0.79 (< 0.001)
Dysfunctional	Ref.	1.04 (0.49)	1.07 (0.04)	1.02 (0.67)	1.04 (0.30)	1.02 (0.76)	1.01 (0.90)
Pain by SF36							
GSI	Ref.	2.05 (0.07)	3.22 (< 0.001)	5.87 (< 0.001)	6.20 (< 0.001)	10.12 (< 0.001)	6.69 (< 0.001)
Gender (female)	Ref.	2.22 (0.05)	1.42 (0.18)	2.41 (0.09)	4.29 (< 0.001)	3.60 (0.15)	3.82 (0.03)
Emotion-focused	Ref.	1.01 (0.86)	1.01 (0.76)	1.02 (0.71)	1.04 (0.42)	1.02 (0.81)	1.06 (0.35)
Problem-focused	Ref.	1.05 (0.47)	0.96 (0.32)	1.11 (0.16)	0.96 (0.39)	1.23 (0.10)	0.97 (0.74)
Dysfunctional	Ref.	1.05 (0.38)	1.07 (0.05)	0.98 (0.77)	1.05 (0.28)	1.03 (0.73)	1.07 (0.23)
Pain by SIBDQ							
GSI	Ref.	2.39 (0.03)	4.04 (< 0.001)	5.57 (< 0.001)	5.53 (< 0.001)	6.20 (< 0.001)	19.91 (< 0.001)
Gender (female)	Ref.	1.16 (0.72)	1.21 (0.50)	1.43 (0.44)	1.93 (0.07)	0.73 (0.62)	3.06 (0.08)
Emotion-focused	Ref.	0.97 (0.49)	1.06 (0.09)	0.98 (0.72)	1.01 (0.88)	0.97 (0.62)	1.10 (0.14)
Problem-focused	Ref.	1.04 (0.52)	0.91 (0.01)	1.08 (0.23)	0.96 (0.41)	0.88 (0.21)	0.79 (< 0.001)
Dysfunctional	Ref.	1.01 (0.91)	1.06 (0.06)	1.00 (0.99)	1.10 (0.01)	1.11 (0.14)	0.99 (0.83)

[1]Statistical differences from the reference value (No pain) are shown. GSI: General Severity Index.

why more patients reported mild pain intensity with SF-36 compared with the other measures. It is also possible that our method of recoding the 6 items in SF-36 to 4 scores corresponding to the questions of P-HBI may account for some of this difference. On the other hand, we recoded the SIBDQ as well, from 7 items to 4 scores, and still its agreement with P-HBI was very good. Thus, the longer recall period may be the explanation: that patients tend to become accustomed to pain over time and discount its intensity. In all, we showed that the combined use of the pain questions from all 3 measures was a useful tool to assess the severity of pain in this CD cohort. The use of the pain questions from the Harvey-Bradshaw Index (similar to P-HBI with an additional question regarding the presence or absence of an abdominal mass) and the SIBDQ was previously reported in a study of opiate use in CD patients in the United States, but no attempt was made to standardize these respective scores[33].

Pain in CD is treated with a variety of analgesics including nonsteroidal anti-inflammatory drugs, opiates and more recently cannabis preparations[12,33,34]. Pain in CD patients is reported as often being undertreated, as was found in a recent large Swiss study[8]. Treatment of pain is often neglected in the patient whose disease is controlled. Unfortunately the ethical limitations of our protocol did not allow of investigation of pain treatments in the cohort.

Predictors of pain

Predictors of abdominal pain in CD have been little investigated. In a pediatric CD cohort in the United States it was shown by multivariate analysis that pain was predicted by depression, weight loss and abdominal tenderness[14]. However, this cohort was composed entirely of subjects suffering from depression, which is known to exacerbate symptoms like pain in chronic illnesses[35]. In a Scandinavian study performed on distressed adults with CD, use of the SF-36 measure revealed that personality impacted on the pain subscale[36]. These two studies did not relate to patients without diagnosed confounding conditions. Our study is the first detailed attempt to our knowledge to unravel the factors that are associated with increased severity of pain in CD patients without psychological or psychiatric comorbidities. By using a self-selected large community cohort presenting all stages of the disease course we were able to investigate patients who are representative of the average patients attending outpatient facilities for on-going medical care. By using a broad spectrum of psycho-social questionnaires we were able to relate the measures of psychological stress, coping strategies, family functioning, satisfaction with life, and functioning at work and at leisure to the intensity of pain captured by the three pain questions. In the univariate analysis, working patients reported less intense pain than those unemployed, and in fact close to 40% of workers had no pain at all. Consistent with this finding, patients with a poorer economic status reported more pain by all three pain measures. Patients with a higher level of satisfaction with life score experienced significantly less pain. In the multinomial logistic regression analysis, stress as measured by the GSI was the variable most related to pain, with the odds ratio increasing progressively as the pain intensity rose. Gender behaved in a fairly similar fashion, with females having more intense pain than males, but with lower odds ratios. These observations show convincingly that the level of stress experienced by patients, as well as gender issues, requires careful clinical consideration in CD cases presenting with pain. It is well known that current smokers have a worse course of CD than non-smokers[37]. Our study adds to this knowledge by the new finding that current smokers experience significantly more pain than non-smokers.

It is well documented that CD patients are less productive than healthy controls and have more periods off work[38]. The literature on this topic has focused in general on the role of medical treatments, particularly the more successful biologic therapy, as well as abdominal surgery in improving the ability of these patients to work. The present study is the first documentation of the association of pain with both work impairment and a lower socioeconomic state. Women are reported to have more severe CD than men[39]. Women with CD also have a reduced quality of life compared with men[40]. Our study however is the first to explore the differences in pain severity and the impact of pain on several psycho-social variables. In healthy individuals and patients with CD there are no gender differences in satisfaction with life[41,42]. The present study indicates that patients with a greater satisfaction of life are healthier, with less pain.

Pain and coping measures

Coping with chronic diseases is an important mental resource to improve patients' well-being, but the variety of measures has resulted in a plethora of concepts regarding coping strategies[43]. We studied disease-coping strategies in relationship to pain using the COPE instrument, which clearly separates emotion-focused, problem-focused and dysfunctional coping strategies and avoids any overlap of component questions[24]. By univariate analysis we found that the dysfunctional coping strategy was significantly correlated with the intensity of pain in all three pain measures. This is not surprising, since this is in fact a negative coping mechanism which does not promote better control of the disease. In the regression analysis we found that dysfunctional coping was associated with mild or moderate pain by all three pain measures. The positively-orientated coping strategies, emotion-focused and problem-focused, showed few correlations with pain intensity. This is contrary to what we expected and the matter requires further investigation. Nevertheless,

these findings regarding coping mechanisms present a message for clinicians treating patients with pain: namely, that prompt referral to a psychologist versed in these matters may assist CD patients to cope correctly with their illness and may actually lead to reduction of their pain level, particularly when dysfunctional coping strategies are identified and averted.

The strengths of our study include the use of a large representative cohort and a series of well-accepted psycho-social instruments. The consistencies of the three pain questions demonstrate the validity of this method of assessing pain. One limitation was the use of recall tools, although a recent publication regarding patients with inflammatory bowel disease did find that patient recall was quite adequate for research purposes[44]. The lack of access to detailed clinical material was another limitation. Thus, we could not relate our findings to specific phenotypes of CD by the Montreal classification, nor were we able to document any treatments given for pain and relate them to our research. Furthermore, we could not determine the direction of the reported associations because of the cross-sectional design of our study. Future work should thus include long-term follow up of patients and knowledge of their phenotypic classification and analgesic medication. Moreover, an interventional program will be required to evaluate whether medical and psychological therapy can alleviate pain and its associations in these patients.

In conclusion, the pain questions in the P-HBI, SF-36 and SIBDQ, although differing in their focus, were related a variety of psycho-social pathologies in our CD cohorts. These are associations or correlations and of course cannot imply causality in a cross-sectional study. We suggest that clinicians apply these three simple questions in the busy clinic setting to determine the severity of pain even in those patients who appear to be in remission. In fact, patients could fill in this information in two or three minutes while waiting to be seen. This simple strategy may identify patients in need of psychological treatment.

ACKNOWLEDGMENTS

The following physicians assisted with patient ascertainment: Iris Dotan, Yehuda Chowers, Dan Turner, Abraham Eliakim, Shomron Ben-Horin, Alexander Fich, Arik Segal, Gil Ben-Yakov, Naim Abu-Freha, Daniella Munteanu, Alexander Rozental, Alexander Mushkalo, Nava Gaspar and Vitaly Dizengof.

COMMENTS

Background

Pain is a very prominent symptom in Crohn's disease (CD), and often is very disabling for patients and requires specific medication. Since patients with CD have socio-psychological disturbances, the authors wished to examine whether these have any associations with pain. Authors wished to determine if knowledge of such associations could be useful to the physicians who manage such patients.

Research frontiers

Intensive perusal of the literature indicated that subject matter of this article has not been researched previously.

Innovations and breakthroughs

Authors found that the pain questions forming part of three commonly used questionnaires in the clinical assessment of these patients were associated with demographic, social and psychological characteristics in these patients.

Applications

The authors suggest that the findings in this study serve as a guide in the clinical and psychological assessment of patients with Crohn's disease.

Terminology

The research makes use of a variety of questionnaires which are well known in psychology but are less familiar to physicians. These are described in detail in the methods section of the paper.

Peer-review

The reviewers of this paper have emphasized that pain is only one of several symptoms in these patients, that socio-demographics impact on this symptom, that patients filling in questionnaires by hardcopy or the internet might represent subsets of patients with important social and disease characteristics, that the use of questionnaires in translation requires validation, that medication type could influence the pain symptom and needs to be considered.

REFERENCES

1 **Bielefeldt K**, Davis B, Binion DG. Pain and inflammatory bowel disease. *Inflamm Bowel Dis* 2009; **15**: 778-788 [PMID: 19130619 DOI: 10.1002/ibd.20848]

2 **Farrokhyar F**, Marshall JK, Easterbrook B, Irvine EJ. Functional gastrointestinal disorders and mood disorders in patients with inactive inflammatory bowel disease: prevalence and impact on health. *Inflamm Bowel Dis* 2006; **12**: 38-46 [PMID: 16374257 DOI: 10.1097/01.MIB.0000195391.49762.89]

3 **Minderhoud IM**, Oldenburg B, Wismeijer JA, van Berge Henegouwen GP, Smout AJ. IBS-like symptoms in patients with inflammatory bowel disease in remission; relationships with quality of life and coping behavior. *Dig Dis Sci* 2004; **49**: 469-474 [PMID: 15139501 DOI: 10.1023/B:DDAS.0000020506.84248.f9]

4 **Agnarsson U**, Björnsson S, Jóhansson JH, Sigurdsson L. Inflammatory bowel disease in Icelandic children 1951-2010. Population-based study involving one nation over six decades. *Scand J Gastroenterol* 2013; **48**: 1399-1404 [PMID: 24164345 DOI: 10.3109/00365521.2013.845799]

5 **Peyrin-Biroulet L**, Sandborn W, Sands BE, Reinisch W, Bemelman W, Bryant RV, D'Haens G, Dotan I, Dubinsky M, Feagan B, Fiorino G, Gearry R, Krishnareddy S, Lakatos PL, Loftus EV, Marteau P, Munkholm P, Murdoch TB, Ordás I, Panaccione R, Riddell RH, Ruel J, Rubin DT, Samaan M, Siegel CA, Silverberg MS, Stoker J, Schreiber S, Travis S, Van Assche G, Danese S, Panes J, Bouguen G, O'Donnell S, Pariente B, Winer S, Hanauer S, Colombel JF. Selecting Therapeutic Targets in Inflammatory Bowel Disease (STRIDE): Determining Therapeutic Goals for Treat-to-Target. *Am J Gastroenterol* 2015; **110**: 1324-1338 [PMID: 26303131 DOI: 10.1038/ajg.2015.233]

6 **Burkhalter H**, Stucki-Thür P, David B, Lorenz S, Biotti B, Rogler G, Pittet V. Assessment of inflammatory bowel disease patient's needs and problems from a nursing perspective. *Digestion* 2015; **91**: 128-141 [PMID: 25677558 DOI: 10.1159/000371654]

7 **Zeitz J**, Ak M, Müller-Mottet S, Scharl S, Biedermann L, Fournier N, Frei P, Pittet V, Scharl M, Fried M, Rogler G, Vavricka S. Pain in IBD Patients: Very Frequent and Frequently Insufficiently Taken

into Account. *PLoS One* 2016; **11**: e0156666 [PMID: 27332879 DOI: 10.1371/journal.pone.0156666]
8 **Srinath AI**, Goyal A, Zimmerman LA, Newara MC, Kirshner MA, McCarthy FN, Keljo D, Binion D, Bousvaros A, DeMaso DR, Youk A, Szigethy EM. Predictors of abdominal pain in depressed pediatric inflammatory bowel disease patients. *Inflamm Bowel Dis* 2014; **20**: 1329-1340 [PMID: 24983975 DOI: 10.1097/MIB.0000000000000104]
9 **Morrison G**, Van Langenberg DR, Gibson SJ, Gibson PR. Chronic pain in inflammatory bowel disease: characteristics and associations of a hospital-based cohort. *Inflamm Bowel Dis* 2013; **19**: 1210-1217 [PMID: 23524595 DOI: 10.1097/MIB.0b013e318280e729]
10 **Spiller RC**. Overlap between irritable bowel syndrome and inflammatory bowel disease. *Dig Dis* 2009; **27** Suppl 1: 48-54 [PMID: 20203497 DOI: 10.1159/000268121]
11 **Levenstein S**, Li Z, Almer S, Barbosa A, Marquis P, Moser G, Sperber A, Toner B, Drossman DA. Cross-cultural variation in disease-related concerns among patients with inflammatory bowel disease. *Am J Gastroenterol* 2001; **96**: 1822-1830 [PMID: 11419836 DOI: 10.1111/j.1572-0241.2001.03878.x]
12 **Storr M**, Devlin S, Kaplan GG, Panaccione R, Andrews CN. Cannabis use provides symptom relief in patients with inflammatory bowel disease but is associated with worse disease prognosis in patients with Crohn's disease. *Inflamm Bowel Dis* 2014; **20**: 472-480 [PMID: 24407485 DOI: 10.1097/01.MIB.0000440982.79036.d6]
13 **Drossman DA**, Li Z, Leserman J, Patrick DL. Ulcerative colitis and Crohn's disease health status scales for research and clinical practice. *J Clin Gastroenterol* 1992; **15**: 104-112 [PMID: 1401820 DOI: 10.1097/00004836-199209000-00005]
14 **Hashash JG**, Ramos-Rivers C, Youk A, Chiu WK, Duff K, Regueiro M, Binion DG, Koutroubakis I, Vachon A, Benhayon D, Dunn MA, Szigethy EM. Quality of Sleep and Coexistent Psychopathology Have Significant Impact on Fatigue Burden in Patients With Inflammatory Bowel Disease. *J Clin Gastroenterol* 2016; Epub ahead of print [PMID: 27775960]
15 **Goodhand JR**, Wahed M, Mawdsley JE, Farmer AD, Aziz Q, Rampton DS. Mood disorders in inflammatory bowel disease: relation to diagnosis, disease activity, perceived stress, and other factors. *Inflamm Bowel Dis* 2012; **18**: 2301-2309 [PMID: 22359369 DOI: 10.1002/ibd.22916]
16 **Reigada LC**, Hoogendoorn CJ, Walsh LC, Lai J, Szigethy E, Cohen BH, Bao R, Isola K, Benkov KJ. Anxiety symptoms and disease severity in children and adolescents with Crohn disease. *J Pediatr Gastroenterol Nutr* 2015; **60**: 30-35 [PMID: 25187105 DOI: 10.1097/MPG.0000000000000552]
17 **Bennebroek Evertsz' F**, Hoeks CC, Nieuwkerk PT, Stokkers PC, Ponsioen CY, Bockting CL, Sanderman R, Sprangers MA. Development of the patient Harvey Bradshaw index and a comparison with a clinician-based Harvey Bradshaw index assessment of Crohn's disease activity. *J Clin Gastroenterol* 2013; **47**: 850-856 [PMID: 23632348 DOI: 10.1097/MCG.0b013e31828b2196]
18 **Ware JE**, Sherbourne CD. The MOS 36-item short-form health survey (SF-36). I. Conceptual framework and item selection. *Med Care* 1992; **30**: 473-483 [PMID: 1593914 DOI: 10.1097/00005650-199206000-00002]
19 **Lewin-Epstein N**, Sagiv-Schifter T, Shabtai EL, Shmueli A. Validation of the 36-item short-form Health Survey (Hebrew version) in the adult population of Israel. *Med Care* 1998; **36**: 1361-1370 [PMID: 9749659 DOI: 10.1097/00005650-199809000-00008]
20 **Irvine EJ**, Zhou Q, Thompson AK. The Short Inflammatory Bowel Disease Questionnaire: a quality of life instrument for community physicians managing inflammatory bowel disease. CCRPT Investigators. Canadian Crohn's Relapse Prevention Trial. *Am J Gastroenterol* 1996; **91**: 1571-1578 [PMID: 8759664]
21 **Burisch J**, Weimers P, Pedersen N, Cukovic-Cavka S, Vucelic B, Kaimakliotis I, Duricova D, Bortlik M, Shonová O, Vind I, Avnstrøm S, Thorsgaard N, Krabbe S, Andersen V, Dahlerup JF, Kjeldsen J, Salupere R, Olsen J, Nielsen KR, Manninen P, Collin P, Katsanos KH, Tsianos EV, Ladefoged K, Lakatos L, Ragnarsson G, Björnsson E, Bailey Y, O'Morain C, Schwartz D, Odes S, Valpiani D, Boni MC, Jonaitis L, Kupcinskas L, Turcan S, Barros L, Magro F, Lazar D, Goldis A, Nikulina I, Belousova E, Fernandez A, Sanroman L, Almer S, Zhulina Y, Halfvarson J, Arebi N, Diggory T, Sebastian S, Lakatos PL, Langholz E, Munkholm P. Health-related quality of life improves during one year of medical and surgical treatment in a European population-based inception cohort of patients with inflammatory bowel disease--an ECCO-EpiCom study. *J Crohns Colitis* 2014; **8**: 1030-1042 [PMID: 24560877 DOI: 10.1016/j.crohns.2014.01.028]
22 **Derogatis LR**, Melisaratos N. The Brief Symptom Inventory: an introductory report. *Psychol Med* 1983; **13**: 595-605 [PMID: 6622612 DOI: 10.1017/S0033291700048017]
23 **Gilbar O**, Ben-Zur H. Adult Israeli community norms for the brief symptom index (BSI). *Int J Stress Manag* 2002; **9**: 1-10 [DOI: 10.1023/A:1013097816238]
24 **Carver CS**. You want to measure coping but your protocol's too long: consider the brief COPE. *Int J Behav Med* 1997; **4**: 92-100 [PMID: 16250744 DOI: 10.1207/s15327558ijbm0401_6]
25 **Nuttman-Shwartz O**, Dekel R. Ways of coping and sense of belonging in the face of a continuous threat. *J Trauma Stress* 2009; **22**: 667-670 [PMID: 19908323 DOI: 10.1002/jts.20463]
26 **Epstein NB**, Baldwin LM, Bishop DS. The McMaster family assessment device. *J Marital Fam Ther* 1983; **9**: 171-180
27 **Slonim-Nevo V**, Mirsky J, Rubinstein L, Nauck B. The impact of familial and environmental factors on the adjustment of immigrants: A longitudinal study. *Journal of Family Issues* 2008; **30**: 92-123 [DOI: 10.1177/0192513X08324575]
28 **Diener E**, Emmons RA, Larsen RJ, Griffin S. The Satisfaction With Life Scale. *J Pers Assess* 1985; **49**: 71-75 [PMID: 16367493 DOI: 10.1207/s15327752jpa4901_13]
29 **Anaby D**, Jarus T, Zumbo B. Psychometric evaluation of the Hebrew language version of the satisfaction with life scale. *Social Indicators Research* 2010; **96**: 267-274 [DOI: 10.1007/s11205-009-9476-z]
30 **Reilly MC**, Zbrozek AS, Dukes EM. The validity and reproducibility of a work productivity and activity impairment instrument. *Pharmacoeconomics* 1993; **4**: 353-365 [PMID: 10146874 DOI: 10.2165/00019053-199304050-00006]
31 Available from: URL: www.reillyassociates.net/WPAI-ANS_V2_0-Hebrew-Israel.doc
32 **Schirbel A**, Reichert A, Roll S, Baumgart DC, Büning C, Wittig B, Wiedenmann B, Dignass A, Sturm A. Impact of pain on health-related quality of life in patients with inflammatory bowel disease. *World J Gastroenterol* 2010; **16**: 3168-3177 [PMID: 20593502 DOI: 10.3748/wjg.v16.i25.3168]
33 **Cheung M**, Khan S, Akerman M, Hung CK, Vennard K, Hristis N, Sultan K. Clinical markers of Crohn's disease severity and their association with opiate use. *J Clin Med Res* 2015; **7**: 33-36 [PMID: 25368699 DOI: 10.14740/jocmr1969w]
34 **Naftali T**, Mechulam R, Lev LB, Konikoff FM. Cannabis for inflammatory bowel disease. *Dig Dis* 2014; **32**: 468-474 [PMID: 24969296 DOI: 10.1159/000358155]
35 **Katon W**. The impact of major depression on chronic medical illness. *Gen Hosp Psychiatry* 1996; **18**: 215-219 [PMID: 8832253]
36 **Boye B**, Lundin KE, Leganger S, Mokleby K, Jantschek G, Jantschek I, Kunzendorf S, Benninghoven D, Sharpe M, Wilhelmsen I, Blomhoff S, Malt UF, Jahnsen J. The INSPIRE study: do personality traits predict general quality of life (Short form-36) in distressed patients with ulcerative colitis and Crohn's disease? *Scand J Gastroenterol* 2008; **43**: 1505-1513 [PMID: 18777439 DOI: 10.1080/00365520802321196]
37 **Veloso FT**. Clinical predictors of Crohn's disease course. *Eur J Gastroenterol Hepatol* 2016; **28**: 1122-1125 [PMID: 27391171 DOI: 10.1097/MEG.0000000000000698]
38 **Büsch K**, da Silva SA, Holton M, Rabacow FM, Khalili H, Ludvigsson JF. Sick leave and disability pension in inflammatory bowel disease: a systematic review. *J Crohns Colitis* 2014; **8**: 1362-1377 [PMID: 25001582 DOI: 10.1016/j.crohns.2014.06.006]

39 **Wagtmans MJ**, Verspaget HW, Lamers CB, van Hogezand RA. Gender-related differences in the clinical course of Crohn's disease. *Am J Gastroenterol* 2001; **96**: 1541-1546 [PMID: 11374696 DOI: 10.1111/j.1572-0241.2001.03755.x]

40 **Saibeni S**, Cortinovis I, Beretta L, Tatarella M, Ferraris L, Rondonotti E, Corbellini A, Bortoli A, Colombo E, Alvisi C, Imperiali G, de Franchis R. Gender and disease activity influence health-related quality of life in inflammatory bowel diseases. *Hepatogastroenterology* 2005; **52**: 509-515 [PMID: 15816468]

41 **Clench-Aas J**, Nes RB, Dalgard OS, Aarø LE. Dimensionality and measurement invariance in the Satisfaction with Life Scale in Norway. *Qual Life Res* 2011; **20**: 1307-1317 [PMID: 21308414 DOI: 10.1007/s11136-011-9859-x]

42 **Sarid O**, Slonim-Nevo V, Pereg A. Satisfaction with Life and Coping in Crohn's Disease: A Gender Perspective (no. 1363). United European Gastroenterology Week: Vienna, 2016

43 **McCombie AM**, Mulder RT, Gearry RB. How IBD patients cope with IBD: a systematic review. *J Crohns Colitis* 2013; **7**: 89-106 [PMID: 22718016 DOI: 10.1016/j.crohns.2012.05.021]

44 **Randell RL**, Long MD, Cook SF, Wrennall CE, Chen W, Martin CF, Anton K, Sandler RS, Kappelman MD. Validation of an internet-based cohort of inflammatory bowel disease (CCFA partners). *Inflamm Bowel Dis* 2014; **20**: 541-544 [PMID: 24451221 DOI: 10.1097/01.MIB.0000441348.32570.34]

P- Reviewer: Garcia-Sanjuan S, Guloksuz S, Lakatos PL
S- Editor: Yu J **L- Editor**: A **E- Editor**: Liu WX

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v23.i6.1090

World J Gastroenterol 2017 February 14; 23(6): 1090-1097
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Prospective Study

Modified docetaxel, cisplatin and capecitabine for stage Ⅳ gastric cancer in Japanese patients: A feasibility study

Osamu Maeda, Ayumu Matsuoka, Ryoji Miyahara, Kohei Funasaka, Yoshiki Hirooka, Masahide Fukaya, Masato Nagino, Yasuhiro Kodera, Hidemi Goto, Yuichi Ando

Osamu Maeda, Ayumu Matsuoka, Yuichi Ando, Department of Clinical Oncology and Chemotherapy, Nagoya University Hospital, Nagoya 466-8560, Japan

Ryoji Miyahara, Kohei Funasaka, Hidemi Goto, Department of Gastroenterology and Hepatology, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan

Yoshiki Hirooka, Department of Endoscopy, Nagoya University Hospital, Nagoya 466-8560, Japan

Masahide Fukaya, Masato Nagino, Division of Surgical Oncology, Department of Surgery, Nagoya University Graduate School of Medicine, 466-8550 Nagoya, Japan

Yasuhiro Kodera, Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, 466-8550 Nagoya, Japan

Author contributions: Maeda O and Ando Y designed the research, performed the protocol treatment and wrote the manuscript; Matsuoka A, Miyahra R, Funasaka K and Fukaya M contributed to performing the protocol treatment; Hirooka Y, Nagino M, Kodera Y and Goto H were involved in patient recruitment and data analysis.

Institutional review board statement: This study was approved by the review boards of Nagoya University Hospital.

Clinical trial registration statement: This study was registered at http://www.umin.ac.jp/ctr/index.htm, registration identification number is UMIN000006009.

Informed consent statement: All patients provided written informed consent.

Conflict-of-interest statement: Kodera Y has received research funding from Chugai Pharmaceutical Co., Ltd., Sanofi, Yakult Honsha Co., Ltd., Takeda Pharmaceutical Co., Ltd., Pfizer Inc., and Bristol-Myers Squib. Goto H has received research funding from Bristol-Myers Squibb, and Takeda Pharmaceutical Co., Ltd. Ando Y has received research funding from Sanofi, Chugai Pharmaceutical Co. Ltd., Takeda Pharmaceutical Co. Ltd., Yakult Honsya Co., Ltd., and Mochida Pharmaceutical Co., Ltd.

Data sharing statement: No additional data are available.

Manuscript source: Invited manuscript

Correspondence to: Osamu Maeda, MD, PhD, Department of Clinical Oncology and Chemotherapy, Nagoya University Hospital, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8560, Aichi, Japan. maeda-o@med.nagoya-u.ac.jp
Telephone: +81-52-7441903
Fax: +81-52-7441903

Received: October 20, 2016
Peer-review started: October 24, 2016
First decision: October 28, 2016
Revised: December 19, 2016
Accepted: January 17, 2017
Article in press: January 17, 2017
Published online: February 14, 2017

Abstract

AIM

To evaluate the feasibility of chemotherapy including fluoropyrimidine, platinum and taxane with modified dosages for unresectable gastric cancer in Japanese patients.

METHODS

We performed a feasibility study of a modified docetaxel, cisplatin and capecitabine (DCX) regimen for stage Ⅳ gastric cancer. In particular, 30 or 40 mg/m^2 of docetaxel on day 1, 60 mg/m^2 of cisplatin on day 1, and 2000 mg/m^2 of capecitabine for 2 wk were administered every three weeks.

RESULTS

Three patients were treated with modified DCX (mDCX) with 30 mg/m^2 docetaxel, and five patients were treated with this regimen with 40 mg/m^2 docetaxel. Grade 3 or 4 neutropenia was observed in six of the eight patients; no patients exhibited febrile neutropenia. Partial response was achieved in four of the eight patients. Three patients underwent gastrectomy, which achieved R0 resection without residual tumors in dissected lymph nodes. In one of these three patients, resected specimens revealed pathological complete response in the primary lesion and in lymph nodes.

CONCLUSION

mDCX was well tolerated by Japanese patients with stage Ⅳ gastric cancer. This regimen might be useful for allowing gastric cancer patients with distant lymph node metastasis to undergo conversion surgery.

Key words: Docetaxel; Cisplatin; Capecitabine; Gastric cancer

Core tip: A combination of fluoropyrimidine and platinum is a standard treatment for unresectable gastric cancer. Although the addition of a taxane to this doublet is expected to improve effectiveness, research has demonstrated that such triplet regimens often cause adverse effects, including neutropenia. To reduce adverse events but maintain therapeutic effectiveness, we devised a triplet regimen with modified dosages. Modified docetaxel, cisplatin and capecitabine treatment was safe and effective for stage Ⅳ gastric cancer. Three of the eight treated patients underwent conversion surgery and achieved long-term survival without recurrence.

Maeda O, Matsuoka A, Miyahara R, Funasaka K, Hirooka Y, Fukaya M, Nagino M, Kodera Y, Goto H, Ando Y. Modified docetaxel, cisplatin and capecitabine for stage Ⅳ gastric cancer in Japanese patients: A feasibility study. *World J Gastroenterol* 2017; 23(6): 1090-1097 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i6/1090.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i6.1090

INTRODUCTION

The prognosis of stage Ⅳ gastric cancer is poor, and the median overall survival time is approximately one year. The standard treatment for Stage Ⅳ gastric cancer is chemotherapy with agents such as fluoropyrimidines and platinum compounds. In Japan, the oral fluoropyrimidine S-1 plus cisplatin (SP) is the standard regimen for HER2-negative advanced gastric cancer because SP was proven to be superior to S-1 alone in a phase Ⅲ randomized trial[1]. Because capecitabine, similarly to S-1, is an effective oral fluoropyrimidine, capecitabine plus cisplatin (XP) is also a possible regimen[2].

The addition of docetaxel to fluoropyrimidine and cisplatin was expected to improve therapeutic efficacy. A combination of docetaxel, cisplatin and 5-fluorouracil (DCF) produced longer overall survival than cisplatin plus 5-fluorouracil; however, the use of DCF is limited due to severe side effects, including hematologic toxicity[3]. Various modified DCF regimens have been tested in attempts to improve tolerability without losing efficacy[4-8]. Research has also examined regimens that replace the infusion of 5-fluorouracil with an oral fluoropyrimidine, such as docetaxel, cisplatin and S-1 (DCS)[9] and docetaxel, cisplatin and capecitabine (DCX)[10]. Although DCX has been reported to be effective for unresectable gastric cancer, this regimen often causes adverse events, including hematologic toxicities[10-12]. We believed that a modification of the doses used for DCX might reduce toxicity but maintain effectiveness. In previous reports, doses of docetaxel used for DCX ranged from 60 mg/m^2 to 75 mg/m^2[10-12]. In certain studies of DCS, the dose of docetaxel was set to 30-40 mg/m^2, and good effectiveness and adequate safety were achieved[13-15]. In the present study, we set the dose of docetaxel to 30 or 40 mg/m^2, which was a lower dose than that used in previous reports on DCX, and evaluated the safety and efficacy of our modified DCX (mDCX) regimen in Japanese patients.

MATERIALS AND METHODS

Patient eligibility

The eligibility criteria included stage Ⅳ unresectable HER2-negative gastric cancer, an age of 20-75 years, Eastern Cooperative Oncology Group performance status 0-1, conserved organ functions, and no prior chemotherapy.

Treatment

The treatment regimen, which consisted of 1000 mg/m^2 capecitabine twice per day on days 1-14, 60 mg/m^2 cisplatin on day 1 and 30 or 40 mg/m^2 docetaxel on day 1, was administered every three weeks. The dosage of docetaxel was 30 mg/m^2 for the first three patients and was planned to increase to 40 mg/m^2 for subsequent patients if no dose-limiting toxicities (DLTs) were observed after the first three patients' first treatment cycle. The treatment was continued until the disease progressed, patients experienced intolerable

Table 1 Patient characteristics and prognoses

	Age	Sex	Macroscopic type	Histopathology	Metastasis	Number of courses	Objective tumor response	Prognosis (mo)		Conversion surgery
1	64	M	3	tub2/por	Liver, LNs	7	PR	21.9	Dead	
2	59	M	3	tub2/por	LNs	5	PR	50.9	Alive	Yes
3	62	M	2	por	Liver, LNs	6	SD	7.4	Dead	
4	65	M	3	por	LNs	5	PR	7.7	Dead	
5	67	F	3	tub2/por	LNs	3	non-CR/non-PD	31.3	Alive	Yes
6	66	M	3	por	LNs	4	non-CR/non-PD	12.0	Dead	
7	62	M	2	por	Liver, LNs	3	SD	5.4	Dead	
8	63	F	2	tub2/por	LNs	4	PR	24.4	Alive	Yes

tub2: Moderately differentiated tubular adenocarcinoma; por: Differentiated adenocarcinoma; LNs: Lymph nodes; PR: Partial response; SD: Stable disease; CR: Complete response.

Table 2 Hematologic and non-hematologic adverse events *n* (%)

	Any grade	Grade 3	Grade 4
Leukopenia	7 (87.5)	2 (25)	0
Neutropenia	7 (87.5)	4 (50)	2 (25)
Anemia	6 (75)	1 (12.5)	0
Thrombocytopenia	7 (87.5)	0	0
Hyperbilirubinemia	3 (37.5)	0	0
Elevated serum aspartate aminotransferase	6 (75)	0	0
Elevated serum alanine aminotransferase	8 (100)	0	0
Elevated serum creatinine	3 (37.5)	0	0
Fever	4 (50)	0	0
Fatigue	2 (25)	0	0
Alopecia	1 (12.5)	0	0
Skin rash	1 (12.5)	0	0
Anorexia	7 (87.5)	4 (50)	0
Diarrhea	4 (50)	2 (25)	0
Nausea	3 (37.5)	0	0
Vomiting	3 (37.5)	0	0
Constipation	1 (12.5)	0	0
Peripheral neuropathy	1 (12.5)	0	0
Infection	1 (12.5)	1 (12.5)	0

side effects, or curative resection was expected.

Treatment was interrupted if a patient developed grade ⩾ 3 hematologic toxicity. If a patient experienced grade 4 neutropenia for more than 5 d or grade 3 febrile neutropenia, the dosage of all agents was decreased to 75% for the next course. If a patient exhibited grade 4 thrombocytopenia, dosages of all agents were decreased to 50%. If a patient had grade ⩾ 2 diarrhea and/or grade ⩾ 2 hand-foot syndrome, the treatment course was interrupted. If creatinine clearance (Ccr) was < 60 mL/min and ⩾ 50 mL/min, cisplatin was decreased to 75%. If Ccr was < 50 mL/min and ⩾ 40 mL/min, cisplatin was decreased to 50%. If Ccr was < 40 mL/min, the treatment protocol was terminated. Supportive treatment, including G-CSF and anti-emetics, was permitted.

Safety and anti-tumor activity assessments

Adverse events were assessed using the National Cancer Institute's CTCAE v4.0. DLTs were defined as adverse events that occurred after the beginning of the first cycle and before the beginning of the second cycle that satisfied any of the following criteria: (1) non-hematologic toxicities ⩾ grade 3 that did not resolve to grade 0 or grade 1 within two consecutive days, except for nausea, vomiting, anorexia and asymptomatic electrolyte imbalance; (2) neutropenia ⩾ grade 3 for > 5 consecutive days; (3) febrile neutropenia (absolute neutrophil count < 1.0×10^9/L and fever ⩾ 38 ℃); (4) grade 4 thrombocytopenia or platelet transfusion; or (5) delay of the treatment cycle for > 2 wk.

Radiological tumor assessments were conducted using computed tomography every eight weeks in accordance with the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1.

RESULTS

Three patients received mDCX with 30 mg/m^2 of docetaxel. Because no DLTs were observed in the first three patients, treatment with 40 mg/m^2 docetaxel was administered to five subsequent patients.

The patients' characteristics and clinical courses are summarized in Table 1, and adverse events are summarized in Table 2. No DLTs were observed after the first treatment cycles in any patient. The relative dose

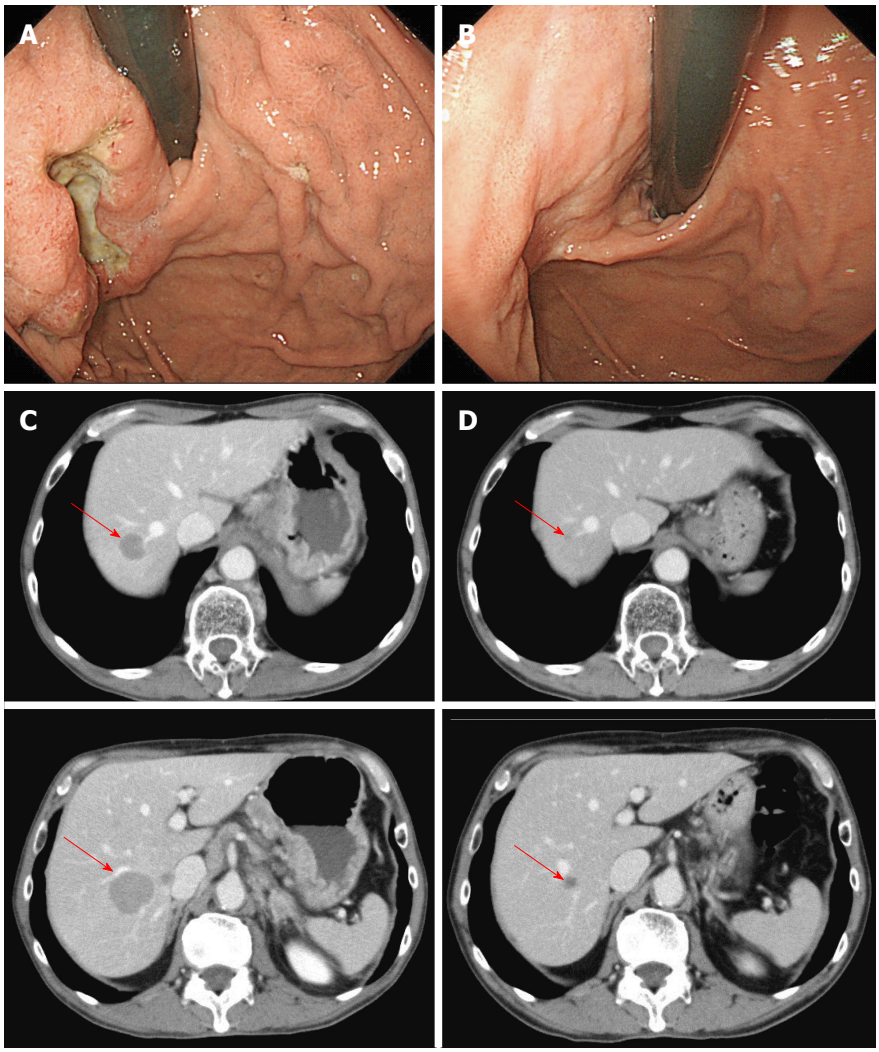

Figure 1 Case 1. Endoscopic findings (A, B) and computed tomography images (C, D) before (A, C) and after (B, D) seven courses of modified docetaxel, cisplatin and capecitabine (DCX) (mDCX).

intensities of DCX were 90.6%, 90.0% and 76.2%, respectively. Four of the eight patients exhibited a partial response (PR). Three patients (cases 2, 5 and 8) underwent gastrectomy with lymph node dissection; for all of these patients, R0 resection was achieved, and no viable tumors were detected in resected lymph nodes (ypN0). Case 2 achieved pathological complete response in both the primary lesion and lymph node metastasis. Regarding case 5, the observed therapeutic effect was grade 2, and she received capecitabine for ten weeks as adjuvant chemotherapy. The clinical courses of three representative cases are presented below.

Case 1

A 64-year-old man had type Ⅲ advanced cancer in the fornix of the stomach and multiple liver metastases (Figure 1). Biopsy specimens were pathologically diagnosed as moderately and poorly differentiated adenocarcinoma. The patient received three courses of mDCX, and the liver metastases shrank, a phenomenon judged to be PR. After five courses, PR was confirmed. After seven courses, shrinkage of the liver metastases was sustained. The treatment protocol was discontinued due to grade 2 sensory peripheral neuropathy. Other adverse events were grade 2 anemia, grade 1 aspartate aminotransferase elevation, grade 1 alanine aminotransferase elevation, and grade 1 anorexia. The patient received post-protocol treatment that included irinotecan and weekly paclitaxel. He died 22 mo after enrollment in the study.

Case 2

A 59-year-old man had type Ⅲ advanced cancer at the small curvature of the angulus with lymph node metastasis along the superior mesenteric artery (#14a; Figure 2). Biopsy specimens were pathologically diagnosed as moderately and poorly differentiated adenocarcinoma. After three courses of mDCX, the lymph node metastasis shrank; after five courses, PR was confirmed. Adverse events included grade 2 leukopenia, grade 3 neutropenia, and grade 2 anemia. The patient underwent subtotal gastrectomy with lymph node dissection. Pathological findings revealed

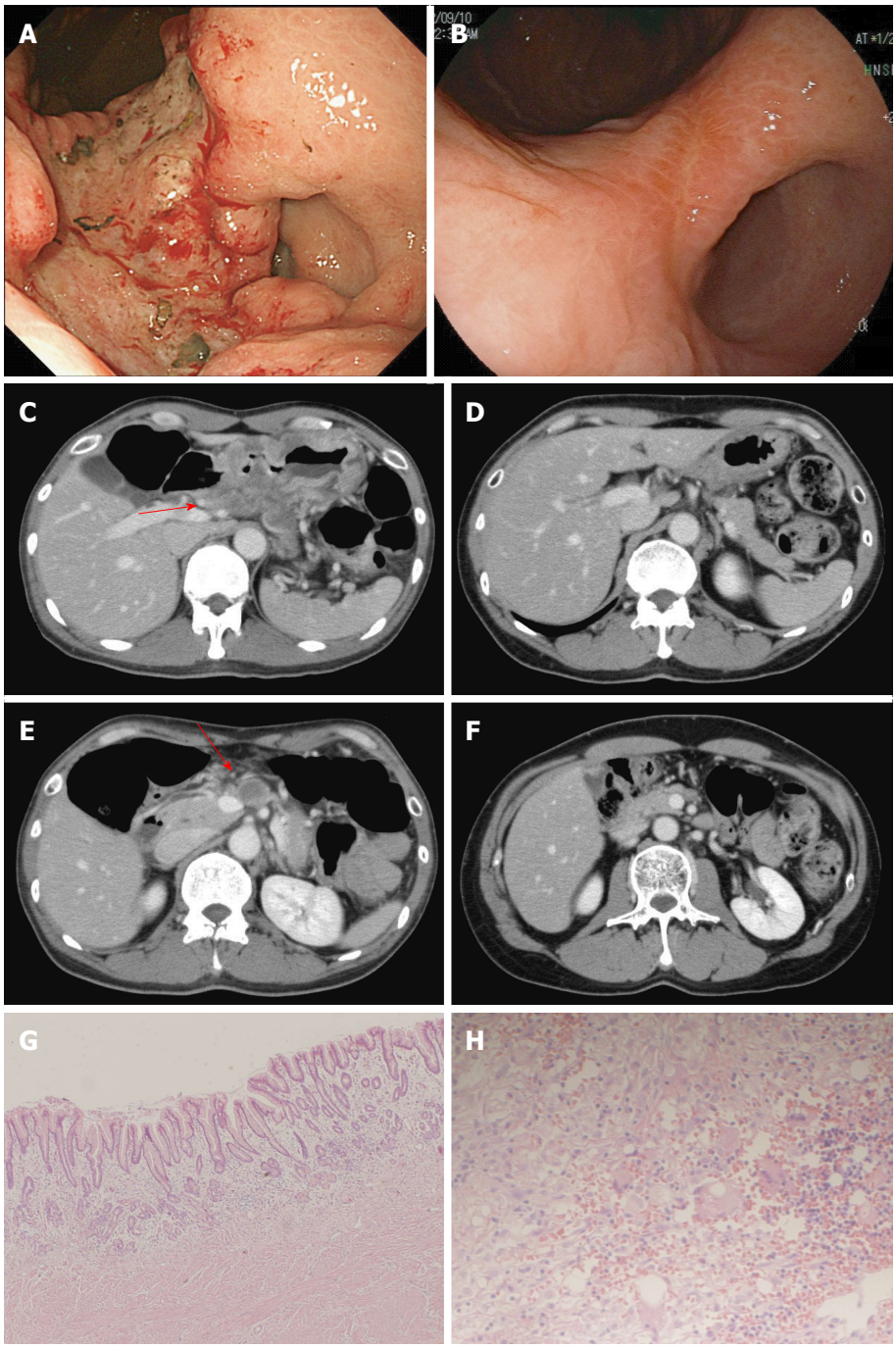

Figure 2 Case 2. Endoscopic findings (A, B) and computed tomography images (C, D, E, F) before treatment (A, C, E) and after (B, D, F) five courses of modified docetaxel, cisplatin and capecitabine (DCX) (mDCX). The primary lesion (A, B), swollen lymph nodes along the common hepatic artery (#8) (C, D) and lymph nodes along the superior mesenteric artery (#14a) (E, F) markedly shrank. Microscopic findings for the resected specimens of the primary lesion (E, magnification × 100) and lymph nodes (F, magnification × 400) revealed no residual tumor.

no residual carcinoma, and the observed therapeutic effect was grade 3. He received S-1 for one year as adjuvant chemotherapy. He remains alive without any findings indicative of recurrence four years after enrollment.

Case 8

A 63-year-old woman had type Ⅲ advanced cancer at the gastric antrum with lymph node metastasis that included left supraclavicular lymph nodes and para-aortic lymph nodes (Figure 3). Biopsy specimens were pathologically diagnosed as moderately and poorly differentiated adenocarcinoma. After two courses of mDCX, the lymph nodes shrank; after four courses, PR was confirmed. Adverse events included grade 2 leukopenia, grade 4 neutropenia, grade 3 anorexia, and grade 3 diarrhea. Because the patient's lymph node metastasis became undetectable by computed tomography, she underwent subtotal gastrectomy with D2 dissection; the para-aortic lymph nodes were not

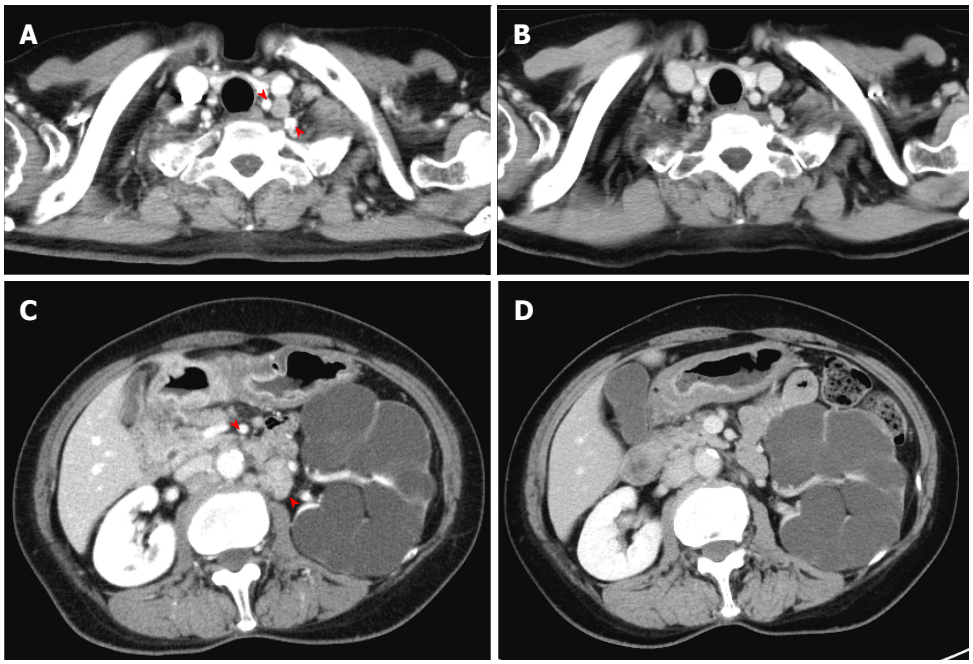

Figure 3 Case 8. Computed tomography images before (A, C) and after (B, D) four courses of modified docetaxel, cisplatin and capecitabine (DCX) (mDCX). Left supraclavicular nodes (A, B) and para-aortic nodes (C, D) became undetectable.

Table 3 Previous reports regarding docetaxel, cisplatin and capecitabine

Ref.	Setting	Capecitabine	Cisplatin	Docetaxel	Interval (d)	Number of patients		Grade 3-4 neutropenia	Febrile neutropenia	RR (95%CI)	PFS (95%CI) (mo)	OS (95%CI) (mo)	R0 resection	pCR
Kang *et al*[10], 2010	Metastatic or recurrent	1875 mg, days 1-14	60 mg, day 1	60 mg, day 1	21	40		62.5%	10%	68% (53%-83%)	7.6 (6.9-8.4)	14.4 (7.3-21.5)		10.0%
Sym *et al*[17], 2010	Neoadjuvant	1875 mg, days 1-14	60 mg, day 1	60 mg, day 1	21	49 (36 resected cases)		69%	4%		R0: 54.3 (0-112.9) Non-R0: 5.1 (3.6-6.6)	R0: not reached Non-R0: 11.5 (7.3-15.7)	63.0%	
Thuss-Patience *et al*[12], 2012	Perioperative	1875 mg, days 1-14	60 mg, day 1	75 mg, day1	21	51	Preoperative	76.5%	21.5%				90.2%	13.7%
							Postoperative	62.9%	11.1%					
Polyzos *et al*[16], 2012	Metastatic	2000 mg, days 2-15	60 mg, day 1	60 mg, day 1	21	36		50%	16%	44.4% (28%-60%)	5 (3-6)[1]	12 (5-24)		
Yoon *et al*[18], 2015	Adjuvant for stage III B-IV	1875 mg, days 1-14	60 mg, day 1	60 mg, day 1	21	46		40%	15%		26.9 (7.5-46.4)[2]	43.9 (29.2-58.7)		

[1]Time to progression; [2]Relapse-free survival. RR: Response rate; PFS: Progression-free survival; OS: Overall survival; pCR: Pathological complete response.

dissected. Pathological findings revealed no residual tumors in the lymph nodes, and the observed therapeutic effect was grade 1b. As adjuvant chemotherapy, she underwent two courses of capecitabine plus oxaliplatin which were discontinued due to grade 2 nausea and fatigue. She subsequently received S-1 for one year. The patient remains alive without any findings indicative of recurrence two years after enrollment.

DISCUSSION

Reports have described the effectiveness of DCX for unresectable gastric cancer[10,16], as preoperative chemotherapy[17], as adjuvant chemotherapy[18] and as perioperative chemotherapy[12]. These reports indicate that DCX is highly effective but also rather toxic, particularly in hematologic respects. To our knowledge, no prior reports have described a DCX trial in Japan.

In the present study, six of the eight patients (75%) experienced grade 3 or grade 4 neutropenia, although no patients experienced febrile neutropenia. Although the modifications to the DCX regimen decreased the dose of docetaxel to 30 or 40 mg/m^2, the frequency of hematologic toxicity in this study was similar to those

reported in prior studies of DCX (neutropenia ⩾ grade 3, 40%-76.5%; Table 3). However, we regarded the observed side effects as tolerable because no DLT was observed after the first cycle, and all patients were able to receive three or more courses of treatment with appropriate supportive care. In addition, the median admission period was five days (data not shown), indicating that for the most part, admission was primarily necessary for hydration due to the administration of cisplatin.

Three of the five patients with only distant lymph node metastasis underwent conversion surgery, and all three patients have remained alive for more than two years without recurrence. Therefore, we believe that intensive treatment with a triplet regimen could be a useful preoperative treatment that enables conversion surgery for patients with distant lymph node metastasis. However, in certain cases, survival time was shorter than one year; in particular, one case died 7.7 mo after the start of treatment, although PR was achieved. It is necessary to identify certain biomarkers to select patients suitable for a triplet regimen. Furthermore, a randomized control study is needed to evaluate whether the proposed triplet regimen is superior to a standard platinum and oral fluoropyrimidine doublet.

In conclusion, mDCX is safe and effective for Stage Ⅳ gastric cancer in Japanese patients.

COMMENTS

Background

For stage Ⅳ gastric cancer, doublet regimens including fluoropyrimidine and platinum agents are standard chemotherapy. The addition of taxanes to the doublet regimen may improve effectiveness but may also increase toxicity.

Research frontiers

The addition of a smaller amount of docetaxel than previously reported to capecitabine and cisplatin was evaluated.

Innovations and breakthroughs

The authors set the dose of docetaxel to 30 or 40 mg/m^2, which was a lower dose than used in previous reports on docetaxel, cisplatin and capecitabine (DCX).

Applications

Intensive treatment with a triplet regimen could be a useful preoperative treatment that allows for conversion surgery in patients with distant lymph node metastasis.

Terminology

DCX: A combination chemotherapy regimen including docetaxel, cisplatin and capecitabine. Conversion surgery: Surgical operation for patients with cancer that was unresectable before chemotherapy and became to be resectable after chemotherapy.

Peer-review

The authors designed the dose of docetaxel to 30 or 40 mg/m^2, which was a lower dose than used in previous reports on DCX, and evaluated the safety and efficacy.

REFERENCES

1 **Koizumi W**, Narahara H, Hara T, Takagane A, Akiya T, Takagi M, Miyashita K, Nishizaki T, Kobayashi O, Takiyama W, Toh Y, Nagaie T, Takagi S, Yamamura Y, Yanaoka K, Orita H, Takeuchi M. S-1 plus cisplatin versus S-1 alone for first-line treatment of advanced gastric cancer (SPIRITS trial): a phase III trial. *Lancet Oncol* 2008; **9**: 215-221 [PMID: 18282805]

2 **Tsuburaya A**, Morita S, Kodera Y, Kobayashi M, Shitara K, Yamaguchi K, Yoshikawa T, Yoshida K, Yoshino S, Sakamoto J. A randomized phase II trial to elucidate the efficacy of capecitabine plus cisplatin (XP) and S-1 plus cisplatin (SP) as a first-line treatment for advanced gastric cancer: XP ascertainment vs. SP randomized PII trial (XParTS II). *BMC Cancer* 2012; **12**: 307 [PMID: 22824079 DOI: 10.1186/1471-2407-12-307]

3 **Van Cutsem E**, Moiseyenko VM, Tjulandin S, Majlis A, Constenla M, Boni C, Rodrigues A, Fodor M, Chao Y, Voznyi E, Risse ML, Ajani JA. Phase III study of docetaxel and cisplatin plus fluorouracil compared with cisplatin and fluorouracil as first-line therapy for advanced gastric cancer: a report of the V325 Study Group. *J Clin Oncol* 2006; **24**: 4991-4997 [PMID: 17075117]

4 **Ozdemir NY**, Abali H, Oksüzoğlu B, Budakoglu B, Uncu D, Güler T, Odabaşi H, Zengin N. The efficacy and safety of reduced-dose docetaxel, cisplatin, and 5-fluorouracil in the first-line treatment of advanced stage gastric adenocarcinoma. *Med Oncol* 2010; **27**: 680-684 [PMID: 19633962 DOI: 10.1007/s12032-009-9268-y]

5 **Keskin S**, Yıldız I, Sen F, Aydogan F, Kilic L, Ekenel M, Saglam S, Sakar B, Disci R, Aykan F. Modified DCF (mDCF) regimen seems to be as effective as original DCF in advanced gastric cancer (AGC). *Clin Transl Oncol* 2013; **15**: 403-408 [PMID: 23054756 DOI: 10.1007/s12094-012-0942-8]

6 **Koca D**, Dogan E, Yardim H, Duzen O, Karaca S. A modified DCF regimen as primary treatment for patients with metastatic gastric cancer. *J BUON* 2013; **18**: 377-384 [PMID: 23818349]

7 **Shah MA**, Janjigian YY, Stoller R, Shibata S, Kemeny M, Krishnamurthi S, Su YB, Ocean A, Capanu M, Mehrotra B, Ritch P, Henderson C, Kelsen DP. Randomized Multicenter Phase II Study of Modified Docetaxel, Cisplatin, and Fluorouracil (DCF) Versus DCF Plus Growth Factor Support in Patients With Metastatic Gastric Adenocarcinoma: A Study of the US Gastric Cancer Consortium. *J Clin Oncol* 2015; **33**: 3874-3879 [PMID: 26438119 DOI: 10.1200/JCO.2015.60.7465]

8 **Wang J**, Xu R, Li J, Bai Y, Liu T, Jiao S, Dai G, Xu J, Liu Y, Fan N, Shu Y, Ba Y, Ma D, Qin S, Zheng L, Chen W, Shen L. Randomized multicenter phase III study of a modified docetaxel and cisplatin plus fluorouracil regimen compared with cisplatin and fluorouracil as first-line therapy for advanced or locally recurrent gastric cancer. *Gastric Cancer* 2016; **19**: 234-244 [PMID: 25604851 DOI: 10.1007/s10120-015-0457-4]

9 **Sato Y**, Takayama T, Sagawa T, Takahashi Y, Ohnuma H, Okubo S, Shintani N, Tanaka S, Kida M, Sato Y, Ohta H, Miyanishi K, Sato T, Takimoto R, Kobune M, Yamaguchi K, Hirata K, Niitsu Y, Kato J. Phase II study of S-1, docetaxel and cisplatin combination chemotherapy in patients with unresectable metastatic gastric cancer. *Cancer Chemother Pharmacol* 2010; **66**: 721-728 [PMID: 20041328 DOI: 10.1007/s00280-009-1215-2]

10 **Kang YK**, Ryu MH, Yoo C, Chang HM, Yook JH, Oh ST, Kim BS, Kim TW. Phase I/II study of a combination of docetaxel, capecitabine, and cisplatin (DXP) as first-line chemotherapy in patients with advanced gastric cancer. *Cancer Chemother Pharmacol* 2011; **67**: 1435-1443 [PMID: 20811894 DOI: 10.1007/s00280-010-1444-4]

11 **Sym SJ**, Ryu MH, Kang HJ, Lee SS, Chang HM, Lee JL, Kim TW, Yook JH, Oh ST, Kim BS, Kang YK. Phase I study of 3-weekly docetaxel, capecitabine and oxaliplatin combination chemotherapy in patients with previously untreated advanced gastric cancer. *Cancer Chemother Pharmacol* 2010; **66**: 373-380 [PMID: 19936751 DOI: 10.1007/s00280-009-1171-x]

12 **Thuss-Patience PC**, Hofheinz RD, Arnold D, Florschütz A, Daum S, Kretzschmar A, Mantovani-Löffler L, Bichev D, Breithaupt K,

Kneba M, Schumacher G, Glanemann M, Schlattmann P, Reichardt P, Gahn B. Perioperative chemotherapy with docetaxel, cisplatin and capecitabine (DCX) in gastro-oesophageal adenocarcinoma: a phase II study of the Arbeitsgemeinschaft Internistische Onkologie (AIO){dagger}. *Ann Oncol* 2012; **23**: 2827-2834 [PMID: 22734012 DOI: 10.1093/annonc/mds129]

13 **Fushida S**, Fujimura T, Oyama K, Yagi Y, Kinoshita J, Ohta T. Feasibility and efficacy of preoperative chemotherapy with docetaxel, cisplatin and S-1 in gastric cancer patients with para-aortic lymph node metastases. *Anticancer Drugs* 2009; **20**: 752-756 [PMID: 19543076]

14 **Nakayama N**, Koizumi W, Sasaki T, Higuchi K, Tanabe S, Nishimura K, Katada C, Nakatani K, Takagi S, Saigenji K. A multicenter, phase I dose-escalating study of docetaxel, cisplatin and S-1 for advanced gastric cancer (KDOG0601). *Oncology* 2008; **75**: 1-7 [PMID: 18719348 DOI: 10.1159/000151613]

15 **Koizumi W**, Nakayama N, Tanabe S, Sasaki T, Higuchi K, Nishimura K, Takagi S, Azuma M, Ae T, Ishido K, Nakatani K, Naruke A, Katada C. A multicenter phase II study of combined chemotherapy with docetaxel, cisplatin, and S-1 in patients with unresectable or recurrent gastric cancer (KDOG 0601). *Cancer Chemother Pharmacol* 2012; **69**: 407-413 [PMID: 21796483 DOI: 10.1007/s00280-011-1701-1]

16 **Polyzos A**, Felekouras E, Karatzas T, Griniatsos J, Dimitroulis D, Polyzos K, Kontzoglou K, Mantas D, Karavokyros J, Nikiteas N, Tsavaris N, Syrigos K, Vafiadis I. Modified docetaxel-cisplatin in combination with capecitabine as first-line treatment in metastatic gastric cancer. a phase II study. *Anticancer Res* 2012; **32**: 4151-4156 [PMID: 22993377]

17 **Sym SJ**, Chang HM, Ryu MH, Lee JL, Kim TW, Yook JH, Oh ST, Kim BS, Kang YK. Neoadjuvant docetaxel, capecitabine and cisplatin (DXP) in patients with unresectable locally advanced or metastatic gastric cancer. *Ann Surg Oncol* 2010; **17**: 1024-1032 [PMID: 19941081 DOI: 10.1245/s10434-009-0838-1]

18 **Yoon S**, Yoo C, Ryu MH, Kang MJ, Ryoo BY, Park SR, Yook JH, Oh ST, Yoo MW, Kim BS, Kang YK. Phase 2 study of adjuvant chemotherapy with docetaxel, capecitabine, and cisplatin in patients with curatively resected stage IIIB-IV gastric cancer. *Gastric Cancer* 2017; **20**: 182-189 [PMID: 26661592 DOI: 10.1007/s10120-015-0580-2]

P- Reviewer: Arigami T, Chuang SM **S- Editor**: Yu J
L- Editor: A **E- Editor**: Liu WX

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v23.i6.1098

World J Gastroenterol 2017 February 14; 23(6): 1098-1105
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Prospective Study

How to improve patient satisfaction during midazolam sedation for gastrointestinal endoscopy?

Eun Hyo Jin, Kyoung Sup Hong, Young Lee, Ji Yeon Seo, Ji Min Choi, Jaeyoung Chun, Sang Gyun Kim, Joo Sung Kim, Hyun Chae Jung

Eun Hyo Jin, Ji Yeon Seo, Ji Min Choi, Department of Internal Medicine, Healthcare Research Institute, Seoul National University Hospital Healthcare System Gangnam Center, Seoul 135-984, South Korea

Kyoung Sup Hong, Jaeyoung Chun, Sang Gyun Kim, Joo Sung Kim, Hyun Chae Jung, Department of Internal Medicine, Liver Research Institute, Seoul National University College of Medicine, Seoul 110-744, South Korea

Young Lee, Department of Biostatistics and Bioinformatics, Healthcare Research Institute, Seoul National University Hospital Healthcare System Gangnam Center, Seoul 135-984, South Korea

Author contributions: Hong KS was involved in making the conception or design of the work and revising it critically for important intellectual content; Jin EH drafted the manuscript and analyzed and interpreted the data; Lee Y was involved in statistical analysis; Seo JY, Choi JM and Chun J participated in data interpretation and collection; Kim SG, Kim JS and Jung HC revised the manuscript critically for important intellectual content; all authors read and approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the institutional review board of Seoul National University Hospital (IRB No. 1402-083-558).

Informed consent statement: All study participants, or their legal guardian, provided written consent prior to study enrollment.

Conflict-of-interest statement: The authors of this manuscript have no conflicts of interest to disclose.

Data sharing statement: There is no additional data available.

Manuscript source: Unsolicited manuscript

Correspondence to: Kyoung Sup Hong, MD, PhD, Department of Internal Medicine, Liver Research Institute, Seoul National University College of Medicine, 101 Daehak-ro, Jongno-gu, Seoul 110-744, South Korea. kshong1@snu.ac.kr
Telephone: +82-2-20720360
Fax: +82-2-7629662

Received: October 4, 2016
Peer-review started: October 11, 2016
First decision: November 9, 2016
Revised: November 24, 2016
Accepted: December 16, 2016
Article in press: December 19, 2016
Published online: February 14, 2017

Abstract

AIM

To determine the procedure-related factors that affect sedation satisfaction and to make a suggestion to improve it.

METHODS

We prospectively enrolled a total of 456 patients who underwent outpatient endoscopy procedures with midazolam sedation between March 2014 and August 2014. All patients completed both pre- and post-endoscopy questionnaires about sedation expectations and satisfaction.

RESULTS

The study cohort included 167 (36.6%) patients who

underwent esophagogastroduodenoscopy (EGD), 167 (36.6%) who underwent colonoscopy, and 122 (26.8%) who underwent a combined procedure (EGD and colonoscopy). Over 80% of all patients were satisfied with sedation using midazolam. In univariate and multivariate analyses, total procedure time in the EGD group, younger age (≤ 50 years), and longer colonoscopy withdrawal time in the colonoscopy group were related to decreased satisfaction with sedation. However, in active monitoring and intervention group, there was no decrease in grade of satisfaction despite longer procedure time due to more procedures during colonoscopy. Younger age (≤ 50 years), longer inter-procedure time gap, and colonoscopy withdrawal time were related to decreased satisfaction in the combined EGD and colonoscopy group.

CONCLUSION

Midazolam is still a safe and effective sedative for gastrointestinal endoscopy. Satisfaction with sedation depends on several factors including age (≤ 50 years) and procedure time duration. To improve patient satisfaction with sedation, active monitoring of sedation status by the endoscopist should be considered for patients who require long procedure time.

Key words: Conscious sedation; Patient satisfaction; Endoscopy; Midazolam; Surveys and questionnaires

Core tip: This was a prospective study of 456 patients that evaluated procedure-related factors with midazolam sedation satisfaction. Satisfaction with sedation depends on several factors including age (≤ 50 years) and procedure duration. To improve patient satisfaction with sedation, active monitoring of sedation status by an endoscopist should be considered for patients whose procedures take a long time.

Jin EH, Hong KS, Lee Y, Seo JY, Choi JM, Chun J, Kim SG, Kin JS, Jung HC. How to improve patient satisfaction during midazolam sedation for gastrointestinal endoscopy? *World J Gastroenterol* 2017; 23(6): 1098-1105 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i6/1098.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i6.1098

INTRODUCTION

Esophagogastroduodenoscopy (EGD) and colonoscopy are important examinations for screening, diagnosing, and treating a variety of gastrointestinal diseases. Specifically, endoscopy is one of the best surveillance tools for early detection of several cancers, but some patients refuse endoscopic examinations because of fear and anxiety of discomfort during the procedure[1]. Previous studies have reported that conscious sedation endoscopy improves patient satisfaction, reduces fear and discomfort, and increases compliance with repeat endoscopic procedures[2,3]. Recently, conscious sedation endoscopy has become commonplace in clinical practice[4-6].

As more procedures emerge that are appropriate for sedation endoscopy, sedation quality becomes an important factor because it is directly related to patient satisfaction and could have an effect on performance of endoscopy. Thus, satisfaction with sedation has become an important outcome measure and surveys of satisfaction are critical for quality assurance in many endoscopy centers[7]. In previous studies, young age, high level of anxiety, female sex, and increased gag reflex have been proposed as factors related to decreased patient satisfaction with non-sedation endoscopy[8,9]. However, results varied about factors related to satisfaction with sedation in endoscopy[7,10], and no survey of satisfaction with sedation endoscopy has yet been validated.

Worldwide, midazolam is the most commonly used drug for sedation during endoscopy, followed by fentanyl, propofol, and meperidine[4-6]. Midazolam is a short-acting benzodiazepine with anxiolytic, amnestic, and hypnotic effects. Appropriate sedation level could be adjusted by intravenous titration of midazolam. Because it is possible to evaluate the subject's level of sedation by medical staff during procedure through the Richmond Agitation-Sedation Scale[11] or Observer's Assessment of Alertness/Sedation Scale[12]. Flumazenil, a specific benzodiazepine receptor antagonist, can be used to treat benzodiazepine overdoses in emergency situations and to help reverse anesthesia[13]. In the present study, all patients received midazolam for sedation, and meperidine was added for patients undergoing colonoscopy.

The purpose of this study was to evaluate patient satisfaction with conscious sedation endoscopy, to determine procedure-related factors that affect satisfaction with sedation during endoscopic examinations, and to make a suggestion to improve it.

MATERIALS AND METHODS

Patient selection

We prospectively enrolled 466 patients who underwent outpatient endoscopy procedures between March 2014 and August 2014 at Seoul National University Hospital (SNUH), which is a tertiary referral center in Korea. Ten (2.1%) patients were excluded because they did not complete the satisfaction questionnaire. A total of 456 patients were eligible for this study.

All participants provided written informed consent before completing study interviews and undergoing endoscopy. The procedure for our review of clinical records for this study was approved by the Institutional Review Board of SNUH (IRB No. 1402-083-558).

Pre-endoscopy interview

Each patient completed an interview before the endoscopic procedure. An investigator administered a questionnaire in the waiting room after the patient had received explanations of the endoscopic procedure and sedation. The following patient information was recorded: age, sex, body mass index, previous sedation endoscopy, anxiety about procedure, cause of anxiety, and patient expectations of sedation depth according to the Richmond Agitation-Sedation Scale (drowsy, light, or deep sedation)[11]. Before the procedure started, nurses checked and recorded vital signs including oxygen saturation and blood pressure.

Endoscopy procedure

After completing the pre-endoscopy questionnaire, all patients were moved from the waiting room to the endoscopy procedure room. Before EGD, patients received topical anesthesia by pharyngeal spray with lidocaine. All patients underwent examinations with sedation by intravenous midazolam; meperidine at a dose of 25 mg was added for all patients undergoing colonoscopy. The examinations were performed by 14 board-certified endoscopists using an esophagogastroduodenoscope (GIF-260; Olympus, Tokyo, Japan) and/or a colonoscope (CF H260AL; Olympus, Tokyo, Japan). A nurse and an assistant monitored the patient during the procedure by periodically assessing pulse, blood pressure, ventilator status, and neurologic status. Nurses also completed records that included adverse effects of midazolam, the doses and frequency of midazolam injections, and the durations of the procedure and sedation. Three stages of sedation have been described: minimal, moderate, and deep[14]. In our study, most patients underwent endoscopy with moderate sedation referred to as "conscious sedation".

Post-endoscopy questionnaire

After the endoscopy procedure, patients were allowed sufficient time to recover from sedation, and then they completed a post-procedure questionnaire before discharge. Patients subjectively evaluated the depth of sedation and memory loss during the procedure. The questionnaire was self-administered and collected information regarding patient satisfaction with sedation (very satisfied, satisfied, neutral, dissatisfied, or very dissatisfied) and the cause of dissatisfaction, if patients answered "dissatisfied" or "very dissatisfied".

Definitions

Paradoxical response was defined as unexpected movement after midazolam injection. Decreased respiration was defined as oxygen saturation below 88% despite stimulation. In the case of decreased respiration, oxygen was administered *via* nasal prong. Procedure time was subdivided into the following periods: midazolam injection to procedure start, procedure duration, and procedure finish to antidote injection. For colonoscopy procedures, we further divided the procedure time into two periods: insertion time (anal verge to cecum) and withdrawal time (cecum to anal verge). For patients in the combined EGD and colonoscopy group, the inter-procedure time gap was defined as the waiting time from the end of the first endoscopy procedure to the beginning of the second endoscopy procedure.

Statistical analysis

Results are expressed as frequencies and percentages for categorical variables and means for continuous variables. We compared the three procedure groups using the χ^2-test for ordinal variables and analysis of variance for quantitative variables.

Patient satisfaction outcomes were grouped according to satisfaction: very satisfied, satisfied, neutral, dissatisfied, and very dissatisfied. We constructed univariate and multivariate proportional odds logistic models to determine which factors were related to satisfaction in each procedure group. Results with *P* values less than 0.05 were considered statistically significant. Data were analyzed with statistical software R, version 3.2.2.

RESULTS

A total of 456 patients were eligible for this study and completed the post-endoscopy questionnaire. The patient group comprised 224 men and 232 women and the mean age of the group was 57.2 years. The study cohort included 167 (36.6%) patients who underwent EGD, 167 (36.6%) who underwent colonoscopy, and 122 (26.8%) who underwent a combined procedure (EGD and colonoscopy together). The characteristics of the three groups are shown in Table 1. Compared with the other procedure groups, the combined group had slightly higher first and total midazolam doses; the combined group was also more likely to receive more frequent injections and have longer procedure time. The EGD group was the most satisfied with conscious sedation (Figure 1).

In all, 280 (61.4%) patients reported no anxiety before endoscopy; only 149 (32.7%) patients had mild anxiety and 19 (4.2%) patients had moderate anxiety. The most common cause of anxiety was fear of endoscopy procedure (n = 69, 41.1%), followed by fear of abdominal pain during endoscopy (n = 34, 20.2%), fear of insufficient sedation (n = 19, 11.3%), and fear of paradoxical response (n = 9, 5.4%). Most patients (50.2%) expected moderate sedation with movement or eye-opening to voice, followed by light sedation (41.9%) with brief awakenings to voice (Table 2).

In the EGD group, 81 (48.5%) patients were very satisfied, 74 (44.3%) were satisfied, 7 (4.2%) were neutral, and 5 (3.0%) were dissatisfied with sedation

Table 1 Baseline characteristics of patients according to procedure group *n* (%)

	EGD (*n* = 167)	Colonoscopy (*n* = 167)	Combined group[1] (*n* = 122)	*P* value
Sex				0.099
Male	71 (42.5)	89 (53.3)	64 (52.5)	
Female	96 (57.5)	78 (46.7)	58 (47.5)	
Age (yr)				0.878
≤ 50	43 (25.7)	44 (26.3)	29 (76.2)	
> 50	124 (74.3)	123 (73.7)	93 (76.2)	
Body mass index (kg/m^2)	22.6	22.9	23.3 (76.2)	0.245
Previous sedation endoscopy				0.310
Yes	137 (82.5)	135 (80.8)	92 (75.4)	
No	29 (17.5)	32 (19.2)	30 (24.6)	
Midazolam (mg)				
First dose	4.1	4.3	4.4	0.014
Second dose	1.5	1.6	1.7	0.055
Third dose	1.0	1.6	1.5	0.269
Fourth dose	1.5	1.5	1.9	0.881
Total midazolam dose (mg)	4.4	5.0	6.4	< 0.005
No. of midazolam injections	1.2	1.4	2.2	< 0.005
Time (min)				
Midazolam injection to procedure start	5.1	4.5	4.7	0.327
Total procedure time	3.5	23.7	42.4	< 0.005
Inter-procedure gap			19.5	
Procedure finish to antidote injection	12.8	13.1	12.1	0.482
Satisfaction with sedation during endoscopy				0.016
Very satisfied	81 (48.5)	51 (30.5)	46 (37.7)	
Satisfied	74 (44.3)	86 (51.5)	58 (47.5)	
Neutral	7 (4.2)	19 (11.4)	11 (9.0)	
Dissatisfied	5 (3)	11 (6.6)	7 (5.7)	
Very dissatisfied	-	-	-	

[1]Combined group: Esophagogastroduodenoscopy and colonoscopy together. EGD: Esophagogastroduodenoscopy.

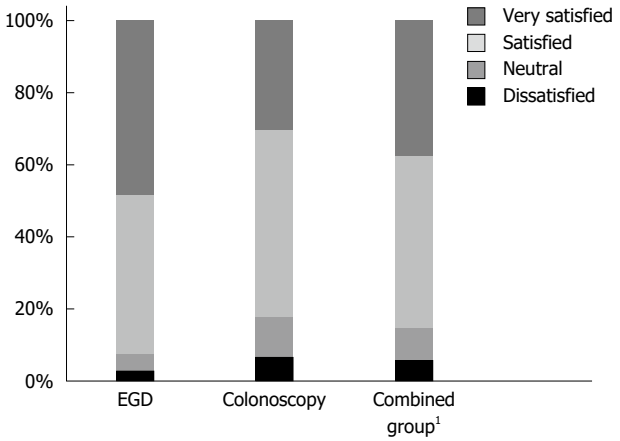

Figure 1 Patient sedation satisfaction according to endoscopy procedure. [1]Combined group: Esophagogastroduodenoscopy and colonoscopy together. EGD: Esophagogastroduodenoscopy.

Table 2 Patient anxiety and expected sedation depth before endoscopy *n* (%)

Anxiety before endoscopy	
No anxiety	280 (61.4)
Mild anxiety	149 (32.7)
Moderate anxiety	19 (4.2)
No answer	8 (1.8)
Cause of anxiety	
Fear of endoscopy procedure	69 (41.1)
Fear of abdominal pain during endoscopy	34 (20.2)
Fear of insufficient sedation	19 (11.3)
Fear of paradoxical response	9 (5.4)
None of the above	36 (21.4)
Expected sedation depth[1]	
Drowsy	19 (4.2)
Light sedation	191 (41.9)
Moderate sedation	229 (50.2)
No answer	9 (2)

[1]Drowsy: Not fully alert, but experiences sustained wakening to voice; Light sedation: Briefly awakens to voice; Moderate sedation: Movement or eye-opening to voice.

according to the post-procedure questionnaire. Total procedure time was only the factor associated with decreased satisfaction (OR = 0.97, *P* = 0.041) in the EGD group (Table 3).

In the colonoscopy group, 51 (30.5%) patients were very satisfied, 86 (51.5%) were satisfied, 19 (11.4%) were neutral, and 11 (6.6%) were dissatisfied with sedation. In our univariate analysis, younger age (≤ 50 years), total midazolam dose, and colonoscopy withdrawal time were associated with decreased patient satisfaction in this group. Age (> 50 years) (OR = 0.38, *P* = 0.005) and colonoscopy withdrawal time (OR = 1.03, *P* = 0.036) were significantly associated with sedation satisfaction in the multivariate analysis (Table 4). In colonoscopy cases, an endoscopist directly commanded nurse to inject additional doses of midazolam under active monitoring of sedation status.

Table 3 Factors associated with sedation satisfaction in the esophagogastroduodenoscopy group

	Univariate analysis				Multivariate analysis			
	Beta	SE (β)	*P* value	OR (95%CI)	Beta	SE (β)	*P* value	OR (95%CI)
Sex (Female)	0.063	0.302	0.834	1.07 (0.59-1.92)				
Body mass index (kg/m^2)	0.001	0.005	0.798	1.00 (0.99-1.01)				
Previous sedation endoscopy	0.642	0.395	0.104	1.90 (0.88-4.12)				
Midazolam, first dose (mg)	-0.094	0.143	0.509	0.91 (0.69-1.20)				
Midazolam, total dose (mg)	-0.022	0.116	0.852	0.98 (0.78-1.23)				
Time (min)								
Midazolam injection to procedure start	-0.033	0.052	0.525	0.98 (0.78-1.23)				
Total procedure time	0.127	0.062	0.041	0.97 (0.87-1.07)	0.127	0.062	0.041	0.97 (0.87-1.07)
Procedure finish to antidote injection	-0.036	0.024	0.127	1.14 (1.01-1.28)				

Table 4 Factors associated with sedation satisfaction in the colonoscopy group

	Univariate analysis				Multivariate analysis			
	Beta	SE (β)	*P* value	OR (95%CI)	Beta	SE (β)	*P* value	OR (95%CI)
Sex (female)	0.197	0.294	0.503	1.22 (0.68-2.17)				
Age (> 50 yr)	-0.937	0.339	0.006	0.39 (0.20-0.76)	-0.956	0.341	0.005	0.38 (0.20-0.75)
Midazolam, first dose (mg)	0.253	0.165	0.127	1.29 (0.93-1.78)				
Midazolam, total dose (mg)	0.223	0.108	0.039	1.25 (1.01-1.54)				
No. of midazolam injections	0.441	0.244	0.070	1.55 (0.96-2.51)				
Time (min)								
Midazolam injection to procedure start	0.009	0.051	0.863	1.01 (0.91-1.12)				
Procedure time								
Colonoscopy insertion time	0.015	0.036	0.670	1.02 (0.95-1.09)				
Colonoscopy withdrawal time	0.030	0.014	0.035	1.03 (1.00-1.06)	0.030	0.014	0.036	1.03 (1.00-1.06)
Procedure finish to antidote injection	0.016	0.020	0.447	1.02 (0.98-1.06)				

These patients were similarly satisfied with sedation despite longer procedure time due to more procedures (28.8 ± 12.2 min *vs* 22.3 ± 12.2 min, *P* = 0.005) (Table 5).

In the combined EGD and colonoscopy group, 46 (37.7%) patients were very satisfied, 58 (47.5%) were satisfied, 11 (9.0%) were neutral, and 7 (5.7%) were dissatisfied with sedation. In our univariate analysis, female sex, younger age (≤ 50 years), total midazolam dose, number of midazolam injections, procedure time, and number of endoscopic mucosal resections were associated with decreased patient satisfaction in the combined group. In the multivariate analysis, age (> 50 years) (OR = 0.38, *P* = 0.022), inter-procedure time gap (OR = 1.02, *P* = 0.027), and colonoscopy withdrawal time (OR = 1.08, *P* = 0.002) were associated with dissatisfaction with sedation (Table 6).

Five (1.1%) patients experienced a paradoxical response, 10 (2.2%) patients complained of pain during the procedure, and 7 patients complained of decreased respiration during the endoscopy procedure. Among these patients, 3 patients with paradoxical response and 2 patients with decreased respiration were given an antidote to the sedative. Of 23 dissatisfied patients, 16 complained of insufficient sedation.

DISCUSSION

Using a multivariate analysis in this prospective study, we found that longer procedure time in EGD, younger age, and longer colonoscopy withdrawal time were procedure-related factors that influenced patient satisfaction with conscious midazolam sedation. Young age, long inter-procedure time, and long colonoscopy withdrawal time were associated with decreased satisfaction in the combined EGD and colonoscopy group, as determined by the multivariate analysis. If a procedure is prolonged, the concerned endoscopist and other health care personnel should pay attention to the sedation status, especially for younger patients.

Few studies have assessed procedure-related factors that affect satisfaction with sedation. In previous studies, endoscopy-associated sedation satisfaction was related to organizational factors such as waiting time, personal considerations, and comfort of the hospital environment[7,10]. Patient factors such as nervousness and chronic use of psychotropic drugs have also been associated with sedation satisfaction[15]. The satisfaction survey mGHAA-9 has been used to evaluate the general satisfaction with hospital systems and subjective aspects of endoscopy centers; however, mGHAA-9 is insufficient to evaluate satisfaction with the sedation itself[3,7].

Previous studies found that female and young patients experienced more discomfort during endoscopy and received more sedatives than male and older patients for achieving similar comfort levels[4,16]. Our findings support the fact that female and younger patients (≤ 50 years) were less satisfied with seda-

Table 5 Patients' satisfaction through active monitoring and intervention by endoscopist during colonoscopy

	Active monitoring (n = 39)	Non-active monitoring (n = 128)	P value
Sex (Male, %)	19 (48.7)	70 (54.7)	0.584
Age (mean ± SD)	56.1 ± 13.0	57.7 ± 13.5	0.514
Proportion of EMR, n (%)	32 (82.1)	51 (39.8)	< 0.001
Satisfaction, n (%)			0.968
Very satisfied	12 (30.8)	39 (30.5)	
Satisfied	20 (51.3)	66 (51.6)	
Fair	5 (12.8)	14 (10.9)	
Unsatisfied	2 (5.1)	9 (7.0%)	
Midazolam, first dose (mg, mean ± SD)	4.5 ± 1.0	4.2 ± 0.9	0.159
Midazolam, total dose (mg, mean ± SD)	5.8 ± 1.2	4.8 ± 1.5	0.002
Midazolam, No. of injections (mean ± SD)	1.6 ± 0.5	1.4 ± 0.7	0.025
Procedure time (min, mean ± SD)	28.8 ± 12.2	22.3 ± 12.2	0.005

Table 6 Factors associated with sedation satisfaction in the combined esophagogastroduodenoscopy and colonoscopy group

	Univariate analysis				Multivariate analysis			
	β	SE (β)	P value	OR (95% CI)	β	SE (β)	P value	OR (95%CI)
Sex (Female)	0.689	0.349	0.049	1.99 (1.00-3.95)				
Age (> 50 yr)	-0.868	0.407	0.033	0.42 (0.19-0.93)	-0.978	0.427	0.022	0.38 (0.16-0.87)
Body mass index (kg/m^2)	-0.001	0.006	0.878	1.00 (0.99-1.01)				
Previous sedation endoscopy	-0.013	0.395	0.974	0.99 (0.46-2.14)				
Midazolam, first dose (mg)	0.105	0.210	0.619	1.11 (0.74-1.68)				
Midazolam, total dose (mg)	0.278	0.113	0.014	1.32 (1.06-1.65)				
No. of midazolam injections	0.690	0.284	0.015	1.99 (1.14-3.48)				
Time (min)								
Midazolam injection to procedure start	0.041	0.033	0.215	1.04 (0.98-1.11)				
Procedure time								
Inter-procedure time gap	0.021	0.010	0.043	1.02 (1.00-1.04)	0.024	0.011	0.027	1.02 (1.00-1.05)
Colonoscopy insertion time	0.084	0.038	0.027	1.09 (1.01-1.17)				
Colonoscopy withdrawal time	0.069	0.025	0.006	1.07 (1.02-1.13)	0.081	0.027	0.002	1.08 (1.03-1.14)
Procedure finish to antidote injection	0.014	0.029	0.637	1.01 (0.96-1.07)				

tion in the combined EGD and colonoscopy group. However, female sex was not a significant factor for dissatisfaction with sedation during endoscopy in our multivariate analysis.

Longer procedure time was strongly associated with dissatisfaction in our analysis. When we divided procedure time for colonoscopy procedures, colonoscopy withdrawal time was associated with sedation satisfaction. When additional procedures such as biopsies and endoscopic mucosal resections were performed, withdrawal time was longer. In colonoscopy cases, an endoscopist directed a nurse to inject additional doses of midazolam while actively monitoring sedation status. Interestingly, over 80% of these patients were satisfied with sedation and there was no decrease in the degree of satisfaction despite longer procedure time due to the additional procedures being carried out (Table 5). However, the endoscopist, as a single variable, was not statistically significant in initial univariate analysis and was not included in multivariate analyses because only one endoscopist was involved in active monitoring of patient groups. Active monitoring and intervention by an endoscopist could be an important way to improve a patient's sedation satisfaction. For active monitoring, endoscopists have to pay close attention to sedation status by observing spontaneous eye opening, verbal arousal, and complaints of pain. As a result of active monitoring, timely dose titrations of midazolam might help maintain the desired conscious sedation during the procedure.

Same-day EGD and colonoscopy are commonly used in clinical practice[17], and carried out in clinical settings when digestive disease is suspected. Performing both EGD and colonoscopy as a combined procedure is convenient for patients, efficient for providers, and saves costs for the health care system[18]. Although the combined procedure group had a longer procedure time than the single-colonoscopy group in our data, patients in the combined group were more satisfied with conscious sedation than those in the colonoscopy group. Patients in the combined group tended to have higher midazolam doses and more midazolam injections than those in the colonoscopy group. This finding is likely because the endoscopist verified the sedation status of the patient and administered additional midazolam before performing the second procedure. In the combined EGD and colonoscopy group, the inter-procedure time gap (the waiting time from the end of the first endoscopy procedure to the start of the second procedure) was related to sedation satisfaction. Therefore, this waiting time should be reduced as much as possible in clinical

practice.

In recent years, the sedative propofol use has increased in community medical practice compared to academic medical practice[19,20]. In a previous study, propofol increased sedation satisfaction by reducing fear and pain compared to other types of sedation[19]. Because propofol provided more rapid recovery than midazolam[21], it has the merit of post-procedure neuropsychologic function over midazolam[22]. Moreover, a previous study showed that propofol was cost-effective in critical illness and emergency situations[23]. However, its cost-effectiveness in outpatient endoscopy is yet unknown. It is important to select sedative medication not only for economic reasons but also for its safe use. The narrow therapeutic window of propofol necessitates close patient monitoring because of the risk of adverse cardiopulmonary events[14]. Therefore, midazolam was still the best option as a sedative during endoscopy in terms of both safety and cost-effectiveness. Administration of another sedative flumazenil results in a safe and cost-effective shortening of the recovery time[24].

This study has some limitations that must be considered. First, we collected the post-procedure survey from patients on site, usually in the recovery room. Patients may have been hesitant to provide responses indicating dissatisfaction in the presence of clinical staff. For this reason, our study showed higher satisfaction scores in on-site surveys than in mail-back surveys[25]. In addition, patients in the recovery room may still have been under the influence of midazolam and, as such, unable to answer all questions accurately. While the patients in this study answered our surveys on the day of the endoscopy examination, previous studies collected such data a few days after the examination *via* telephone surveys or using a mail-back system[7,16]. However, the response rate to telephone or mail back surveys could be lower than that to the on-site survey[25]. Even though the on-site survey has weaknesses, the magnitude of the differences is small, and the on-site method is simple and associated with a higher response rate than mail-back surveys.

Second, the surveys were not anonymous: each survey had the name of the patient and the date of the procedure printed at the top of the questionnaire. This unblinded format could also have led patients to overestimate satisfaction because most patients anticipated a return visit to the hospital to discuss the results of the endoscopy. However, anonymous questionnaires were impossible for this study because we analyzed clinical procedure data such as procedure time and midazolam doses. Third, we used a satisfaction survey that has not been formally validated. A few validated surveys exist for evaluating the general satisfaction of endoscopy, but currently no validated survey specifically evaluates sedation satisfaction.

In conclusion, midazolam is still a safe and effective sedative for gastrointestinal endoscopy. Satisfaction with sedation depends on total procedure time in EGD; younger age and colonoscopy withdrawal time in colonoscopy; and younger age, inter-procedure time gap, and colonoscopy withdrawal time in combined procedures. To improve patient satisfaction with midazolam sedation, active monitoring and intervention by the endoscopist should be considered for patients who require long procedure time.

COMMENTS

Background

The use of endoscopy is important for the early detection of gastrointestinal cancers, but some patients refuse endoscopic examinations owing to fear and anxiety over expected discomfort during the procedure. Conscious sedation endoscopy is the best option to relieve patient discomfort. Therefore, satisfaction with sedation endoscopy is critical for quality assurance in many endoscopy centers. This study was designed to evaluate patient satisfaction with conscious sedation endoscopy, to determine which procedure-related factors affect satisfaction with sedation, and to offer suggestions for improvement.

Research frontiers

In this study, the authors determined which procedure-related factors affect patient satisfaction with sedation during endoscopic examinations. Those factors varied in significance depending on the type of procedure (*e.g.*, esophagogastroduodenoscopy, colonoscopy, and combined group). This outcome suggests that the endoscopist should closely monitor sedation status and pay attention to procedure-related factors, such as procedure time or patient factor (*e.g.*, age), depending on procedure type.

Innovations and breakthroughs

An interesting finding of this study was that active monitoring and intervention by an endoscopist could be an important way to improve patient sedation satisfaction. In addition, midazolam was still found to be a safe and effective medication for conscious sedation.

Applications

The results of this study could help an endoscopist make decisions concerning midazolam titration and when to administer additional doses of midazolam.

Terminology

Midazolam is a short-acting benzodiazepine with anxiolytic, amnestic, and hypnotic effects. Propofol is an intravenous sedative-hypnotic agent used in the induction and maintenance of anesthesia.

Peer-review

A pleasure to read about this interesting topic regarding the patient/customer's perception of adequate sedation that corresponds to the use of drug. A discussion regarding cost comparison of the drugs may add another dimension to drug selection by the Endoscopist/Medical center.

REFERENCES

1 **Siegel R**, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin* 2012; **62**: 10-29 [PMID: 22237781 DOI: 10.3322/caac.20138]

2 **Abraham NS**, Fallone CA, Mayrand S, Huang J, Wieczorek P, Barkun AN. Sedation versus no sedation in the performance of diagnostic upper gastrointestinal endoscopy: a Canadian randomized controlled cost-outcome study. *Am J Gastroenterol* 2004; **99**: 1692-1699 [PMID: 15330904 DOI: 10.1111/j.1572-0241.2004.40157.x]

3 **Loftus R**, Nugent Z, Graff LA, Schumacher F, Bernstein CN, Singh H. Patient satisfaction with the endoscopy experience and

willingness to return in a central Canadian health region. *Can J Gastroenterol* 2013; **27**: 259-266 [PMID: 23712300]
4 **Childers RE**, Williams JL, Sonnenberg A. Practice patterns of sedation for colonoscopy. *Gastrointest Endosc* 2015; **82**: 503-511 [PMID: 25851159 DOI: 10.1016/j.gie.2015.01.041]
5 **Froehlich F**, Harris JK, Wietlisbach V, Burnand B, Vader JP, Gonvers JJ. Current sedation and monitoring practice for colonoscopy: an International Observational Study (EPAGE). *Endoscopy* 2006; **38**: 461-469 [PMID: 16767580 DOI: 10.1055/s-2006-925368]
6 **Porostocky P**, Chiba N, Colacino P, Sadowski D, Singh H. A survey of sedation practices for colonoscopy in Canada. *Can J Gastroenterol* 2011; **25**: 255-260 [PMID: 21647459]
7 **Ko HH**, Zhang H, Telford JJ, Enns R. Factors influencing patient satisfaction when undergoing endoscopic procedures. *Gastrointest Endosc* 2009; **69**: 883-891, quiz 891.e1 [PMID: 19152911 DOI: 10.1016/j.gie.2008.06.024]
8 **Mulcahy HE**, Kelly P, Banks MR, Connor P, Patchet SE, Farthing MJ, Fairclough PD, Kumar PJ. Factors associated with tolerance to, and discomfort with, unsedated diagnostic gastroscopy. *Scand J Gastroenterol* 2001; **36**: 1352-1357 [PMID: 11761029]
9 **Campo R**, Brullet E, Montserrat A, Calvet X, Moix J, Rué M, Roqué M, Donoso L, Bordas JM. Identification of factors that influence tolerance of upper gastrointestinal endoscopy. *Eur J Gastroenterol Hepatol* 1999; **11**: 201-204 [PMID: 10102233]
10 **Yacavone RF**, Locke GR, Gostout CJ, Rockwood TH, Thieling S, Zinsmeister AR. Factors influencing patient satisfaction with GI endoscopy. *Gastrointest Endosc* 2001; **53**: 703-710 [PMID: 11375575 DOI: 10.1067/mge.2001.115337]
11 **Sessler CN**, Gosnell MS, Grap MJ, Brophy GM, O'Neal PV, Keane KA, Tesoro EP, Elswick RK. The Richmond Agitation-Sedation Scale: validity and reliability in adult intensive care unit patients. *Am J Respir Crit Care Med* 2002; **166**: 1338-1344 [PMID: 12421743 DOI: 10.1164/rccm.2107138]
12 **Chernik DA**, Gillings D, Laine H, Hendler J, Silver JM, Davidson AB, Schwam EM, Siegel JL. Validity and reliability of the Observer's Assessment of Alertness/Sedation Scale: study with intravenous midazolam. *J Clin Psychopharmacol* 1990; **10**: 244-251 [PMID: 2286697]
13 **Whitwam JG**, Amrein R. Pharmacology of flumazenil. *Acta Anaesthesiol Scand Suppl* 1995; **108**: 3-14 [PMID: 8693922]
14 **Lichtenstein DR**, Jagannath S, Baron TH, Anderson MA, Banerjee S, Dominitz JA, Fanelli RD, Gan SI, Harrison ME, Ikenberry SO, Shen B, Stewart L, Khan K, Vargo JJ. Sedation and anesthesia in GI endoscopy. *Gastrointest Endosc* 2008; **68**: 815-826 [PMID: 18984096 DOI: 10.1016/j.gie.2008.09.029]
15 **Peña LR**, Mardini HE, Nickl NJ. Development of an instrument to assess and predict satisfaction and poor tolerance among patients undergoing endoscopic procedures. *Dig Dis Sci* 2005; **50**: 1860-1871 [PMID: 16187188 DOI: 10.1007/s10620-005-2952-7]
16 **Seip B**, Bretthauer M, Dahler S, Friestad J, Huppertz-Hauss G, Høie O, Kittang E, Nyhus S, Pallenschat J, Sandvei P, Stallemo A, Svendsen MV, Hoff G. Patient satisfaction with on-demand sedation for outpatient colonoscopy. *Endoscopy* 2010; **42**: 639-646 [PMID: 20669075 DOI: 10.1055/s-0030-1255612]
17 **Urquhart J**, Eisen G, Faigel DO, Mattek N, Holub J, Lieberman DA. A closer look at same-day bidirectional endoscopy. *Gastrointest Endosc* 2009; **69**: 271-277 [PMID: 18725159 DOI: 10.1016/j.gie.2008.04.063]
18 **El-Serag HB**, Xu F, Biyani P, Cooper GS. Bundling in medicare patients undergoing bidirectional endoscopy: how often does it happen? *Clin Gastroenterol Hepatol* 2014; **12**: 58-63 [PMID: 23911874 DOI: 10.1016/j.cgh.2013.07.021]
19 **Kilgert B**, Rybizki L, Grottke M, Neurath MF, Neumann H. Prospective long-term assessment of sedation-related adverse events and patient satisfaction for upper endoscopy and colonoscopy. *Digestion* 2014; **90**: 42-48 [PMID: 25139268 DOI: 10.1159/000363567]
20 **Faulx AL**, Vela S, Das A, Cooper G, Sivak MV, Isenberg G, Chak A. The changing landscape of practice patterns regarding unsedated endoscopy and propofol use: a national Web survey. *Gastrointest Endosc* 2005; **62**: 9-15 [PMID: 15990813]
21 **Patterson KW**, Casey PB, Murray JP, O'Boyle CA, Cunningham AJ. Propofol sedation for outpatient upper gastrointestinal endoscopy: comparison with midazolam. *Br J Anaesth* 1991; **67**: 108-111 [PMID: 1859744]
22 **Ulmer BJ**, Hansen JJ, Overley CA, Symms MR, Chadalawada V, Liangpunsakul S, Strahl E, Mendel AM, Rex DK. Propofol versus midazolam/fentanyl for outpatient colonoscopy: administration by nurses supervised by endoscopists. *Clin Gastroenterol Hepatol* 2003; **1**: 425-432 [PMID: 15017641]
23 **Hohl CM**, Nosyk B, Sadatsafavi M, Anis AH. A cost-effectiveness analysis of propofol versus midazolam for procedural sedation in the emergency department. *Acad Emerg Med* 2008; **15**: 32-39 [PMID: 18211311 DOI: 10.1111/j.1553-2712.2007.00023.x]
24 **Mathus-Vliegen EM**, de Jong L, Kos-Foekema HA. Significant and safe shortening of the recovery time after flumazenil-reversed midazolam sedation. *Dig Dis Sci* 2014; **59**: 1717-1725 [PMID: 24563235 DOI: 10.1007/s10620-014-3061-2]
25 **Lin OS**, Schembre DB, Ayub K, Gluck M, McCormick SE, Patterson DJ, Cantone N, Soon MS, Kozarek RA. Patient satisfaction scores for endoscopic procedures: impact of a survey-collection method. *Gastrointest Endosc* 2007; **65**: 775-781 [PMID: 17466197 DOI: 10.1016/j.gie.2006.11.032]

P- Reviewer: Hay JM, Kumaran SV, Triantafillidis JK
S- Editor: Qi Y **L- Editor**: Wang TQ **E- Editor**: Liu WX

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v23.i6.1106

World J Gastroenterol 2017 February 14; 23(6): 1106-1112
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Coexisting tubular adenoma with a neuroendocrine carcinoma of colon allowing early surgical intervention and implicating a shared stem cell origin

Mahmoud L Soliman, Ashish Tiwari, Qing Zhao

Mahmoud L Soliman, Qing Zhao, Department of Pathology and Laboratory Medicine, Boston University Medical Center, Boston, MA 02118, United States

Ashish Tiwari, Department of Gastroenterology, Boston University Medical Center, Boston, MA 02118, United States

Author contributions: Soliman ML and Zhao Q contributed to the acquisition of data, writing, and revision of this manuscript; Tiwari A provided the endoscopic picture in Figure 1 and wrote the figure legend; all authors have read and approved the final version of the manuscript for publication.

Institutional review board statement: This case report was exempt from the Institutional Review Board standards at Boston University

Informed consent statement: Consent was not obtained but the presented data are anonymized and risk of identification is minimal.

Conflict-of-interest statement: The authors declare no conflicts of interest.

Manuscript source: Unsolicited manuscript

Correspondence to: Qing Zhao, MD, PhD, Assistant Professor, Department of Pathology and Laboratory Medicine, Boston University Medical Center, 670 Albany Street, 6th floor, Room 670, Boston, MA 02118,
United States. grace.zhao@bmc.org
Telephone: +1-617-4145339
Fax: +1-617-4145314

Received: October 28, 2016
Peer-review started: October 28, 2016
First decision: December 01, 2016
Revised: December 17, 2016
Accepted: January 4, 2017
Article in press: January 4, 2017
Published online: February 14, 2017

Abstract

High-grade colonic neuroendocrine carcinomas (NECs) are uncommon but extremely aggressive. Their co-existence with tubular adenoma (TA) has rarely been reported. We present a 68-year-old man who was found on routine colonoscopy to have multiple colorectal TAs and an ulcerated lesion in the ascending colon. Microscopically, a poorly-differentiated invasive carcinoma juxtaposed with a TA was identified. Differential diagnosis included a poorly-differentiated adenocarcinoma, medullary carcinoma, high-grade NEC and lymphoma. The immunohistochemical profile showed positive staining for keratins, synaptophysin and chromogranin but negative for LCA, CDX2, CK7, CK20, TTF-1 and PSA, supporting the NEC diagnosis. Upon subsequent laparoscopic right hemicolectomy, the tumor was identified as a 3.0 cm umbilicated and ulcerated mass with an adjacent TA. Both TA and NEC showed positive staining for β-catenin indicating a shared colonic origin. The mitotic counts (77/10 high power fields) and a high proliferation rate (75% by Ki-67) corroborated a high-grade stratification. Mutational analysis indicated a wild-type *BRAF* and *KRAS* with mismatch repair proficiency. The AJCC (7th edition) pathologic stage is pT3, pN0, pMx. The patient received adjuvant chemotherapy with cisplatin/etoposides for three cycles and will be followed up for a year to detect recurrence. In conclusion, the co-existence of TA with high grade-NEC in our case allowed early identification

and intervention of the otherwise asymptomatic but aggressive tumor. In addition, the finding of a high-grade NEC within a large TA in this case suggests a link between the two lesions and could represent a shared stem cell origin.

Key words: Neuroendocrine carcinoma; Tubular adenoma; Colorectal; Colocalization

Core tip: This is a case report of a patient with a high-grade large cell neuroendocrine carcinoma in the ascending colon with an overlying tubular adenoma discovered during routine colonoscopic screening in absence of clinical symptoms. This is a unique case where the contiguity of the neuroendocrine carcinoma to the tubular adenoma allowed for the diagnosis of the otherwise asymptomatic high-grade carcinoma. Being aware of this association bears practical implication where it can be conducive to the early and correct diagnosis of invasive cancer. In addition, we review the literature citing pertinent cases.

Soliman ML, Tiwari A, Zhao Q. Coexisting tubular adenoma with a neuroendocrine carcinoma of colon allowing early surgical intervention and implicating a shared stem cell origin. *World J Gastroenterol* 2017; 23(6): 1106-1112 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i6/1106.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i6.1106

INTRODUCTION

Neuroendocrine tumors (NET) is a distinct group of neoplasms that arises from enterochromaffin/neuroendocrine cells throughout the body and display unique histomorphology. The NETs are composed of neuroendocrine secretory granules with neurosecretory capacity that can be detected immunohistochemically on tissue sections and serologically in peripheral blood, *i.e.,* chromogranin A, which can be used as tumor surrogate marker. The 2010 World Health Organization (WHO) classification, divides NET into well- and poorly-differentiated categories. The well-differentiated NET include low-grade (G1) and intermediate-grade (G2), whereas the poorly-differentiated tumors are high-grade (G3) and are called neuroendocrine carcinoma (NEC), including large cell and small cell types[1]. Both mitotic count and Ki-67 labeling index for measuring proliferation rate are required for tumor classification.

NETs in gastrointestinal tract account for 2% of all GI malignancies, and majority of them are well- to moderately-differentiated. High-grade NECs are extremely rare in GI tract, but they are extremely aggressive with poor prognosis when compared to colorectal adenocarcinoma of similar pathological stages due to their advanced and widely metastatic disease at the time of diagnosis[2,3]. The coexistence of tubular adenoma (TA) with high-grade NECs has been reported in only a few articles in the GI tracts such as in the ampulla of Vater[4], stomach[5] and rectum[6,7]. Most of the reported cases showed liver or lymph nodes metastases at the time of diagnosis. We present a unique case of a large cell high-grade NEC of ascending colon that was identified during a routine colonoscopic surveillance, due to its coexistence with a TA.

CASE REPORT

A 68-year-old male undergoing a routine colonoscopy was found to have multiple TAs measuring 5-8 mm, including one in ascending colon with surrounding ulcerated, irregular and slightly raised mucosa (Figure 1A) which was also biopsied. Under microscopic examination, an infiltrating poorly-differentiated malignant tumor was identified in the ulcerated lesion in close vicinity with an overlying TA. The tumor demonstrated pathological findings suggestive of a high-grade malignancy such as increased mitotic figures and apoptosis. The differential diagnosis included a poorly-differentiated adenocarcinoma, medullary carcinoma, NEC, high-grade lymphoma and sarcoma. Immunohistochemical studies performed on formalin-fixed paraffin-embedded tissue sections revealed positive staining for keratin Cam5.2, neuroendocrine markers synaptophysin and chromogranin but negative for lymphoma (LCA/CD45), colorectal adenocarcinoma (CDX2, CK20, CK7), lung adenocarcinoma (TTF-1) and prostatic adenocarcinoma (PSA) markers. A diagnosis of high-grade NEC large cell type with associated TA was rendered. Further evaluation by computed tomography (CT) and magnetic resonance imaging (MRI) for tumor staging revealed no metastatic disease or lymphadenopathy. Three months after the biopsy, the patient underwent laparoscopic right hemicolectomy. A 3-cm umbilicated tumor mass was identified in the proximal ascending colon with slightly raised border and central ulceration (Figure 1B). The tumor was grossly and microscopically seen invading into and through the muscularis propria into subserosal soft tissue, but not the serosa. The microscopic examination revealed solid sheets and nests of high-grade tumor cells with medium to large-sized vesicular nuclei and prominent nucleoli as well as prominent lymphocytic infiltration; a pattern morphologically consistent with the large-cell variant of high-grade NEC (Figure 2A and B). Moreover, the residual overlying TA is again identified in the raised mucosal surface with no high-grade dysplasia (Figure 2A). In addition to the classic morphology of a high-grade large-cell neuroendocrine carcinoma, the diagnosis was confirmed using immunohistochemical studies showing positive staining of the tumor cells for synaptophysin and chromogranin (Figure 3A

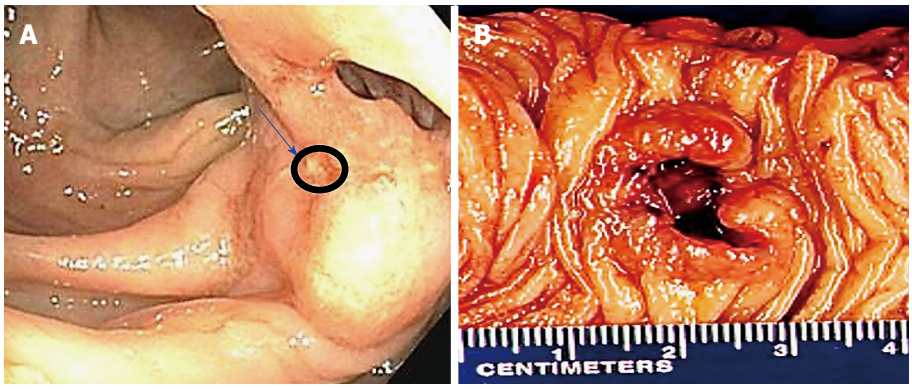

Figure 1 A 68-year-old male undergoing a routine colonoscopy was found to have multiple tubular adenomas measuring 5-8 mm. A: The endoscopic picture shows an ulcerated non-circumferential medium-sized slighted raised polyp/lesion (arrow and circle) in the ascending colon measuring 0.3 cm in diameter; B: Gross picture of the specimen with the ascending colon showing an ulcerating mass with raised borders and clear resection and radial margins.

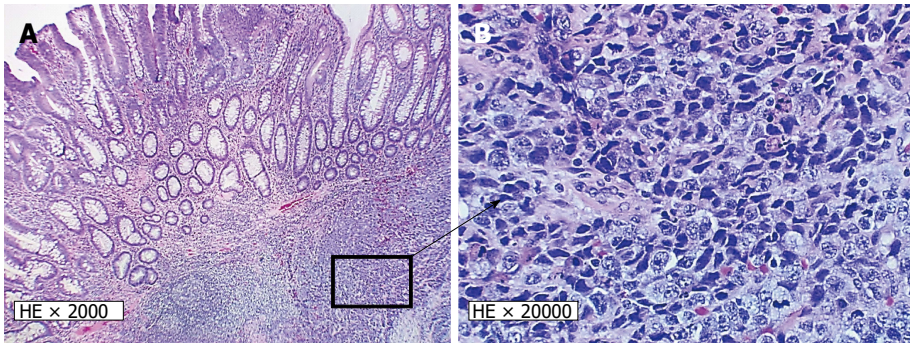

Figure 2 Hematoxylin and eosin staining of the tumor sections at × 2000 (A) and × 20000 (B). A: Shows the tubular adenoma component juxtaposed to the underlying high-grade large-cell neuroendocrinal carcinoma; B: Higher magnification showing medium to large-sized tumor cells with vesicular nuclei, prominent nucleoli consistent with large-cell neuroendocrine carcinoma.

and B). The number of mitotic figures [up to 77/10 high power fields (hpf)], the presence of focal tumor necrosis and the Ki-67 proliferation index of 75% (Figure 3D) confirmed the stratification as a high-grade NEC. Mutational analysis studies indicated that the NEC tumor cells expressed wild type *BRAF* and *KRAS* in addition to intact expression of mismatch repair proteins (MLH1, MSH2, PMS2 and MSH6) and therefore negative screening for Lynch syndrome. Immunohistochemical studies showed strong and diffuse positive staining for CAM5.2 and nuclear staining for β-catenin in the NEC tumor cells and TA (Figure 3C, E and F), indicating both components are related. No regional lymph node metastases were noted in 24 examined lymph nodes. According to the American Joint Committee on Cancer Staging Manual, 7th edition, the tumor is staged as pT3, pN0, pMx. The patient received adjuvant chemotherapy with cisplatin/etoposides for three cycles and has not shown any recurrence. The patient will be followed up for a year to detect recurrence.

Discussion and literature review

We described a clinical scenario of a 68-year-old male patient who was found to have a mixed high-grade large cell NEC with an overlying TA discovered during routine colonoscopic screening in absence of clinical symptoms. The invasive large cell NEC was located underneath the TA, deeply involving the muscularis propria, without liver or distant metastatic disease or lymph nodes involvement at the time of primary tumor resection. The patient received postsurgical chemotherapy due to the high-grade nature of the carcinoma. As of the time of the article preparation, no recurrence was identified. Long-term follow up will be necessary to report any potential recurrence. We suspect that the clinical outcome will be shaped by the behavior of the clinically predominant tumor, which is neuroendocrine tumor in the present case.

The sequence of adenoma to adenocarcinoma in colorectal cancer is well established and both are believed to originate from the same precursor cells. A mixed or collision tumor is composed of both adenocarcinoma and NEC. The notion that the two malignancies as well as the TA originate from common multipotent stem cells simultaneously differentiating into glandular and neuroendocrine lineages is likely and plausible. However, when a TA is mixed with a high-grade NEC, their association becomes less clear. Following the examination of genome-wide loss of heterozygosity, it

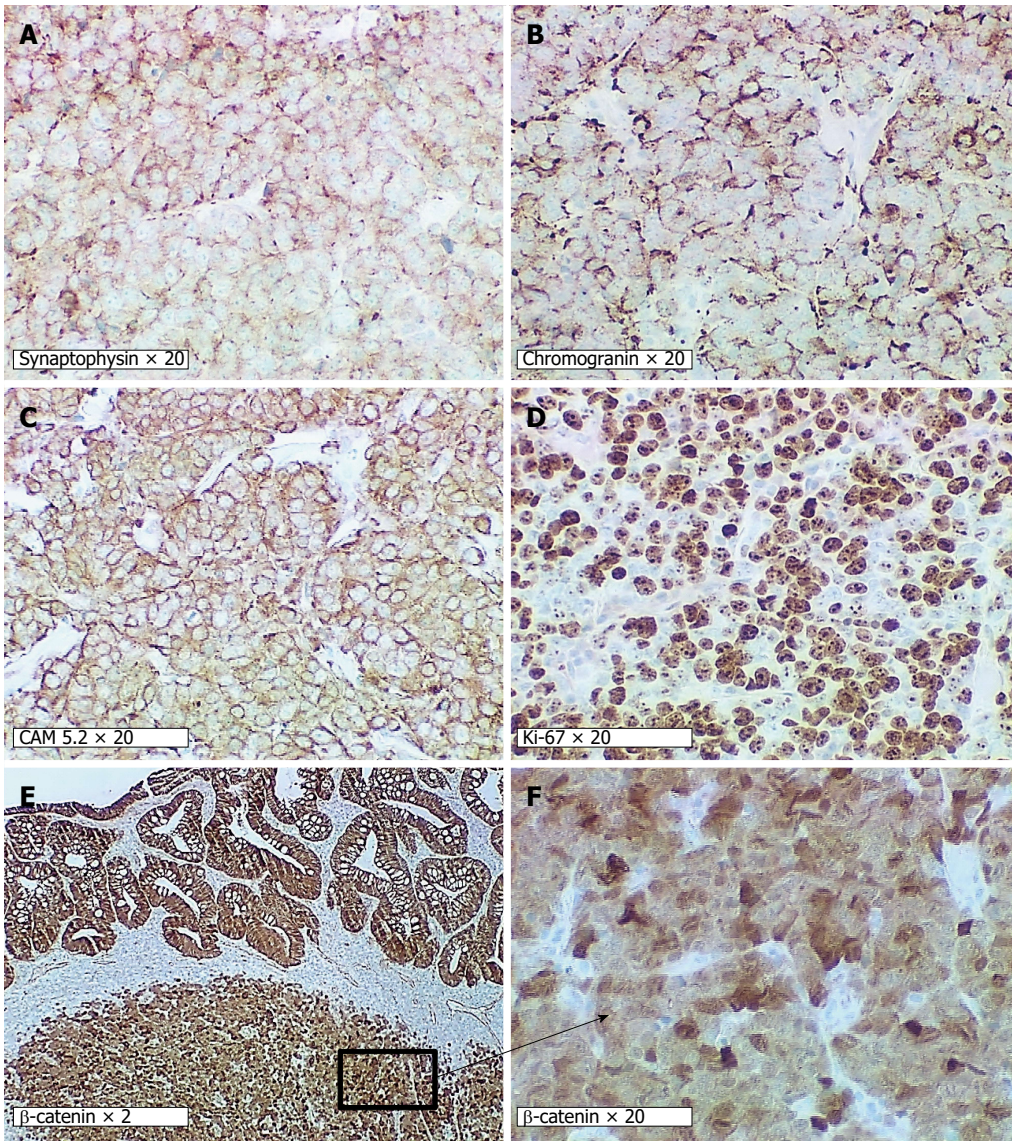

Figure 3 High-grade large-cell neuroendocrine carcinoma. Immunohistochemical profile of the tumor showing positive staining for synaptophysin (A) and chromogranin (B) in the neuroendocrine carcinoma component. Both the glandular and neuroendocrine components stained positive for CAM5.2 (C) and β-catenin (E and F). The Ki-67 labeling index is approximately 75% positive in stained tumor cells (D).

was proposed that the common genetics of the glandular and neuroendocrine components indicate origin from a single precursor[8]. This suggests that the two components of mixed tumors arise from multi-potential stem cells and show bi-phenotypic differentiation after carcinogenesis is initiated[9,10]. An alternative hypothesis is that mixed tumors are a neuroendocrine phenotype of dedifferentiated tubular adenocarcinoma[11]. Certain cases are reported of cecum and rectosigmoid collision adenocarcinoma and neuroendocrine tumors which were found to metastasize to lymph nodes as juxtaposed glandular and neuroendocrine components[12,13]. Likewise, a case of collision tumor located in transverse colon (adenocarcinoma and large-cell NEC) presented three years after resection with a retroperitoneal metastasis with 50% NEC and 50% adenocarcinoma[14]. These latter examples support the counter argument against the double primaries theory.

Poorly differentiated NEC may lose their intestinal differentiation and show negative immunostaining for CK20 and CDX2 as in our case. However, we found CK20 and CDX2 to be positive in the TA component as expected. The dedifferentiation and/or loss of intestinal differentiation may be a late event.

The current case presented as an ulcerative mass in the right colon, with TA (30% of the mass) juxtaposed with NEC (70% of the mass). In a review of 67 previously reported cases and 23 new cases, mixed neoplasms were found to present as a polypoid growth (57%), mass (30%) and ulcerating lesion (9%). The glandular component varied from adenoma (17%) to adenoma with well- or moderately-differentiated adenocarcinoma (35%), or adenoma with poorly-

differentiated adenocarcinoma (48%). Furthermore, 56% of mixed colo-rectal tumors arise in the right colon with the tumors being 58% collision tumors (two distinct neoplastic components), 42% composite (*i.e.,* intermixed neoplastic components) and less than 1% amphicrine (where individual cells demonstrate both exocrine and endocrine features) and with mean age of presentation of 61.9[15]. Nevertheless, mixed neoplasms have also been reported in various locations like the gallbladder[16], pancreatic ampulla[17], stomach[10] and cecum[18]. Based on the WHO 2010 classification, the neuroendocrine component in the present case is G3 based on the mitotic count of 77/10 hpf and the Ki-67 index of 75%, which far exceed the 20/10 hpf and 20% proliferation index, respectively used to grade NECs.

The patient under study has not presented with symptoms suggestive of carcinoid syndrome. Whereas many neuroendocrine tumors present with secretory syndromes characterized by flushing, diarrhea, wheezes, sweating, palpitations and right-sided heart valve lesions, gastrointestinal neuroendocrine tumors are less likely to present with carcinoid syndrome[19]. Serum chromogranin A elevation can be detected in patient with neuroendocrine tumor in the absence of carcinoid syndromes.

The large cell NEC is a poorly-differentiated neoplasm that is quite and belongs to the poorest prognostic subgroup among primary colorectal cancers. In a study of 6495 patients with colorectal cancer, only 0.65% had NEC and only 0.2% had large cell NEC. Despite its rarity, it is important to differentiate colorectal NEC from other tumor types because patients may benefit from alternative cytotoxic chemotherapy[20]. In this regard, unlike the colorectal adenocarcinoma, large cell NEC is managed primarily with platinum based chemotherapy such as cisplatin/etoposide and cisplatin/irinotecan. Based on various studies, the median survival rate for patients with colorectal NEC is between 5 and 11 mo with one-year survival rate between 10% and 15%. Patients with colorectal large cell neuroendocrine carcinoma were found an overall median survival of 9.4 mo[20].

Well-differentiated neuroendocrine tumors (also known as carcinoid) or microcarcinoid tumor nests had been reported in association with colonic tubular or tubulovillous adenoma. Those neuroendocrine components are well-differentiated either forming tumor nodules or scattered in the lamina propria within the adenoma lesion[21,22]. High-grade NECs arising in association with tubulovillous or tubular adenoma have only been seen in a handful of cases[23-26]. Whereas more than 50% of cases were found to have liver, bone or nodal metastasis at the time of diagnosis even when the tumor was microscopic, some primary tumors showed invasion limited to the muscularis mucosa. Therefore, colorectal NECs tend to behave aggressively and the prognosis is extremely poor with survival lasting a few months. Our case represents a unique situation where the presence of a TA warranted a polypectomy and endoscopic evaluation led to the adjacent ulcerated mucosa to be noticed and biopsied. In other words, the contiguity of the NEC to the TA allowed for early diagnosis of the otherwise asymptomatic NEC. Being aware of this association bears practical implication where it can be conducive to the early and correct diagnosis of high-grade tumor such as the NEC in the current case, and avoiding overlooking other fragments distinct from the adenoma.

Another implication to be born in mind is that superficial biopsy specimens may not provide the adequate representation of underlying neuroendocrine tumor found in the mixed tumor. Even when present in the biopsy sample, pathologists must be vigilant of the possibility of a mixed tumor and resist the tendency to diagnose an adenoma before careful inspection of the tissue for additional lesions. The overlying large TA or tubulovillous adenomas may mask a deep invasive tumor, making it imperative to collect adequate and deep tissue samples. Furthermore, surgical pathologist must also be aware of the differential diagnosis of poorly differentiated neoplasms that may involve an adenoma. This includes poorly-differentiated adenocarcinoma, medullary carcinoma, high-grade neuroendocrine carcinoma and lymphoma. Because the prognosis and management varies significantly amongst these entities, careful attention must be paid to the morphology of the tumor cells and general architecture. In addition, a panel of immunohistochemical stains that includes neuroendocrine, lymphocytic and adenocarcinoma markers and DNA mismatch repair enzyme screening should be considered to use for rendering the correct diagnosis.

COMMENTS

Case characteristics

An asymptomatic 68-year-old male patient undergoing routine colonoscopy.

Clinical diagnosis

Multiple ascending colon tubular adenomas, one of which is surrounded by an ulcerated and irregular mucosa of a suspicious lesion.

Differential diagnosis

The tumor demonstrated morphological features of high-grade and poorly differentiated malignancy. Broad differential diagnoses included a poorly-differentiated adenocarcinoma, medullary carcinoma, neuroendocrine carcinoma, high-grade lymphoma and sarcoma.

Laboratory diagnosis

Laboratory results were unremarkable.

Imaging diagnosis

Computed tomography and magnetic resonance imaging evaluation for tumor staging revealed no metastatic disease or lymphadenopathy.

Pathological diagnosis

Immunohistochemical studies performed on formalin-fixed paraffin-embedded tissue sections revealed positive staining of the tumor cells for the neuroendocrine markers (synaptophysin and chromogranin) but negative staining for lymphoma (LCA/CD45), colorectal adenocarcinoma (CDX2, CK20, CK7) and prostatic adenocarcinoma markers, rendering a diagnosis of high-grade neuroendocrine large-cell type with associated tubular adenoma.

Treatment

Laparoscopic right hemicolectomy followed by three cycles of adjuvant chemotherapy with cisplatin/etoposides.

Term explanation

Neuroendocrine carcinomas are malignant neoplasms of the enterochromaffin tissue.

Experiences and lessons

For patients undergoing colonoscopy screening, all biopsy fragments should be examined at all levels with high suspicion in order not to miss indolent high grade malignancies.

Peer-review

Colocalization of tubular adenoma and high-grade neuroendocrine carcinoma is rarely encountered; early diagnosis of a high-grade albeit asymptomatic neuroendocrine carcinoma carries a better long-term survival benefit.

REFERENCES

1 **Klimstra DS**. Pathologic Classification of Neuroendocrine Neoplasms. *Hematol Oncol Clin North Am* 2016; **30**: 1-19 [PMID: 26614366 DOI: 10.1016/j.hoc.2015.08.005]

2 **Yao JC**, Hassan M, Phan A, Dagohoy C, Leary C, Mares JE, Abdalla EK, Fleming JB, Vauthey JN, Rashid A, Evans DB. One hundred years after "carcinoid": epidemiology of and prognostic factors for neuroendocrine tumors in 35,825 cases in the United States. *J Clin Oncol* 2008; **26**: 3063-3072 [PMID: 18565894 DOI: 10.1200/JCO.2007.15.4377]

3 **Ilett EE**, Langer SW, Olsen IH, Federspiel B, Kjær A, Knigge U. Neuroendocrine Carcinomas of the Gastroenteropancreatic System: A Comprehensive Review. *Diagnostics* (Basel) 2015; **5**: 119-176 [PMID: 26854147 DOI: 10.3390/diagnostics5020119]

4 **Sun JH**, Chao M, Zhang SZ, Zhang GQ, Li B, Wu JJ. Coexistence of small cell neuroendocrine carcinoma and villous adenoma in the ampulla of Vater. *World J Gastroenterol* 2008; **14**: 4709-4712 [PMID: 18698690 DOI: 10.3748/wjg.v14.i29.4709]

5 **De Marco L**, Carlinfante G, Botticelli L, Di Maira PV, Putrino I, Cavazza A. Mixed neoplasia of the stomach: description of a case of tubular adenoma combined with carcinoid. *Pathologica* 2003; **95**: 214-216 [PMID: 14577207]

6 **Yoshikane H**, Arakawa D, Kawashima H, Sakakibara A, Hidano H, Takahashi H, Yokoi T. Small neuroendocrine carcinoma of the rectum entirely covered by an adenomatous component. *Endoscopy* 2001; **33**: 298 [PMID: 11293775 DOI: 10.1055/s-2001-12807]

7 **Khamidullina GA**, Kapuller LL, Sereda EN, Izbagambetov NA, Zharkov NV. Small-cell rectal carcinoma coexisted with tubular-villous adenoma. *Arkh Patol* 2006; **68**: 37-39 [PMID: 17144530]

8 **Kim KM**, Kim MJ, Cho BK, Choi SW, Rhyu MG. Genetic evidence for the multi-step progression of mixed glandular-neuroendocrine gastric carcinomas. *Virchows Arch* 2002; **440**: 85-93 [PMID: 11942581]

9 **Kitajima T**, Kaida S, Lee S, Haruta S, Shinohara H, Ueno M, Suyama K, Oota Y, Fujii T, Udagawa H. Mixed adeno(neuro)endocrine carcinoma arising from the ectopic gastric mucosa of the upper thoracic esophagus. *World J Surg Oncol* 2013; **11**: 218 [PMID: 24139488 DOI: 10.1186/1477-7819-11-218]

10 **Kim JJ**, Kim JY, Hur H, Cho YK, Han SU. Clinicopathologic significance of gastric adenocarcinoma with neuroendocrine features. *J Gastric Cancer* 2011; **11**: 195-199 [PMID: 22324009 DOI: 10.5230/jgc.2011.11.4.195]

11 **Gurzu S**, Kadar Z, Bara T, Bara T, Tamasi A, Azamfirei L, Jung I. Mixed adenoneuroendocrine carcinoma of gastrointestinal tract: report of two cases. *World J Gastroenterol* 2015; **21**: 1329-1333 [PMID: 25632209 DOI: 10.3748/wjg.v21.i4.1329]

12 **Pecorella I**, Memeo L, Ciardi A, Rotterdam H. An unusual case of colonic mixed adenoendocrine carcinoma: collision versus composite tumor. A case report and review of the literature. *Ann Diagn Pathol* 2007; **11**: 285-290 [PMID: 17630114 DOI: 10.1016/j.anndiagpath.2006.03.011]

13 **Meşină C**, Vasile I, Ciobanu D, Calotă F, Gruia CL, Streba L, Mogoantă SŞ, Pârvănescu H, Georgescu CV, Tarniţă DN. Collision tumor of recto-sigmoidian junction - case presentation. *Rom J Morphol Embryol* 2014; **55**: 643-647 [PMID: 25178338]

14 **Minaya-Bravo AM**, Garcia Mahillo JC, Mendoza Moreno F, Noguelares Fraguas F, Granell J. Large cell neuroendocrine - Adenocarcinona mixed tumour of colon: Collision tumour with peculiar behaviour. What do we know about these tumours? *Ann Med Surg* (Lond) 2015; **4**: 399-403 [PMID: 26635955 DOI: 10.1016/j.amsu.2015.10.004]

15 **Li Y**, Yau A, Schaeffer D, Magliocco A, Gui X, Urbanski S, Waghray R, Owen D, Gao ZH. Colorectal glandular-neuroendocrine mixed tumor: pathologic spectrum and clinical implications. *Am J Surg Pathol* 2011; **35**: 413-425 [PMID: 21317713 DOI: 10.1097/PAS.0b013e3182093657]

16 **Paniz Mondolfi AE**, Slova D, Fan W, Attiyeh FF, Afthinos J, Reidy J, Pang Y, Theise ND. Mixed adenoneuroendocrine carcinoma (MANEC) of the gallbladder: a possible stem cell tumor? *Pathol Int* 2011; **61**: 608-614 [PMID: 21951672 DOI: 10.1111/j.1440-1827.2011.02709.x]

17 **Huang Z**, Xiao WD, Li Y, Huang S, Cai J, Ao J. Mixed adenoneuroendocrine carcinoma of the ampulla: two case reports. *World J Gastroenterol* 2015; **21**: 2254-2259 [PMID: 25717267 DOI: 10.3748/wjg.v21.i7.2254]

18 **Jain A**, Singla S, Jagdeesh KS, Vishnumurthy HY. Mixed adenoneuroendocrine carcinoma of cecum: a rare entity. *J Clin Imaging Sci* 2013; **3**: 10 [PMID: 23607079 DOI: 10.4103/2156-7514.107995]

19 **Wang YH**, Lin Y, Xue L, Wang JH, Chen MH, Chen J. Relationship between clinical characteristics and survival of gastroenteropancreatic neuroendocrine neoplasms: A single-institution analysis (1995-2012) in South China. *BMC Endocr Disord* 2012; **12**: 30 [PMID: 23194346 DOI: 10.1186/1472-6823-12-30]

20 **Bernick PE**, Klimstra DS, Shia J, Minsky B, Saltz L, Shi W, Thaler H, Guillem J, Paty P, Cohen AM, Wong WD. Neuroendocrine carcinomas of the colon and rectum. *Dis Colon Rectum* 2004; **47**: 163-169 [PMID: 15043285]

21 **Pulitzer M**, Xu R, Suriawinata AA, Waye JD, Harpaz N. Microcarcinoids in large intestinal adenomas. *Am J Surg Pathol* 2006; **30**: 1531-1536 [PMID: 17122508 DOI: 10.1097/01.pas.0000213295.88778.00]

22 **Ito H**, Ito M, Tahara E. Minute carcinoid arising in gastric tubular adenoma. *Histopathology* 1989; **15**: 96-99 [PMID: 2767625]

23 **Aponte Aponte IE**, Caceres W. Anal large cell neuroendocrine carcinoma with tubulovillous adenoma coexistence: A case report. *Bol Asoc Med P R* 2014; **106**: 27-28 [PMID: 26148395]

24 **Kuratate S**, Inoue S, Chikakiyo M, Kaneda Y, Harino Y, Hirose T, Yagi T, Saitoh S, Sumitomo M, Fujino R, Satake N. Coexistent poorly-differentiated neuroendocrine cell carcinoma and non-invasive well-differentiated adenocarcinoma in tubulovillous adenoma of the rectum: report of a case. *J Med Invest* 2010; **57**: 338-344 [PMID: 20847536]

25 **Ispas C**, Yu J, Tarantino DR, Lara JF. Pathologic quiz case: a 44-year-old woman with a tubulovillous adenoma of the colon and liver and bone lesions. Small cell (neuroendocrine) carcinoma of the colon with metastasis and an associated, overlying villous

adenoma. *Arch Pathol Lab Med* 2005; **129**: 412-414 [PMID: 15737043 DOI: 10.1043/1543-2165(2005)1292.0.CO;2]
26 **Ubiali A**, Benetti A, Papotti M, Villanacci V, Rindi G. Genetic alterations in poorly differentiated endocrine colon carcinomas developing in tubulo-villous adenomas: a report of two cases. *Virchows Arch* 2001; **439**: 776-781 [PMID: 11787850]

P- Reviewer: Liu SH **S- Editor**: Qi Y
L- Editor: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v23.i6.1113

World J Gastroenterol 2017 February 14; 23(6): 1113-1118
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Pancreatic endometrial cyst mimics mucinous cystic neoplasm of the pancreas

Michael A Mederos, Nicole Villafañe, Sadhna Dhingra, Carlos Farinas, Amy McElhany, William E Fisher, George Van Buren II

Michael A Mederos, Nicole Villafañe, Amy McElhany, William E Fisher, George Van Buren II, Department of General Surgery, Baylor College of Medicine, Houston, TX 77030, United States

Sadhna Dhingra, Department of Pathology, Baylor College of Medicine, Houston, TX 77030, United States

Carlos Farinas, Department of Radiology, Baylor College of Medicine, Houston, TX 77030, United States

Author contributions: Mederos MA and Villafañe N designed the report and wrote the paper; Amy M assisted in writing and editing the paper; Fisher WE assisted in writing and editing the paper; Van Buren II G performed the surgery, collected the patient's clinical data, designed the report and wrote the paper; Dhingra S reviewed the pathology, selected the images, and wrote the paper; Farinas C reviewed the imaging, selected the images, and wrote the paper.

Institutional review board statement: After approval from the institutional review board through Baylor College of Medicine tissue and clinical information was obtained.

Informed consent statement: The patient provided informed consent for the study.

Conflict-of-interest statement: None of the authors have any conflict of interest.

Manuscript source: Invited manuscript

Correspondence to: George Van Buren II, MD, Assistant Professor, Department of General Surgery, Baylor College of Medicine, 6620 Main St, Suite 1350, Houston, TX 77030, United States. george.vanburen@bcm.edu
Telephone: +1-7137982262
Fax: +1-713798258

Received: September 30, 2016
Peer-review started: September 30, 2016
First decision: December 19, 2016
Revised: January 13, 2017
Accepted: January 17, 2017
Article in press: January 17, 2017
Published online: February 14, 2017

Abstract

Pancreatic cysts include a variety of benign, premalignant, and malignant lesions. Endometrial cysts in the pancreas are exceedingly rare lesions that are difficult to diagnose pre-operatively. This report describes the findings in a 43-year-old patient with a recent episode of acute pancreatitis who presented with a large cyst in the tail of the pancreas. Imaging demonstrated a loculated pancreatic cyst, and cyst fluid aspiration revealed an elevated amylase and carcinoembryonic antigen. The patient experienced an interval worsening of abdominal pain, fatigue, diarrhea, and a 15-pound weight loss 3 mo after the initial episode of pancreatitis. With concern for a possible pre-malignant lesion, the patient underwent a laparoscopic distal pancreatectomy with splenectomy, which revealed a 16 cm × 12 cm × 4 cm lesion. Final histopathology was consistent with an intra-pancreatic endometrial cyst. Here we discuss the overlapping imaging and laboratory features of pancreatic endometrial cysts and mucinous cystic neoplasms of the pancreas.

Key words: Pancreatic cyst; Pancreatic endometrial cyst; Endometriosis; Mucinous cystic neoplasm of the

pancreas; Distal pancreatectomy

Core tip: Intra-pancreatic endometrial cysts are an exceedingly rare entity. Imaging and laboratory assessments are valuable tools for diagnosing pancreatic cysts. However, pre-operative diagnosis of pancreatic endometrial cysts remains problematic. These lesions have overlapping radiographic and laboratory features with premalignant lesions, such as mucinous cystic neoplasms. Due to the diagnostic similarities between these rare endometrial cysts and the more common mucinous cystic lesions, the optimal diagnostic and therapeutic option is resection.

Mederos MA, Villafañe N, Dhingra S, Farinas C, McElhany A, Fisher WE, Van Buren II G. Pancreatic endometrial cyst mimics mucinous cystic neoplasm of the pancreas. *World J Gastroenterol* 2017; 23(6): 1113-1118 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i6/1113.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i6.1113

INTRODUCTION

Pancreatic cysts encompass a variety of benign, premalignant, and malignant lesions. Accurate pre-operative diagnosis of these cysts can pose a challenge[1-4]. Imaging, laboratory findings, and patient history often assist in classification of the lesion[1-4], however, definitive diagnosis is often made with surgical resection. Intra-pancreatic endometrial cysts are a rare entity. The most common sites of endometriosis are the pelvic organs[5]; however, endometriosis of the upper abdominal organs has been described[1,6-15]. A literature review identified 12 previous cases of pancreatic endometriosis[1,6-15]. Each of these patients underwent surgical resection because a malignant or pre-malignant lesion could not be ruled out. Due to the rarity of these lesions, there are no established radiographic or laboratory studies to help differentiate endometrial cysts from the more common and precarious premalignant pancreatic lesions, especially in the pre-menopausal age range.

CASE REPORT

A 43-year-old obese Caucasian female presented to clinic for surgical evaluation of a pancreatic cyst. Three months prior to this visit, the patient was admitted to an outside hospital with one day of severe epigastric pain. She was found to have an elevated serum amylase and diagnosed with acute pancreatitis. Her imaging with a computed tomography (CT) scan during her initial hospitalization for pancreatitis revealed an 8 cm × 10 cm pancreas cyst with wall thickening and loculation in the pancreatic tail abutting the posterior stomach, spleen, and left renal hilum. Further evaluation with endoscopic ultrasound (EUS) demonstrated a 10 cm × 6 cm mass, and fine needle aspiration of brown fluid revealed a high amylase (> 1000 U/L) and elevated carcinoembryonic antigen (CEA) (951 ng/mL). Serum CEA and cancer antigen 19-9 were within normal limits.

At this clinic visit, the patient reported worsening abdominal pain, fatigue, diarrhea, anorexia, and a 15-pound weight loss since her initial episode of pancreatitis. She denied having fever, blood in the stool, or hematemesis. Her medical history was significant for type 2 diabetes, hyperlipidemia, non-alcoholic fatty liver disease, and endometriosis. She had a past surgical history of an uncomplicated caesarian section. She denied abdominal trauma, cyclical/intermittent pain, chronic alcoholism, or episodes of pancreatitis prior to the recent episode; she had no history of previous medical or surgical therapy for her endometriosis. The patient had regular monthly menstral periods with no exacerbation of pain. There was no family history of pancreatic cancer, but her mother did have gallstone pancreatitis.

Repeat imaging at this visit revealed interval enlargement of the lesion. Axial, coronal and sagittal CT images (Figures 1 and 2) demonstrated a 10.0 cm × 11.1 cm × 16.5 cm macrocystic, thin walled, well circumscribed fluid density mass with a few internal septations and locules, which arises from and replaces the pancreatic body and tail. This mass abuts the stomach in its superior aspect and the spleen in its lateral aspect. Post-contrast images did not show evidence of internal enhancement. Mural nodules or other solid components were not identified.

The initial presentation of a cystic lesion in the setting of pancreatitis can be concerning for pancreatic pseudocyst; however, the presence of the cystic lesion at the time of the initial presentation of pancreatitis, the interval enlargement, multilocular appearance, and elevated cystic CEA were concerning for a premalignant pancreatic cystic lesion. Thus, a decision was made to proceed with resection as opposed to a drainage procedure.

Diagnostic laparoscopy revealed a large, 16 cm cyst in the pancreatic tail adherent to the spleen, left kidney, and diaphragm. Significant fatty liver disease and omental inflammation were noted. The ovaries, Fallopian tubes, and uterus appeared normal without evidence of endometriosis. A laparoscopic distal pancreatectomy and splenectomy was then performed.

Gross evaluation of the specimen revealed a 16 cm × 12 cm × 4 cm unilocular cyst containing gray-green cloudy fluid. The cyst wall was smooth, trabeculated, gray-brown with focal areas of hemorrhage. No papillary projections were seen. Histological examination showed cyst wall lined by benign cubo-columnar epithelial lining resting on a cellular stroma composed of bland spindle cells associated with thin walled blood

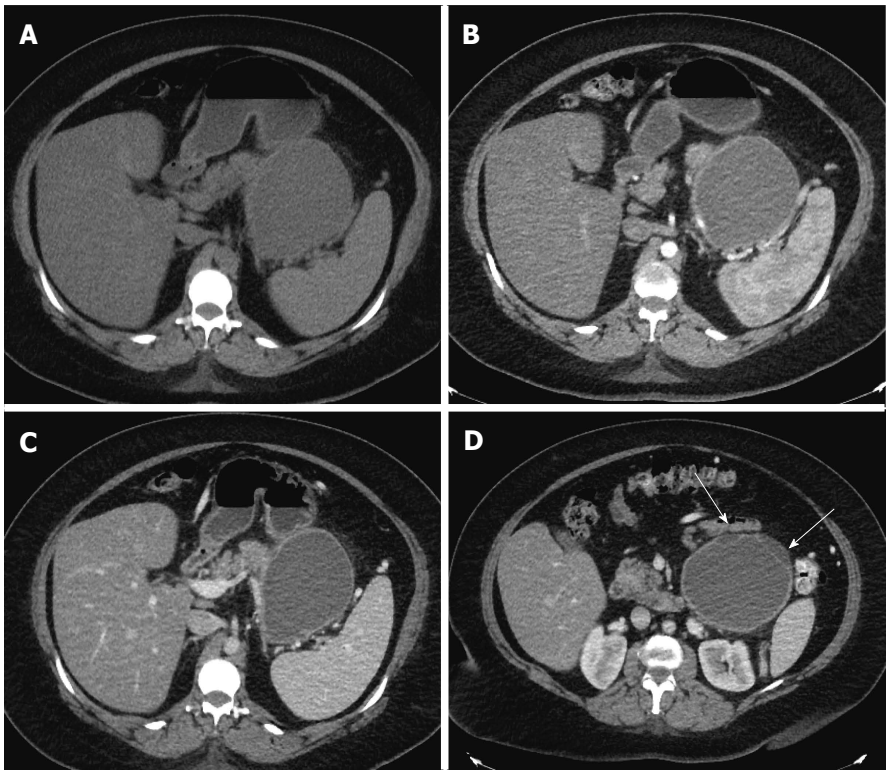

Figure 1 Axial computed tomography images. Axial computed tomography (CT) images in unenhanced (A), pancreatic (B) and portal venous phase (C) show a well circumscribed, thin walled, large fluid density cystic lesion arising from and replacing the pancreatic body and tail, which abuts the spleen. The lesion does not exhibit post-contrast enhancement. Axial CT image at a lower level in portal venous phase (D) shows several thin septations and small loculations (arrows).

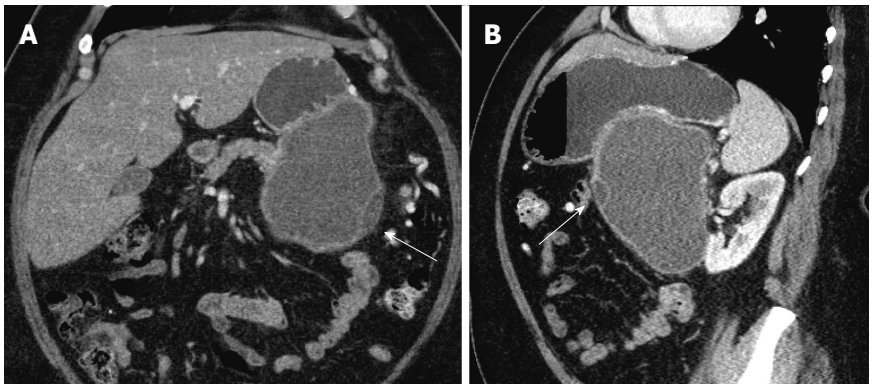

Figure 2 Coronal (A) and sagittal (B) images in portal venous phase show the same lesion abutting the stomach in its superior aspect. Thin septations and small loculations are again noted (arrows).

vessels (Figure 3). This cyst was considered intrapancreatic because pancreatic tissue was present in the wall of the cyst without a fat plane. The spindloid stroma enclosed few scattered benign glands and groups of hemosiderin laden macrophages (Figure 4). Immunohistochemical evaluation revealed the spindle cell stroma to be positive for CD10 (Figure 5), estrogen receptor and negative for inhibin (Figure 6). These features were consistent with cystic endometriosis.

After surgery, the patient had an uncomplicated hospital course, and she received post-splenectomy vaccinations. Her post-operative recovery was uncomplicated. On further questioning, the patient relayed a distant history of endometriosis. She claimed endometrial pain in the past however at the time of the operation she was having regular menstrual periods with her last menstrual period three weeks prior to the operation. At 16 mo from surgery the patient was doing well without any abdominal pain recurrence.

DISCUSSION

Pancreatic cysts include a variety of benign, premalignant, and malignant lesions. The expanded differential diagnosis for this case includes Mucinous cystic neoplasm (MCN) of the pancreas, pancreatic pseudocyst,

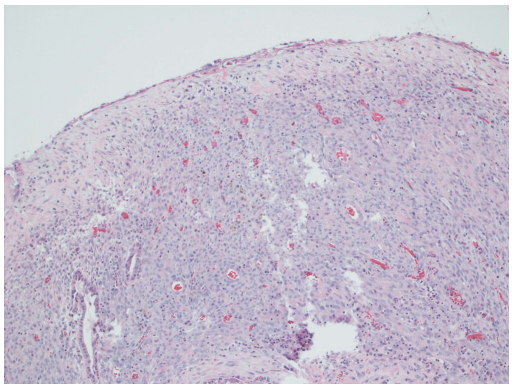

Figure 3 Cyst wall lined by bland cubo-columnar epithelium resting on a layer of cellular spindle cell stroma with thin walled blood vessels. Hematoxylin and eosin staining, magnification × 100.

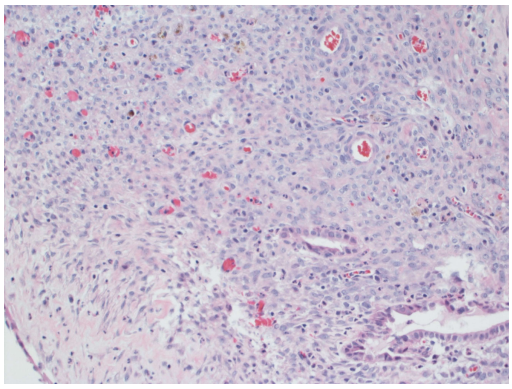

Figure 4 Cellular stroma shows bland spindle cells enclosing few benign glands and hemosiderin laden macrophages. Hematoxylin and eosin staining, magnification × 200.

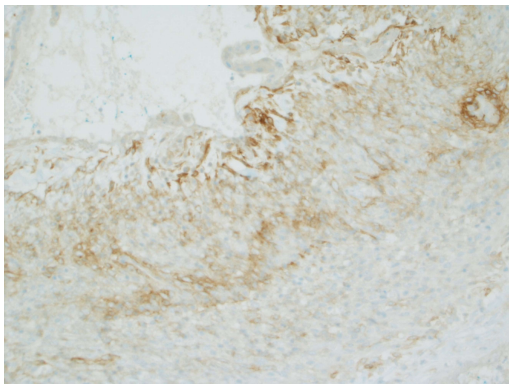

Figure 5 Positive staining for CD10 in the cellular spindloid stroma. Immunohistochemical stain for CD10, magnification × 100.

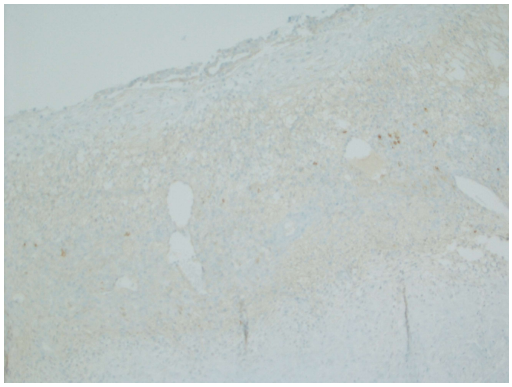

Figure 6 Negative staining for inhibin in the cellular spindloid stroma. Immunohistochemical stain for inhibin, magnification × 100.

serous cystadenoma, serous cystadenocarcinoma, solid pseudopapillary tumor, cystic pancreatic adenocarcinoma, cystic pancreatic neuroendocrine tumor, and ectopic tissue. Endometrial cysts in the pancreas are exceedingly rare lesions that are difficult to diagnose pre-operatively[1,6-15]. This report describes the findings of a giant endometrial cyst of the pancreas in a patient with a recent episode of acute pancreatitis.

Endometriosis is the presence of endometrial glands and stroma outside the uterus. This disease is characterized by recurrent pain that is commonly associated with the menstrual cycle. There are multiple theories for the pathogenesis of endometriosis, which include direct extension, menstrual regurgitation, lymphatic/vascular spread, and coelomic metaplasia[5]. The most common sites of endometriosis are the pelvic organs[5]; however, endometriosis of the upper abdominal organs has been described[1,6-15]. Endometrial cysts of the pancreas remain an exceedingly rare entity. There have been a total of only 12 cases described in the literature[1,6-15]. The most common presumptive diagnosis was a neoplastic cyst based on imaging and laboratory assessments. Pre-operative suspicion for an endometrial cyst was low in all but one case[14]. In that case report, the patient did have a gynecologic history of endometriosis as well as a previous caesarian section for her one child birth. She had never required any medical or surgical therapy for her disease. She did not relate any of her abdominal symptoms to her menstrual cycle or hormones.

The initial impression of the lesion was that it was likely a MCN of the pancreas due to its location in the pancreatic tail. A pancreatic pseudocyst was less likely since the temporal relationship with the episode of acute pancreatitis was not standard. Classically, pseudocysts develop after episodes of acute pancreatitis in a progressive fashion from peri-pancreatic fluid collections, to walled off fluid collections, to pancreatic pseudocysts[16,17]. Pancreatic pseudocyst formation occurs over a six to eight-week period after the initial episode of pancreatitis. These lesions are round fluid collections with a relatively thick and well-enhancing wall of fibrous tissue[16,17]. Radiologically, they present as extra-pancreatic lesions without septation, loculations, solid components, or wall calcification[16]. They may communicate with the pancreatic duct on magnetic resonance pancreatography or EUS[2]. Cyst fluid analysis often reveals elevations in amylase and lipase, but normal CEA levels[2-4,16,18]. In this case, the pancreatic cystic lesion was present at the time of the initial diagnosis of the episode of pancreatitis, and the patient had no previous episodes of pancreatitis.

The incorrect temporal relationship of the cyst to the pancreatitis and abnormally elevated cyst CEA were concerning for an alternative diagnosis.

MCN are lesions with malignant potential that are predominantly seen in middle-aged females[2,16]. About 90% of these lesions present in the tail of the pancreas and can exceed 20 cm in the greatest dimension[16]. On imaging, MCNs have a smooth contour and wall enhancement[2]. They may present with multiloculations, thick internal septae, papillary or nodular excrescences, and wall calcifications[2,16]. Multiple loculations and size > 5 cm have been shown to correlate with an invasive component and malignant transformation[16]. Histologically, the MCN is lined by mucinous epithelium with varying degree of dysplasia, resting on a layer of cellular ovarian-like stroma[17,19]. The ovarian-like stroma on microscopy can resemble endometrial stroma[20]; however, by immunohistochemistry the ovarian-like stroma with MCN is often positive for inhibin, estrogen receptor and progesterone receptor[1,19]. The stroma is negative for CD10, which is an immunohistochemical marker of endometrial stroma[20]. An elevated cyst fluid CEA often helps to differentiate mucinous from non-mucinous lesions[3,4,16]. In one study, a cyst fluid CEA cutoff of 109.9 ng/mL was both sensitive (81%) and specific (98%) for mucinous lesions[4]. According to another systematic review, a CEA > 800 ng/mL is strongly associated with a mucinous lesion with a specificity of 98%[3]. In this patient, the cyst was 16 cm in the largest dimension with loculations. Cyst aspiration demonstrated an elevated CEA of 951 ng/mL. These findings, in addition to the patient's age and gender, supported a presumptive diagnosis of a premalignant mucinous cyst. Classically, MCNs do not have elevated cyst amylase levels due to the fact that the lesions are not directly connected to the pancreatic duct[2-4,16-18]. In this setting, the EUS did reveal an elevated amylase which confounded the diagnosis of a possible MCN. Furthermore, MCNs can be confused with pseudocysts intraoperatively and histopathologically[18]. MCNs may contain purulent material and demonstrate ovarian stroma that resembles the granulation tissue of pseudocysts[2,18]. However, given the elevated CEA, we felt the most likely preoperative diagnosis MCN.

In all but one case reported in the literature, the presenting symptom with pancreatic endometrial cysts was abdominal pain[1,6-15]. Less than half of these cases correlated pain with the patients' menstrual cycles, and only one case had a presumptive diagnosis of endometriosis[14]. In that report, the authors suggest pancreatic endometriosis can be recognized with magnetic resonance imaging (MRI) based on a hyperintense T1 signal with fat suppression, which is suggestive of blood components. Although the MRI was suggestive of endometriosis, the authors noted that the findings were not conclusive. EUS with fine needle aspiration is a reasonable test when imaging is equivocal. In this present case report, the elevated cyst fluid CEA warranted surgical resection. An elevated cyst fluid CEA was reported in only one other report of a pancreatic endometrial cyst (940 ng/mL)[1].

In conclusion, a wide variety of benign, premalignant, and malignant pancreatic cysts exist. Pancreatic endometrial cysts remain a very rare entity. Currently, there is no reliable method of detecting endometrial disease of the pancreas pre-operatively. Pancreatic endometrial cysts can present with elevated cyst CEA. Due to the diagnostic similarities between these rare endometrial cysts and mucinous cystic lesions, the optimal diagnostic and therapeutic option is resection.

COMMENTS

Case characteristics

A 43-year-old obese Caucasian female with a history of type 2 diabetes, hyperlipidemia, non-alcoholic fatty liver disease, and endometriosis presented for surgical evaluation of a large pancreatic cyst discovered during an admission for acute pancreatitis.

Clinical diagnosis

Mild left-sided tenderness to palpation without rebound or palpable masses.

Differential diagnosis

Mucinous cystic neoplasm of the pancreas, pancreatic pseudocyst, serous cystadenoma, serous cystadenocarcinoma, solid pseudopapillary tumor, cystic pancreatic adenocarcinoma, cystic pancreatic neuroendocrine tumor, or ectopic tissue

Laboratory diagnosis

Cyst fluid analysis from endoscopic ultrasound with fine needle aspiration demonstrated an elevated elevated carcinoembryonic antigen and amylase of 951 ng/mL and > 1000 U/L, respectively.

Imaging diagnosis

Computed tomography demonstrated a 10.0 cm × 11.1 cm × 16.5 cm macrocystic, well-circumscribed fluid density mass containing a few internal septations and locules within the pancreatic body and tail.

Pathological diagnosis

Intrapancreatic cystic endometriosis.

Treatment

Laparoscopic distal pancreatectomy.

Related reports

Endometrial cysts in the pancreas are exceedingly rare lesions that are difficult to diagnose pre-operatively. This entity is easily confused for a mucinous cystic neoplasm or some other pre-malignant lesion due to similar radiographic and laboratory features.

Term explanation

Endometriosis is the presence of endometrial glands and stroma outside the uterus.

Experiences and lessons

Pancreatic endometriosis is easily confused for a malignant or pre-malignant lesion based on imaging and laboratory studies. This diagnosis should be considered in the differential in pre-menopausal women experiencing recurrent or worsening abdominal pain in the setting of a pancreatic cyst on imaging.

Without a reliable method to diagnose pancreatic endometriosis pre-operatively, surgical resection is recommended.

Peer-review

A wide variety of benign, premalignant, and malignant pancreatic cysts exist. Pancreatic endometrial cysts remain a very rare entity. Due to the diagnostic similarities between these rare endometrial cysts and mucinous cystic lesions, the optimal diagnostic and therapeutic option is resection.

REFERENCES

1 **Assifi MM**, Nguyen PD, Agrawal N, Dedania N, Kennedy EP, Sauter PK, Prestipino A, Winter JM, Yeo CJ, Lavu H. Non-neoplastic epithelial cysts of the pancreas: a rare, benign entity. *J Gastrointest Surg* 2014; **18**: 523-531 [PMID: 24449000 DOI: 10.1007/s11605-014-2459-7]

2 **Kucera JN**, Kucera S, Perrin SD, Caracciolo JT, Schmulewitz N, Kedar RP. Cystic lesions of the pancreas: radiologic-endosonographic correlation. *Radiographics* 2012; **32**: E283-E301 [PMID: 23150863 DOI: 10.1148/rg.327125019]

3 **van der Waaij LA**, van Dullemen HM, Porte RJ. Cyst fluid analysis in the differential diagnosis of pancreatic cystic lesions: a pooled analysis. *Gastrointest Endosc* 2005; **62**: 383-389 [PMID: 16111956 DOI: 10.1016/S0016-5107(05)01581-6]

4 **Cizginer S**, Turner BG, Bilge AR, Karaca C, Pitman MB, Brugge WR. Cyst fluid carcinoembryonic antigen is an accurate diagnostic marker of pancreatic mucinous cysts. *Pancreas* 2011; **40**: 1024-1028 [PMID: 21775920 DOI: 10.1097/MPA. 0b013e318 21bd62f]

5 **Giudice LC**. Clinical practice. Endometriosis. *N Engl J Med* 2010; **362**: 2389-2398 [PMID: 20573927 DOI: 10.1056/NEJMcp1000274]

6 **Marchevsky AM**, Zimmerman MJ, Aufses AH, Weiss H. Endometrial cyst of the pancreas. *Gastroenterology* 1984; **86**: 1589-1591 [PMID: 6714583 DOI: 10.1016/S0016-5085(84)80177-8]

7 **Goswami AK**, Sharma SK, Tandon SP, Malik N, Mathur RP, Malik AK, Bapna BC. Pancreatic endometriosis presenting as a hypovascular renal mass. *J Urol* 1986; **135**: 112-113 [PMID: 3941442]

8 **Verbeke C**, Härle M, Sturm J. Cystic endometriosis of the upper abdominal organs. Report on three cases and review of the literature. *Pathol Res Pract* 1996; **192**: 300-304; discussion 305 [PMID: 8739477 DOI: 10.1016/S0344-0338(96)80235-4]

9 **Sumiyoshi Y**, Yamashita Y, Maekawa T, Sakai T, Shirakusa T. A case of hemorrhagic cyst of the pancreas resembling the cystic endometriosis. *Int Surg* 2000; **85**: 67-70 [PMID: 10817436]

10 **Lee DS**, Baek JT, Ahn BM, Lee EH, Han SW, Chung IS, Sun HS, Park DH. A case of pancreatic endometrial cyst. *Korean J Intern Med* 2002; **17**: 266-269 [PMID: 12647644 DOI: 10.3904/kjim. 2002.17.4.266]

11 **Tunuguntla A**, Van Buren N, Mathews MR, Ehrenfried JA. Endometriosis of the pancreas presenting as a cystic pancreatic neoplasm with possible metastasis. *South Med J* 2004; **97**: 1020-1021 [PMID: 15558937 DOI: 10.1097/01.SMJ.0000129937.38887.87]

12 **Oishi M**, Hashida H, Yuba Y, Takabayashi A. Pancreatic endometrial cyst: report of a case. *Surg Today* 2011; **41**: 1011-1015 [PMID: 21748624 DOI: 10.1007/200595-010-4400-3]

13 **Loja Oropeza D**, Alvizuri Escobedo J, Vilca Vasquez M, Altamirano Bautista J. Endometriosis of the pancreas. *Rev Gastroenterol Peru* 2009; **29**: 55-60 [PMID: 19424410]

14 **Monrad-Hansen PW**, Buanes T, Young VS, Langebrekke A, Qvigstad E. Endometriosis of the pancreas. *J Minim Invasive Gynecol* 2012; **19**: 521-523 [PMID: 22748958 DOI: 10.1016/j.jmig. 2012.03.011]

15 **Plodeck V**, Sommer U, Baretton GB, Aust DE, Laniado M, Hoffmann RT, Platzek I. A rare case of pancreatic endometriosis in a postmenopausal woman and review of the literature. *Acta Radiol Open* 2016; **5**: 2058460116669385 [PMID: 27733937 DOI: 10.1177/2058460116669385]

16 **Kosmahl M**, Kloppel G. Pancreatic cystic lesions and neoplasms. In: Johnson CD, Imrie CW. Pancreatic disease: basic science and clinical management. London: Springer, 2004: 133-143

17 **Rockacy M**, Khalid A. Update on pancreatic cyst fluid analysis. *Ann Gastroenterol* 2013; **26**: 122-127 [PMID: 24714589]

18 **Volkan Adsay N**. Cystic lesions of the pancreas. *Mod Pathol* 2007; **20** Suppl 1: S71-S93 [PMID: 17486054 DOI: 10.1038/modpathol. 3800706]

19 **Yeh MM**, Tang LH, Wang S, Robert ME, Zheng W, Jain D. Inhibin expression in ovarian-type stroma in mucinous cystic neoplasms of the pancreas. *Appl Immunohistochem Mol Morphol* 2004; **12**: 148-152 [PMID: 15354741 DOI: 10.1097/00129039-200406000 -00009]

20 **Sumathi VP**, McCluggage WG. CD10 is useful in demonstrating endometrial stroma at ectopic sites and in confirming a diagnosis of endometriosis. *J Clin Pathol* 2002; **55**: 391-392 [PMID: 11986349 DOI: 10.1136/jcp.55.5.391]

P- Reviewer: Jeng KS, Li CH, Wani IA, Zavras A
S- Editor: Yu J **L- Editor**: A **E- Editor**: Liu WX

Published by **Baishideng Publishing Group Inc**
8226 Regency Drive, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

ISSN 1007-9327